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REVIEW

Convergent strategies in biosynthesis

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This review article focuses on how nature sometimes solves the same problem in the biosynthesis of small molecules but using very different approaches. Four examples, involving isopentenyl diphosphate, menaquinone, lysine, and aromatic polyketides, are highlighted that represent different strategies in convergent metabolism.

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1 Introduction

Many microorganisms grow in the presence of glucose and inorganic salts. They generate energy by catabolism *via* glycolysis, the citrate cycle, and oxidative phosphorylation. Moreover, they simultaneously synthesise universal small compounds, such as amino acids, nucleic acids, lipids and vitamins, from intermediate compounds produced during catabolism.

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The biosynthetic pathways of these compounds have been established with model microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, as representatives of prokaryotes and lower eukaryotes, respectively. For a long time, the biosynthetic pathways established in these systems were believed to be common among all microorganisms. However, prokaryotes and lower eukaryotes sometimes utilise different pathways to synthesise the same small bioactive compounds, especially for the biosyntheses of cofactors (vitamins), as recently summarised by Begley.[1] In the case of thiamine biosynthesis, the thiazole and pyrimidine moieties are separately biosynthesized and coupled to afford thiamine phosphate in both prokaryotic bacteria and lower eukaryotes.[1,2] However, eukaryotic biosynthetic pathways for the formation of both thiazole and pyrimidine moieties are quite different from those of prokaryotic bacteria. Quinolinic acid, on the other hand, is known to be a common precursor in the biosynthesis of the pyrimidine ring of NAD in both prokaryotic bacteria and eukaryotes. Biosynthetic routes to quinolinic acid, however, are quite different from each other in prokaryotes and eukaryotes.[1,2] When focusing on only prokaryotic bacteria or eukaryotes, some (micro)organisms use alternative biosynthetic pathways. In the former case, both *E. coli* and *Bacillus subtilis* biosynthesize pyridoxal phosphate (PLP) *via* distinct pathways. In *E. coli*, the pyridine ring of PLP is formed by condensation of 1-deoxy-D-xylulose 5-phosphate and 3-amino-1-hydroxyacetone-1-phosphate, whereas it is formed by

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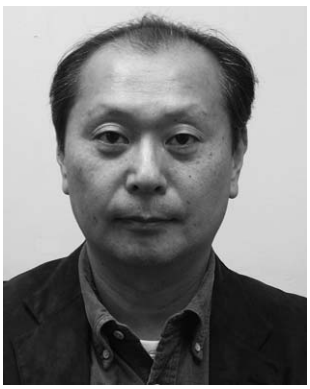
Tohru Dairi received his B.Sc. (1983) and M.Sc. (1985) degrees from Nagoya University. From 1985 to 1994, he was at Kyowa Hakko Kogyo, Co. Ltd. After receiving his Ph. D from The University of Tokyo (1982), he joined Toyama Prefectural University and studied genes and enzymes essential for the biosynthesis of natural products, especially isoprenoids produced by actinomycetes. Since 2010 he has been a professor at Hokkaido University. His research interests are novel microbial primary pathways, unique secondary metabolic pathways, and enzymes involved in these pathways.



Makoto Nishiyama

Makoto Nishiyama received his B.Sc. (1984), M.Sc. (1986), and Ph.D. (1991) degrees from The University of Tokyo. He was appointed as an Assistant Professor at The University of Tokyo in 1988, and promoted to Associate Professor in 1994 and to Professor in 2003 in the Biotechnology Research Center of The University of Tokyo. He has been studying structure, function, and regulation of enzymes involved in amino acid biosynthesis.

Tomohisa Kuzuyama

Tomohisa Kuzuyama was born in Yokosuka, Japan, in 1966. He completed his PhD with a thesis entitled "Studies on the fosfomycin biosynthesis" under the supervision of Professor Haruo Seto in the Graduate School of Agricultural and Life Sciences, The University of Tokyo in 1995. In the same year, he was appointed as an assistant professor in the Institute of Molecular and Cellular Biosciences, The University of Tokyo was where he began to study the mevalonate pathway and the 2-C-methyl-D-erythritol 4-phosphate pathway in bacteria. He joined Professor Joseph P. Noel's laboratory as a visiting scientist from 2003 through 2004. He was then promoted to an associate professor in the Biotechnology Research Center, The University of Tokyo in 2004. His current research interests aim at a comprehensive understanding of biosynthetic machinery for terpenoids.

Isao Fujii

Isao Fujii received his Ph.D. from The University of Tokyo (Prof. Ushio Sankawa) in 1983, and carried out postdoctoral research at The Ohio State University (Prof. Heinz G. Floss). Then, he was appointed as an Assistant Professor at The University of Tokyo in 1985, and was promoted to Associate Professor in 1997. He moved to Iwate Medical University, School of Pharmacy, as a Professor in 2007. He has been studying the biosynthesis of microbial polyketides at enzyme and gene levels. Recently, he is focusing on heterologous expression of fungal polyketide synthases and their functional analyses.

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the condensation of glyceraldehyde 3-phosphate, ribulose phosphate, and ammonia in *B. subtilis*.[1,3] In the latter case, both animals and plants synthesize L-ascorbic acid, albeit by distinct pathways.[4] These examples are briefly summarized in Table 1.

Recent advances in genome sequencing have revealed the presence or absence of orthologues of the genes responsible for known biosynthetic pathways to common small molecules. For example, the biosynthetic pathway for the formation of isopentenyl diphosphate (IPP) from acetate (the so-called mevalonic acid (MVA) pathway) was established mainly in *S. cerevisiae*. Curiously, most prokaryotic microorganisms lack orthologues of MVA pathway genes, even though IPP is essential in most bacteria for isoprenoid biosynthesis. This observation was one of the rationales to search for an alternative pathway to IPP that ultimately led to the discovery of the independent 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. The biosynthetic pathway to menaquinone (MK), which is an essential compound for respiration in microorganisms, represents a similar example of a genomics-guided discovery of a new pathway to an old molecule. Amino acids are also known to be biosynthesised by convergent paths and we focus on lysine synthesis in lower eukaryotes and prokaryotes that utilise the α-aminoadipate pathway and the diaminopimelate pathway, respectively. While these examples all involve primary metabolites, secondary (natural product) metabolites are also known to arise from convergent pathways. For example, aromatic polyketides, such as 1,3,6,8-tetrahydroxynaphthalene (T4HN), which is a well-known precursor in melanin biosynthesis, is made in prokaryotic streptomycetes and eukaryotic fungi *via* type III polyketide synthases (PKS) and iterative type I PKSs, respectively. In this review, we highlight the convergent strategies employed in nature for the biosynthesis of these four molecules, as recent examples of evolutionary equivalence.

2 IPP biosynthesis in microorganisms

2.1 Introduction

Isoprenoids are found in all organisms where they serve important biological functions, such as hormone signalling (steroids) in mammals, antioxidation (carotenoids) in plants, electron transport (ubiquinone or menaquinone), and cell wall biosynthesis intermediates (lipid II) in bacteria. All isoprenoids are synthesised by the consecutive condensation of the five-carbon monomer IPP (**1**) to its isomer dimethylallyl diphosphate (DMAPP; **2**), both of which are essential metabolic precursors for isoprenoid biosynthesis. Two distinct metabolic pathways exist for IPP and DMAPP biosynthesis: the classical MVA pathway, found ubiquitously in mammals and archaea, and the MEP pathway, discovered in 1990s. In this section, recent studies

Table 1 Cofactors (vitamins) biosynthesized by two distinct pathways

Cofactor (vitamin)	Moieties	Starting compounds	Intermediates [a]	Organisms
Thiamine[1]	Pyrimidine	5-Aminoimidazole ribotide	Hydroxymethyl pyrimidine pyrophosphate	Prokaryotic bacteria
		Pyridoxine (PLP) Histidine	? [b]	Eukaryote yeast & plants
	Thiazole	Glyceraldehyde 3-phosphate Pyruvate Glycine NifS (sulfur donor)	Thiazole phosphate carboxylate	Prokaryotic bacteria
		Glycine Cysteine C_5 unit	? [b]	Eukaryote yeast & plants
NAD[1]	Pyridine ring	Dihydroxyacetone phosphate Aspartate	Quinolinic acid	Prokaryotic bacteria
		Tryptophan	Quinolinic acid	Eukaryote yeast & human. Some prokaryotic bacteria
PLP[1,2]	Pyridine ring	D-Erythrose 4-phosphate 1-Deoxy-D-xylulose 5-phosphate	3-Amino-1-hydroxyacetone-1-phosphate	*Escherichia coli* (Gammaproteobacteria)
		Glyceraldehyde 3-phosphate Ribulose phosphate NH_3	—[c]	*Bacillus subtilis* (more common pathway)
L-Ascorbic acid[3]	—	D-Glucose 6-phosphate	UDP-D-glucose D-Glucuronic acid L-Glulono-1,4-lactone *etc*	Animals
		D-Glucose 6-phosphate	GDP-D-mannose L-Galactose L-Galatono-1,4-lactone *etc*	Plants

[a] Representative intermediates were shown. [b] Details of the reaction remained unclear. [c] Pyridine ring is directly formed from the starting compounds.

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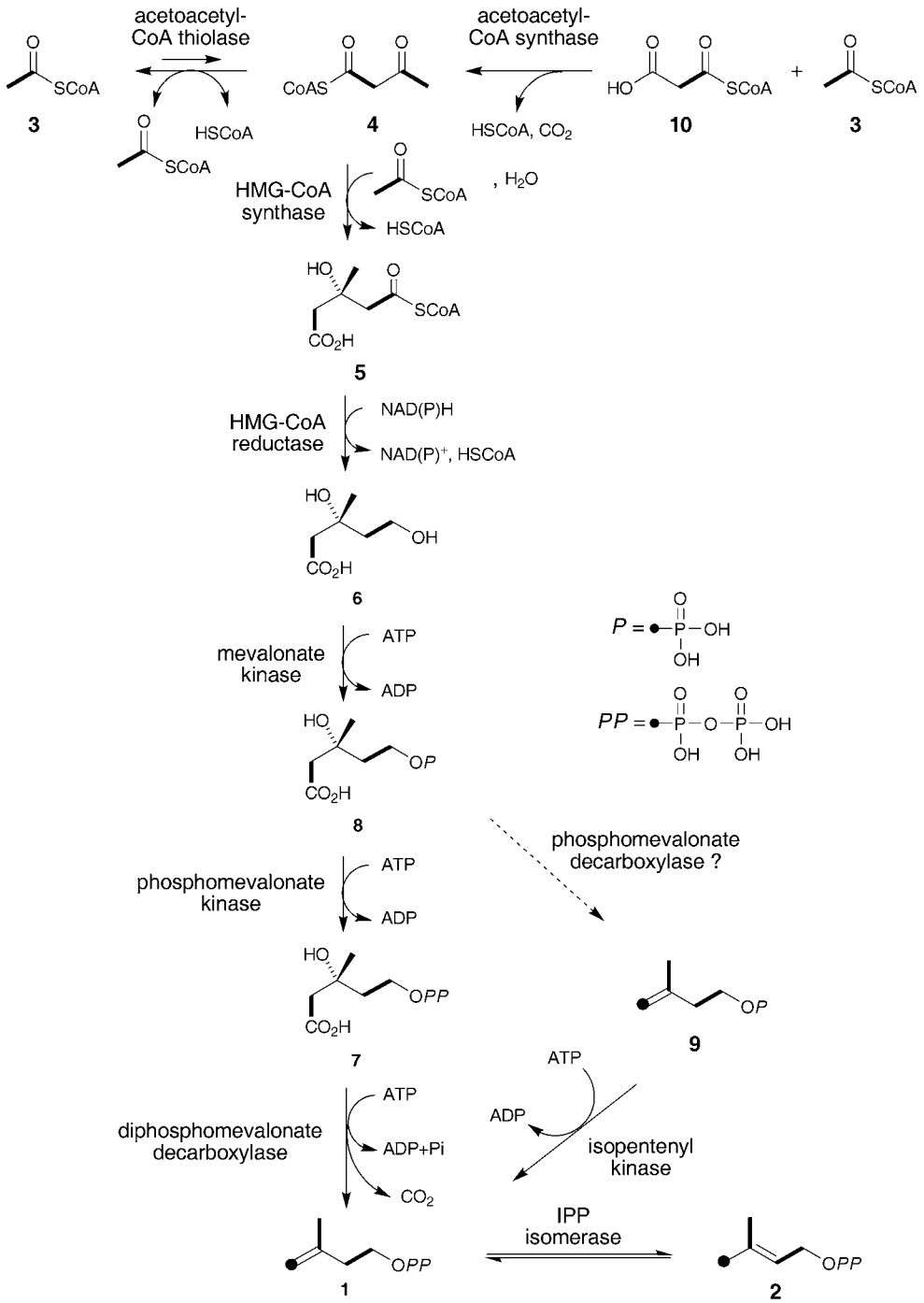
on the classical MVA pathway and the newly elucidated MEP pathway are elaborated.

2.2 MVA pathway

2.2.1 Classical pathway. The originally described MVA pathway consists of the following seven steps: acetoacetyl-CoA synthesis, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthesis, reduction of HMG-CoA, two phosphorylations of MVA, decarboxylation of diphosphomevalonate, and isomerisation of IPP. The biosynthetic pathway for IPP and DMAPP biosynthesis is summarised in Scheme 1. Eukaryotes and bacteria share a common pathway, but the pathway in archaea differs slightly in the final steps, compared to those of eukaryotes and bacteria (see 2.2.5). In addition, recent studies revealed that certain actinobacteria use unusual acetoacetyl-CoA synthesising enzymes (see 2.2.3).[5]

Usually, the MVA pathway begins with a thioester-dependent Claisen condensation reaction between two molecules of acetyl-CoA (**3**), catalysed by acetoacetyl-CoA thiolase (EC 2.3.1.9), to give acetoacetyl-CoA (**4**). A second condensation reaction with a third molecule of **3** then yields the six-carbon compound,



Scheme 1 MVA pathway in bacteria and archaea.

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(3*S*)-HMG-CoA (**5**). This condensation reaction, which is an aldol-like process, is catalysed by HMG-CoA synthase (EC 2.3.3.10). **5** is then reduced to MVA (**6**) by HMG-CoA reductase (EC 1.1.1.34 and EC 1.1.1.88), the rate-limiting enzyme of the MVA pathway. This enzyme is classified into either class I or class II, based on its amino acid sequence (see 2.2.4).

6 is converted into diphosphomevalonate (**7**) *via* phosphomevalonate (**8**) by two consecutive phosphorylation reactions involving ATP hydrolysis, catalysed by MVA kinase (EC 2.7.1.36) and phosphomevalonate kinase (EC 2.7.4.2). **7** is then converted into **1** by diphosphomevalonate decarboxylase (EC 4.1.1.33). In this step, the release of CO_2 from **7** occurs during the hydrolysis of ATP to ADP and inorganic phosphate. Finally, **1** is converted into its isomer **2** by the action of IPP isomerase (EC 5.3.3.2). There are two types of IPP isomerases, which have no amino acid sequence similarity (see 2.2.6).[6]

2.2.2 Gene clusters for the MVA pathway. Fig. 1 shows the organisation of the genes responsible for the MVA pathway in 10 bacteria and the archaeon, *Methanococcus jannaschii*.

In high GC Gram-positive members of the Actinobacteria, including *Streptomyces* sp. strain CL190,[7] *Streptomyces* sp. strain KO-3988,[8] *Streptomyces annulatus*,[9] *Kitasatospora griseola*,[10] *Actinoplanes* sp. strain A40644,[11] and *Nocardia farcinica*,[12] the six MVA pathway genes (except the acetoacetyl-CoA thiolase-encoding gene) are clustered in a single locus on their genomes, and the order and extent of the genes are identical. For example, the gene cluster of *Streptomyces* sp. strain CL190 contains six open reading frames (*nphT1* to *nphT6*) encoding the enzymes that catalyse the formation of **1** and **2** *via* **6** from **4**. Also, in *Borrelia garinii*,[13] a Gram-negative spirochete bacterium that is a causative agent of Lyme borreliosis, and *Paracoccus zeaxanthinifaciens*,[14] a Gram-negative marine bacterium that produces the yellow carotenoid zeaxanthin, the six MVA pathway genes are clustered in a single locus. However, the structures of these clusters are different from those of high GC Gram-positive members of the Actinobacteria. *Enterococcus faecalis* has two loci with two kinase genes: the diphosphomevalonate decarboxylase gene and the IPP isomerase gene grouped in one locus, and the HMG-CoA synthase and HMG-CoA reductase genes on the second locus.[15,16] In addition, the *E. faecalis* HMG-CoA reductase gene is fused with the acetoacetyl-CoA thiolase gene to encode a dual-function protein (MvaE) that catalyses both the acetoacetyl-CoA thiolase and the HMG-CoA reductase reactions.[15] In *Staphylococcus aureus*, the six MVA pathway genes are encoded in three loci.[17] In the archaea *M. jannaschii*, the four MVA pathway genes are scattered throughout the genome, and the phosphomevalonate kinase and diphosphomevalonate decarboxylase genes are missing.[18]

2.2.3 Acetoacetyl-CoA synthesising enzymes. The MVA pathway starts with the synthesis of **4**, which was first reported to be biosynthesised *via* a thioester-dependent Claisen condensation reaction between two molecules of **3** catalysed by acetoacetyl-CoA thiolase (EC 2.3.1.9). Therefore, acetoacetyl-CoA thiolase was expected to exist in the MVA pathway gene cluster of Actinobacteria. However, a homologue of acetoacetyl-CoA thiolase, involved in the conversion of **3** to **4**, is absent. Instead, an open reading frame (for example, *nphT7* of *Streptomyces* sp. strain CL190) that shares homology with β-ketoacyl-(acyl carrier protein (ACP)) synthase (KAS) III flanks the gene cluster (Fig. 1).

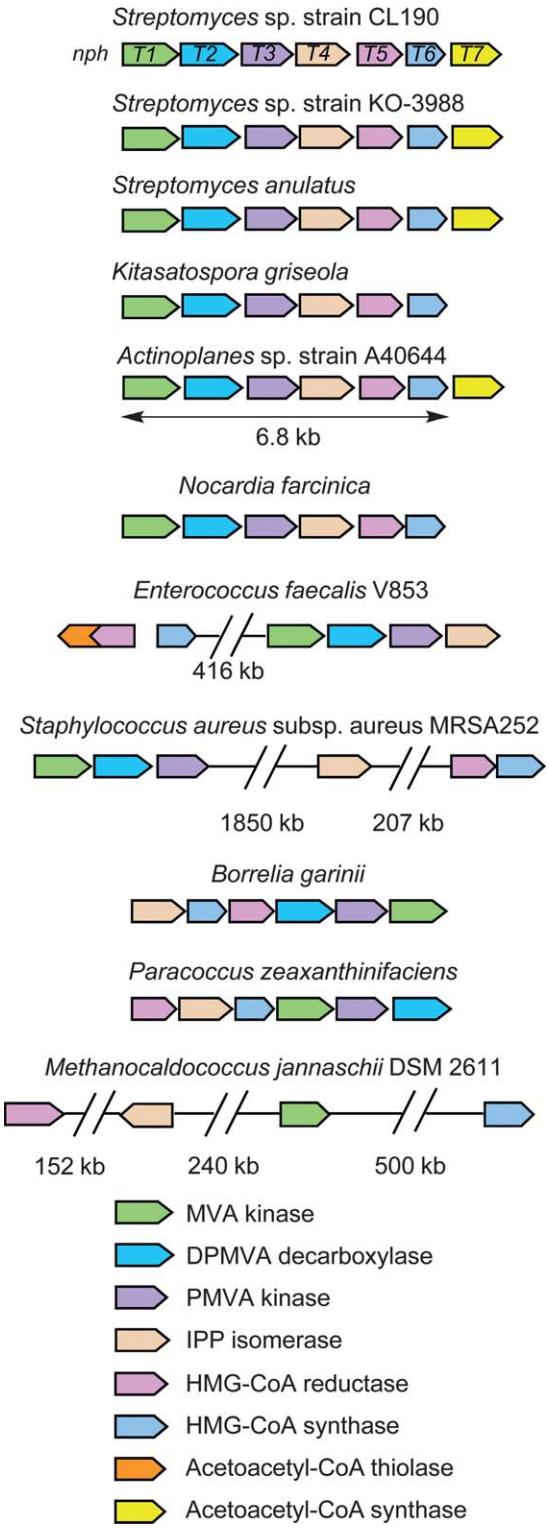

Fig. 1 MVA pathway gene clusters.

Recent studies demonstrated that NphT7 catalyses a single condensation of **3** and malonyl-CoA (**10**) to give **4** and CoA.[5] NphT7 was not able to synthesise acetoacetyl-CoA through the condensation of two molecules of **3**. Based on the enzymatic

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properties of NphT7, it was proposed that it was a novel "acetoacetyl-CoA synthase" of the thiolase superfamily. In addition, co-expression of the *nphT7* gene with the HMG-CoA synthase and HMG-CoA reductase genes in the heterologous host *Streptomyces albus* resulted in a 3.5-fold higher production of **6** than when only the HMG-CoA synthase and HMG-CoA reductase genes were expressed. This result suggests that *nphT7* could be used to significantly increase the production of commodity isoprenoids, such as carotenoids, taxol, and artemisinin.

BLAST searches using the NphT7 sequence as a query sequence revealed that NphT7 homologues are widespread in bacteria.[5] Of the homologues, some are located in the biosynthetic gene clusters of unidentified natural products, suggesting that NphT7-like enzymes are involved in the biosynthesis of terpene-based natural products.

2.2.4 Two classes of HMG-CoA reductases. HMG-CoA reductase (HMGR) is the rate-limiting enzyme in the MVA pathway. Recent progress in genome analysis unveiled an extensive body of information regarding the HMGR sequence. Comparison of amino acid sequences and phylogenetic analysis of representative HMGRs from eukaryotes, archaea, and bacteria revealed two distinct classes, I (EC 1.1.1.34) and II (EC 1.1.1.88).[19–21]

2.2.4.1 Class I HMG-CoA reductase. Class I HMGRs are found in eukaryotes, archaea, and high GC Gram-positive members of the actinobacteria. Class I HMGRs that have been characterised include those found in humans, rats, hamsters, yeasts, plants, *Haloferax volcanii*,[22,23] *Sulfolobus solfataricus*,[24] and some *Streptomyces* spp.[25,26] Class I HMGRs utilise two NADPH equivalents to reductively deacylate the thioester group of **5** to the primary alcohol of **6**:

(*S*)-HMG-CoA **5** + 2NADPH + $2H^+$ → (*R*)-MVA **6** + CoA + $2NADP^+$

In all archaea, HMGR is an essential enzyme for the synthesis of isoprenoid glycerol ethers, the main components of cell membranes.[27] The recombinant HMGR gene from *H. volcanii* was characterised.[22,23] Except for its optimal enzyme activity under high ionic strength conditions, all the catalytic properties of *H. volcanii* HMGR were comparable with those of HMGR from higher eukaryotes. *H. volcanii* HMGR uses NADPH as a coenzyme and exhibits stereoselectivity for (*S*)-HMG-CoA. The HMGR from the thermophilic archaeon *S. solfataricus* P2 was also characterised.[24]Although *S. solfataricus* HMGR shows optimal activity at 85 °C and pH 5.5, the enzyme exhibits substrate specificities typical for a eukaryotic HMGR.

Bacterial class I HMGR was first characterised from *Streptomyces* sp. strain CL190, a high GC Gram-positive member of the Actinobacteria.[25] This biosynthetic enzyme utilises NADPH and its inhibition profile against statins, such as pravastatin and mevinolin, resembles those of eukaryotic enzymes.

Genome analysis revealed that class I HMGR genes exist in pathogenic bacteria, including *Vibrio cholera*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus*.[28] In contrast, no other genes for the MVA pathway, other than the HMGR gene, are found in their genomes. Instead, these bacteria carry all the genes required for the MEP pathway. Thus, these bacteria must exclusively utilise the MEP pathway for isoprenoid biosynthesis. Why these pathogenic bacteria carry the HMGR gene remains unknown.

2.2.4.2 Class II HMG-CoA reductase. Class II HMGRs are found in bacteria and archaea. Class II HMGRs that have been characterised include those found in *Pseudomonas mevalonii*,[29] *Staphylococcus aureus*,[17] *E. faecalis*,[15] *Archaeoglobus fulgidus*,[30] and *Listeria monocytogenes*.[31] The inhibition of class I and class II HMGRs by statins has been extensively investigated and has revealed that they have different sensitivities. Although class I HMGRs are strongly inhibited by statins at nanomolar concentrations, class II HMGRs are greater than four orders of magnitude less sensitive to statins.[21]

A bacterial class II HMGR was first obtained from *P. mevalonii*, which can grow on **6** as the sole carbon source.[29] *P. mevalonii* HMGR plays a biodegradative role *in vivo*, converting **6** to **5**. Unlike class I HMGRs, which utilise NADPH as the coenzyme exclusively, the class II HMGR from *P. mevalonii* utilises NADH as the coenzyme.[32] *P. mevalonii* HMGR is one of the best characterised enzymes among the HMGRs reported.

Although *P. mevalonii* HMGR has a biodegradative role, other bacterial class II HMGRs have biosynthetic roles in the production of isoprenoid compounds. A biosynthetic class II HMGR was first characterised from the Gram-positive pathogen *S. aureus*.[17] Unlike *P. mevalonii* HMGR, which utilises only NADH, *S. aureus* HMGR can utilise either NADH or NADPH as the coenzyme, although NADPH is preferred.

Gram-positive cocci use the MVA pathway, which is essential for growth. Analysis of genome sequences of enterococci revealed the presence of the *mvaE* gene that encodes both acetoacetyl-CoA thiolase and a class II HMGR in a single protein.[15] The MvaE protein was the first example of an HMG-CoA reductase fused to another enzyme. Western blotting analysis using enterococcal extracts indicated that the *mvaE* gene product was expressed in *Enterococcus faecium*, *E. faecalis*, and *E. hirae*. The fusion protein catalysed both the acetoacetyl-CoA thiolase and HMGR reactions. The K_m values, pH profiles and K_i values of *E. faecalis* HMGR,were comparable to those of other class II HMGRs. In addition, the enzyme exclusively used NADPH as the coenzyme.

The class II HMGR gene was also isolated from *L. monocytogenes*, a Gram-positive rod-shaped bacterium which causes listeriosis.[31] While the purified enzyme utilises both NADH and NADPH for catalysis, NADPH is the preferred coenzyme. *L. monocytogenes* HMGR was inhibited by micromolar concentrations of lovastatin and mevastatin. The hyperthermophilic archaeon *Archaeoglobus fulgidus*, which grows in hydrothermal environments, harbours a class II HMGR instead of a class I HMGR, as found in other archaea.[30] Unlike *P. mevalonii* HMGR, *A. fulgidus* HMGR exhibited dual coenzyme specificity. While significant activity was observed using NADPH, NADH was the preferred coenzyme.

2.2.4.3 Class II HMGRs as a new molecular target for antibacterial drugs. Class II HMGRs are present in clinically important human pathogens, including *Borrelia burgdorferi*,

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Streptococcus pyogenes, *Streptococcus pneumoniae*, *S. aureus*, *E. faecalis*, and *L. monocytogenes*. Disruption of the *mvaA* gene that encodes *S. aureus* HMGR demonstrated that the gene is essential for the growth of this organism, and growth of the *mvaA* null mutant was severely attenuated in the mouse haematogenous pyelonephritis infection model.[17] Since the enterococci are a major cause of nosocomial infections, the eubacterial class II HMGRs are a promising molecular target for the development of antibiotics against multi-drug-resistant strains.

2.2.5 Late steps in archaea. The archaebacterium *Methanocaldococcus jannaschii* has slightly different late steps in the MVA pathway compared with those of eukaryotes and bacteria (Scheme 1). In "the archaeal MVA pathway", **1** is synthesised from isopentenyl phosphate (IP; **9**) by the action of IP kinase, whose gene is located 586 kb upstream of the HMG-CoA reductase gene.[18] This kinase is also found in several other archaea, including *Methanobacterium thermautotrophicum*, *Methanosarcina mazei*, *Thermoplasma acidophilum* and *Aeropyrum pernix*. The recombinant IP kinase was biochemically characterized[33] and the crystal structures were solved.[34,35] However, the enzyme responsible for the synthesis of **9** remains unknown. White and co-workers propose that an unidentified phosphomevalonate decarboxylase may catalyse the decarboxylation of MVA phosphate to give **9** (Scheme 1).[18]

2.2.6 Two types of IPP isomerases. 1 produced through the MVA pathway must be converted into **2**, because **2** is required as a priming allylic substrate, by generating an allylic carbocation intermediate, to start the prenyl-chain elongation reactions for polyprenyl diphosphates, such as geranyl diphosphate and farnesyl diphosphate. IPP isomerase (EC 5.3.3.2) catalyses the interconversion reaction between **1** and **2**. Based on their amino acid sequences and coenzyme requirements, IPP isomerases are classified as either type 1 or type 2 enzymes.[6]

2.2.6.1 Type 1 IPP isomerase. Type 1 IPP isomerase from *S. cerevisiae* catalyses the isomerase reaction in the presence of a divalent metal cation such as Mg^{2+} or Mn^{2+}.[36] Yeast IPP isomerase is completely inhibited by thiol-blocking reagents, such as iodoacetamide, suggesting that a cysteine residue is involved in the catalytic mechanism.[37] Similarly, IPP isomerase activity in *E. coli*, which is also dependent on a metal divalent cation and inhibited by iodoacetamide, was the first reported example of the enzyme from bacteria.[38]

2.1.6.2 Type 2 IPP isomerase. The first type 2 IPP isomerase was isolated from *Streptomyces* sp. strain CL190 in 2001,[6] more than 40 years after the identification of the type 1 isomerases. The type 2 IPP isomerase from CL190 has no sequence homology with the type 1 enzymes, suggesting that the two types of IPP isomerase are evolutionarily independent. The type 2 enzyme requires FMN and NAD(P)H, in addition to a metal divalent cation, for enzyme activity. This curious cofactor requirement is considered to have made the *in vitro* detection of this enzyme difficult, and is the main reason why this type of IPP isomerase was overlooked for so long. The pro-*R* stereo-specificity of the enzyme at the C-2 of IPP is the same as that of the type 1 IPP isomerase,[39] although the reaction specificity of hydrogen exchange at the C-2 and C-4 of IPP is much more stringent.[40]

Although the role of FMN in the type 2 IPP isomerase reaction was controversial, recent studies on the crystal structures of the type 2 IPP isomerase from the *Sulfolobus shibatae* suggests that FMN acts as a general acid–base catalyst without playing any redox roles.[41]

2.3 MEP pathway

2.3.1 Pathway and enzymes. Reviews describing the discovery and biochemistry of the MEP pathway were previously published in *Natural Product Reports*.[42,43] Furthermore, numerous reviews covering the MEP pathway and its enzymes have been published, including "Isoprenoid biosynthesis as a novel target for antibacterial and antiparasitic drugs"[44] and "The MEP pathway: a new target for the development of herbicides, antibiotics and antimalarial drugs".[45]

The initial step of the MEP pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP; **11**) by a transketolase-type condensation of pyruvate (**12**) and D-glyceraldehyde 3-phosphate (D-GAP; **13**), catalysed by DXP synthase (EC 2.2.1.7) in a thiamine diphosphate-dependent manner.[46–48] This enzyme is also essential for the synthesis of vitamins B_1 and B_6 in bacteria.[48] In the second step, the intramolecular rearrangement and NADPH-dependent reduction of DXP simultaneously occur to form MEP (**14**).[49] This reaction is catalysed by NADPH-dependent DXP reductoisomerase (EC 1.1.1.267).[50–52] **14** is finally converted into both **1** and **2** by the action of the following five enzymes, MEP cytidylyltransferase (EC 2.7.7.60),[53,54] 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME; **15**) kinase (EC 2.7.1.148),[55,56] 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP; **16**) synthase (EC 4.6.1.12),[57,58] iron-sulphur (Fe–S) cluster-containing 4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBDP) synthase (EC 1.17.7.1),[59] and Fe–S cluster-containing HMBDP reductase (EC 1.17.1.2).[60] The reaction mechanisms of the latter two enzymes are controversial and there have been several catalytic mechanisms proposed for both of the enzymes.[61,62] The crystal structures of all the enzymes in the MEP pathway, except for HMBDP synthase, have been determined.[63–68] The complete MEP pathway for **1** and **2** biosynthesis is illustrated in Scheme 2.

It has been suggested that the genomes of a number of bacteria contain the all the genes of the MEP pathway except those encoding DXP reductoisomerase. Recent studies revealed that an uncharacterised protein from a pathogenic bacterium, *Brucella abortus*, with no homology to DXP reductoisomerase, simultaneously catalyses the intramolecular rearrangement and NADPH-dependent reduction of **11** to form **14**, as well as DXP reductoisomerase.[69] This use of two different enzymes for the same reaction is similar to the usage of two types of IPP isomerase.[6] The DXP reductoisomerase-like enzyme from *B. abortus* belongs to the homoserine dehydrogenase family and has homologues widely distributed in bacteria.[69]

2.3.2 Inhibitors. The MEP pathway is present in most bacteria, including pathogens, as well as in plant chloroplasts, and the apicoplasts of Apicomplexan protozoa, such as *Plasmodium* and *Toxoplasma*.[43] Because the MEP pathway is absent

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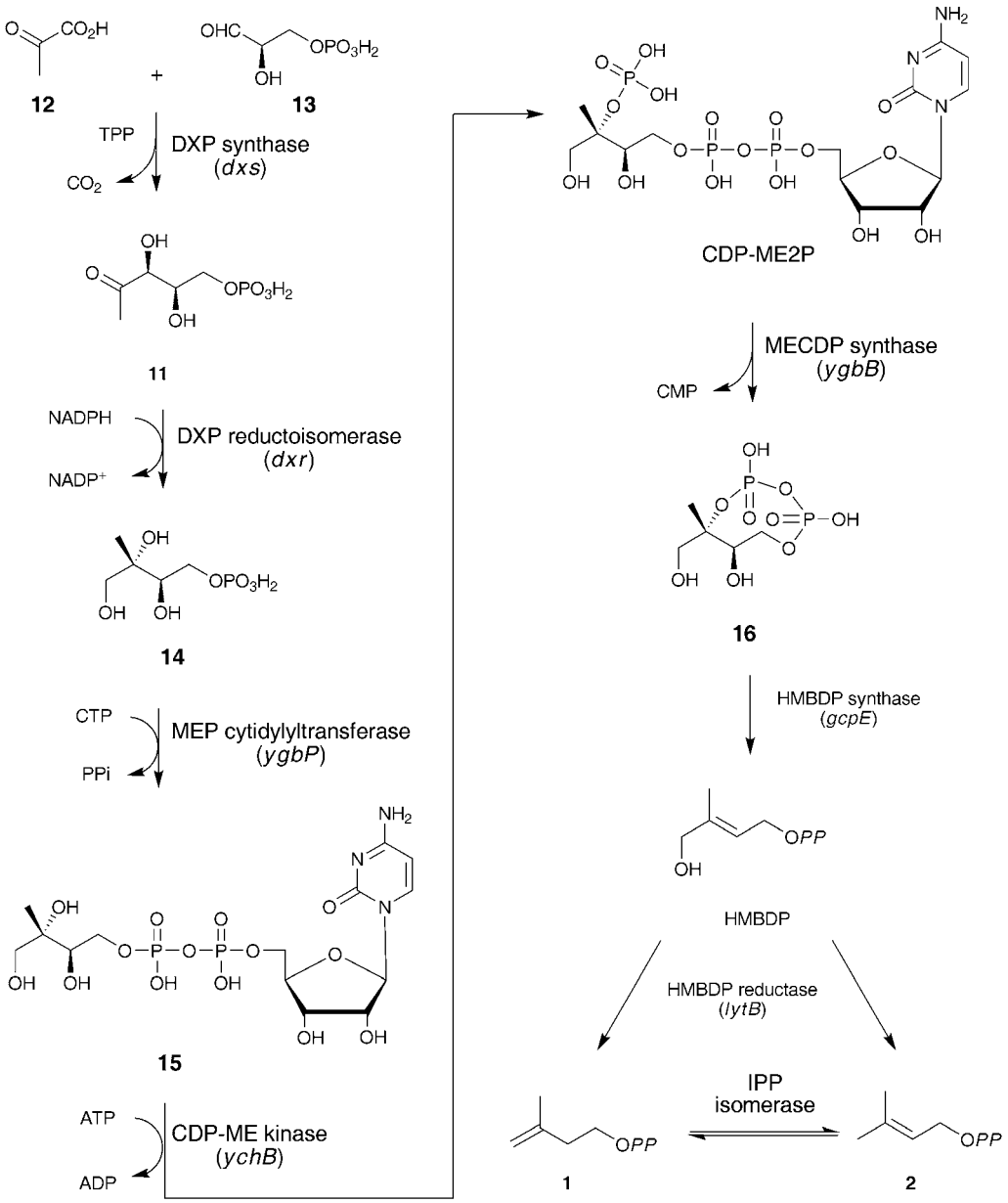

Scheme 2 MEP pathway.

from mammals, all MEP pathway enzymes represent effective targets for the development of antibacterial and antimalarial drugs, and herbicides. In fact, it has been demonstrated that the knock-out mutant of DXP reductoisomerase is lethal in *E. coli*.[70]

The natural product fosmidomycin (**17**), which was isolated from *Streptomyces lavendulae* in 1980,[71] is a potent and specific inhibitor of DXP reductoisomerase (Fig. 2).[72] In addition, **17** and its derivative inhibited *Plasmodium falciparum* DXP reductoisomerase, and consequently these inhibitors cured mice infected with the rodent malaria parasite *Plasmodium vinckei*.[73] **17** inhibited the homoserine dehydrogenase homologue from *B. abortus* as well as DXP reductoisomerase.[69]

Ketoclomazone (2-(2-chlorobenzyl)-4,4-dimethyl-isoxazolidine-3,5-dione; **18**) is a derivative of a soil-applied herbicide, clomazone, also known as dimethazone or FMC 57020 (Fig. 2).[74] **18** causes significantly lower chlorophyll and carotenoid levels in barley leaves. In addition, 20 μM of **18** inhibited 47% of the DXP synthase activity in crude extracts from *E. coli* expressing a plant *Catharanthus roseus* DXP

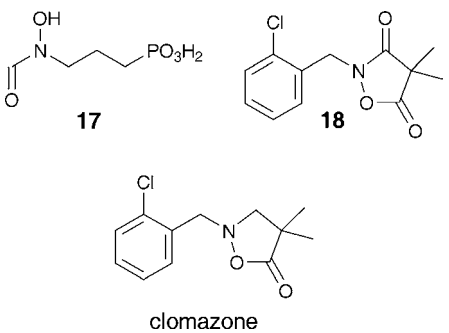

Fig. 2 Inhibitors of the MEP pathway.

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synthase gene,[75] suggesting that **18** is an inhibitor of DXP synthase. Therefore, **18** and clomazone have been used as chemical tools to suppress the MEP pathway in plants.[76,77] In contrast, **18** has not been reported to have antibacterial activity, although most bacteria, including pathogens, utilise the MEP pathway for isoprenoid biosynthesis.[45] However, recent studies demonstrated that **18** inhibits DXP synthases from *E. coli* and *Haemophilus influenzae* and exhibits antibacterial activity against these bacteria.[78] The kinetics of inhibition by **18** suggests that **18** binds to an unidentified inhibitor-binding site that differs from the substrate-binding sites for **12** and **13** on DXP synthase.

The antibacterial activity of **18** is not as potent as that of **17** and ampicillin, possibly because **18** was developed as a herbicide. However, derivatisation of **18** might improve its antibacterial activity. For example, a target-based approach was reported to identify possible *Mycobacterium tuberculosis* DXP synthase inhibitors based on the structure of a known transketolase inhibitor 3-(4-chloro-phenyl)-5-benzyl-4*H*-pyrazolo[1,5-*a*]pyrimidin-7-one.[79] Two analogues of the inhibitor identified in the approach showed antibacterial activity against *M. tuberculosis* with minimum inhibitory concentrations of 7.6 and 7.7 μM.

2.4 Diversity of the biosynthesis of IPP and DMAPP

Most bacteria synthesise **1** and **2** using the MEP pathway, whereas almost all archaea and some Gram-positive bacteria, including *Staphylococcus*, *Enterococcus*, and *Streptococcus*, use the MVA pathway. Only a few bacteria use both. The species distribution of the MVA and MEP pathways are not associated with those of the type 1 and type 2 IPP isomerases (Fig. 3). Although most bacteria that use the MEP pathway do not have an IPP isomerase, some have a type 1 enzyme, some have a type 2, and others have both. On the other hand, bacteria using only the MVA pathway usually possess a type 2 IPP isomerase. This is also the case for most archaea. However, the Q-fever pathogen *Coxiella burnetii* has the MVA pathway genes and a type 1 IPP isomerase, and the halophilic archaeon *Halobacterium* sp. NRC-1 possesses both functional type 1 and type 2 IPP isomerases, with the MVA pathway.[80]

2.5 Growth-phase dependent expression of the two pathways

Streptomyces sp. strain CL190, which produces the terpenoid antibiotic naphterpin, possesses the genes for both the MVA and MEP pathways (Fig. 1 and 3).[7,81] Detailed tracer experiments, using ^{13}C-labeled acetate (for the MVA pathway) and glucose (for the MEP pathway) on naphterpin and menaquinone, proved that there was simultaneous operation of the both pathways for IPP biosynthesis in the strain.[82] In contrast, most actinomycetes use only the MEP pathway for IPP biosynthesis. Such examples include *Streptomyces coelicolor* A3(2),[83] *Streptomyces avermitilis*,[84] and *Streptomyces griseus*,[85] whose genome sequences have been deciphered.

Kitasatospora griseola, a terpentecin-producer, also uses both the MVA and MEP pathways for IPP biosynthesis (Fig. 1 and 3).[10,86] Expression analyses of the genes in both the MEP and MVA pathways demonstrated that the MEP pathway genes are expressed before those in the MVA pathway.[86] The production of terpentecin and the expression of the MVA pathway genes appeared to be synchronised in the producer cells. Generally, secondary metabolites, such as naphterpin and terpentecin, are biosynthesised in the late stage of the growth. In addition, the biosynthetic gene cluster of terpentecin flanks the MVA pathway gene cluster.[10,87] These observations strongly suggest that the MVA pathway genes are utilised for the production of the secondary metabolites, such as terpentecin and naphterpin. This suggestion is also supported by a recent report on the biosynthesis of furanonaphthoquinone I produced by *Streptomyces cinnamonensis* DSM 1042.[88] Tracer experiments using ^{13}C-labeled acetate and glycerol on furanonaphthoquinone I proved that there was simultaneous operation of the MVA pathway (*ca* 80%) and the MEP pathway (*ca* 20%) for IPP biosynthesis in the strain.

2.6 Summary

We summarise the two distinctly different metabolic pathways for the synthesis of **1** in microorganisms *via* the MVA and MEP pathways in this section. The MVA pathway starts with the condensation of two molecules of **3**, whereas the MEP pathway starts with the condensation of **12** and **13**. For the synthesis of one molecule of **1**, the MVA pathway utilises one molecule of NADPH and three molecules of ATP, whereas the MEP pathway utilizes one NADPH, one CTP, and one ATP. Thus, these two pathways must have evolved independently in microorganisms. It is intriguing that two types of IPP isomerases and two kinds of DXP reductoisomerases are distributed for the synthesis of **1**. Undiscovered alternative routes for **1** may additionally be buried in enormous quantity of genome sequences. Further analyses of IPP biosynthesis in microorganisms may illustrate the interplay of functional convergence and divergence in the evolution of metabolic pathways.

3 Menaquinone biosynthesis in microorganisms

3.1 Introduction

In prokaryotes, ubiquinone and menaquinone (**19**, MK) are lipid-soluble molecules that shuttle electrons between the membrane-bound protein complexes in the electron transport chain. For example, *E. coli*, a facultative anaerobe, utilises ubiquinone under aerobic conditions, but uses MK when grown anaerobically.[89,90] On the other hand, *B. subtilis*, a Gram-positive aerobe, contains only MK. Thus, MK biosynthesis is essential for survival of *B. subtilis*. In mammalian cells, ubiquinone plays a sole role in the electron transport chain, which is located in the inner mitochondrial membrane. On the other hand, MK functions as an essential vitamin for the biological activation of a family of proteins involved in blood coagulation, bone metabolism, and cell cycle regulation.

Humans are unable to synthesize MK and obtain essential amounts of it *via* diet or from bacteria present in the gut. In contrast, microorganisms have a *de novo* biosynthetic pathway.[89–91] In this review, we first introduce recent studies on the classical pathway established in *E. coli*, and then focus on an alternative pathway (futalosine pathway), which was recently identified in some microorganisms.

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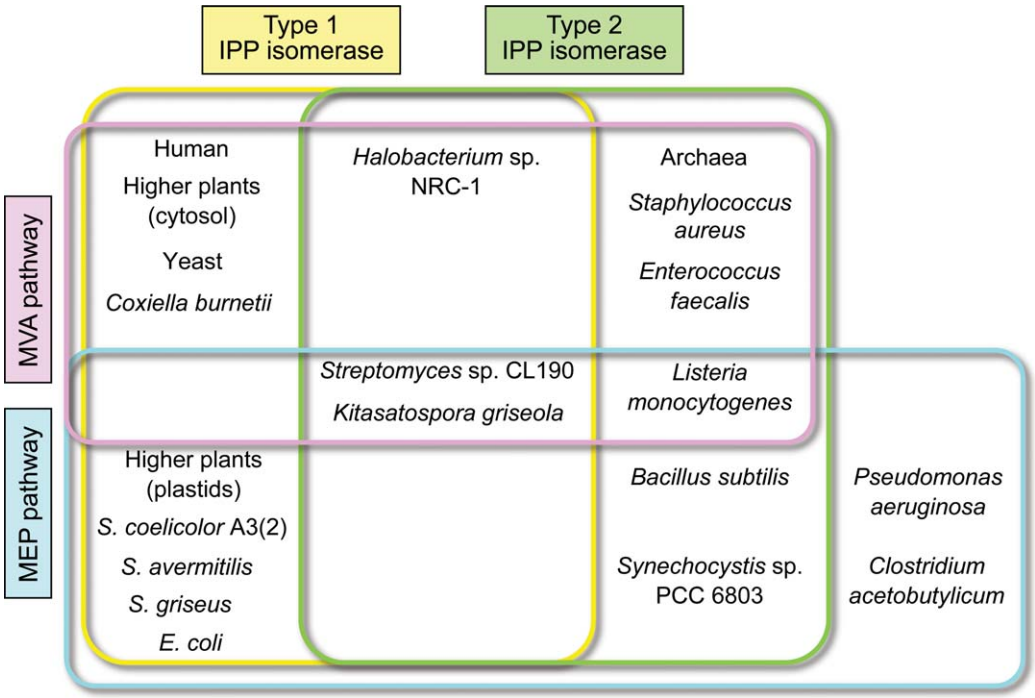

Fig. 3 Distributions of the MVA and MEP pathways and two types of IPP isomerases.

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3.2 Classical pathway

The classical MK biosynthetic pathway was established by pioneering studies in *E. coli* (Scheme 3).[89–91] Chorismate (**20**), derived from the shikimate (**21**) pathway, is initially converted into isochorismate (**22**) by MenF, an isochorismate synthase. Then, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (**23**, SHCHC) is formed from **22** and 2-ketoglutarate (**24**) by MenD, a thiamine-dependent enzyme. **23** is dehydrated by MenC to yield an aromatic compound, *o*-succinylbenzoate (**25**), followed by the attachment of coenzyme A by MenE to yield *o*-succinylbenzoyl-CoA (**26**). **26** is then converted into 1,4-dihydroxy 2-naphthoic acid (**27**) by MenB *via* the elimination of CoA, which is perhaps catalysed by MenH. In the last two steps of the pathway, **19** is synthesised by MenA and MenG, which catalyse the prenylation and *S*-adenosylmethionine-dependent methylation reactions, respectively.

The established pathway was accepted until a few years ago when two successive biosynthetic steps were revised by Guo and co-workers. One was the reaction catalysed by MenD.[92] The MenD product was previously considered to be **23** because it was formed from **22** and **24** by a crude extract of a *menC*$^{-}$*menD*$^{+}$ mutant. However, a time lag between the substrate (**22**) consumption and the product (**23**) formation in an *in vitro* assay with the recombinant enzyme was observed. Based on these experimental results, an intrinsic intermediate, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid (**28**, SEPHCHC) was confirmed to be formed from **22** by MenD. MenD was thus renamed SEPHCHC synthase and the stereochemistry at the C-2 position of **28** was also determined.[93] The structural analyses of MenD have also been reported.[94–96]

The other biosynthetic step in the pathway that was also recently revised, was the step after the formation of **28**.[97,98] **28** was spontaneously converted into **23** at alkaline pH but not at physiological pH. Moreover, SHCHC synthase activity was clearly detected in the crude extract of *E. coli*. These results suggested the presence of an enzyme that specifically catalysed the conversion of **28** into **23**. To identify the enzyme, the known MK biosynthetic enzymes, MenF, MenD, MenC, MenE, MenB, and MenH were overproduced in *E. coli*, and used in a SHCHC synthase assay. Consequently, MenH, which was previously proposed to facilitate the hydrolysis of 1,4-dihydroxy-2-naphthoic acid-CoA, exhibited a high level of activity in catalyzing the formation of **23** from **28**. To determine whether MenH was a bifunctional enzyme catalyzing separate steps in **19** biosynthesis, chemically synthesised 1,4-dihydroxy-2-naphthoic acid-CoA was used in an *in vitro* assay. However, MenH was unable to hydrolyze this compound. Taking these results together, MenH was concluded to be a monofunctional enzyme that catalyses the conversion of **28** into **23**. Very recently, the activity of the MenB enzyme from *E. coli* was shown to be dependent on an exogenous bicarbonate anion.[99]

3.3 Alternative pathway (futalosine pathway)

3.3.1 Evidence of the presence of an alternative pathway. *Streptomyces* strains produce MK with various lengths of prenyl side chain that have been used in taxonomic studies as a chemical marker. In 2002 and 2003, the complete genome sequences of *S. coelicolor* A3(2)[83] and *Streptomyces avermitilis*[84] were determined. Very curiously, there were few orthologues of the *men* genes as previously established for MK biosynthesis in *E coli*, as described above, except for two genes, *menA* and *menG*, whose products catalyse the prenylation and methylation reactions, respectively. In addition to these two microorganisms, the

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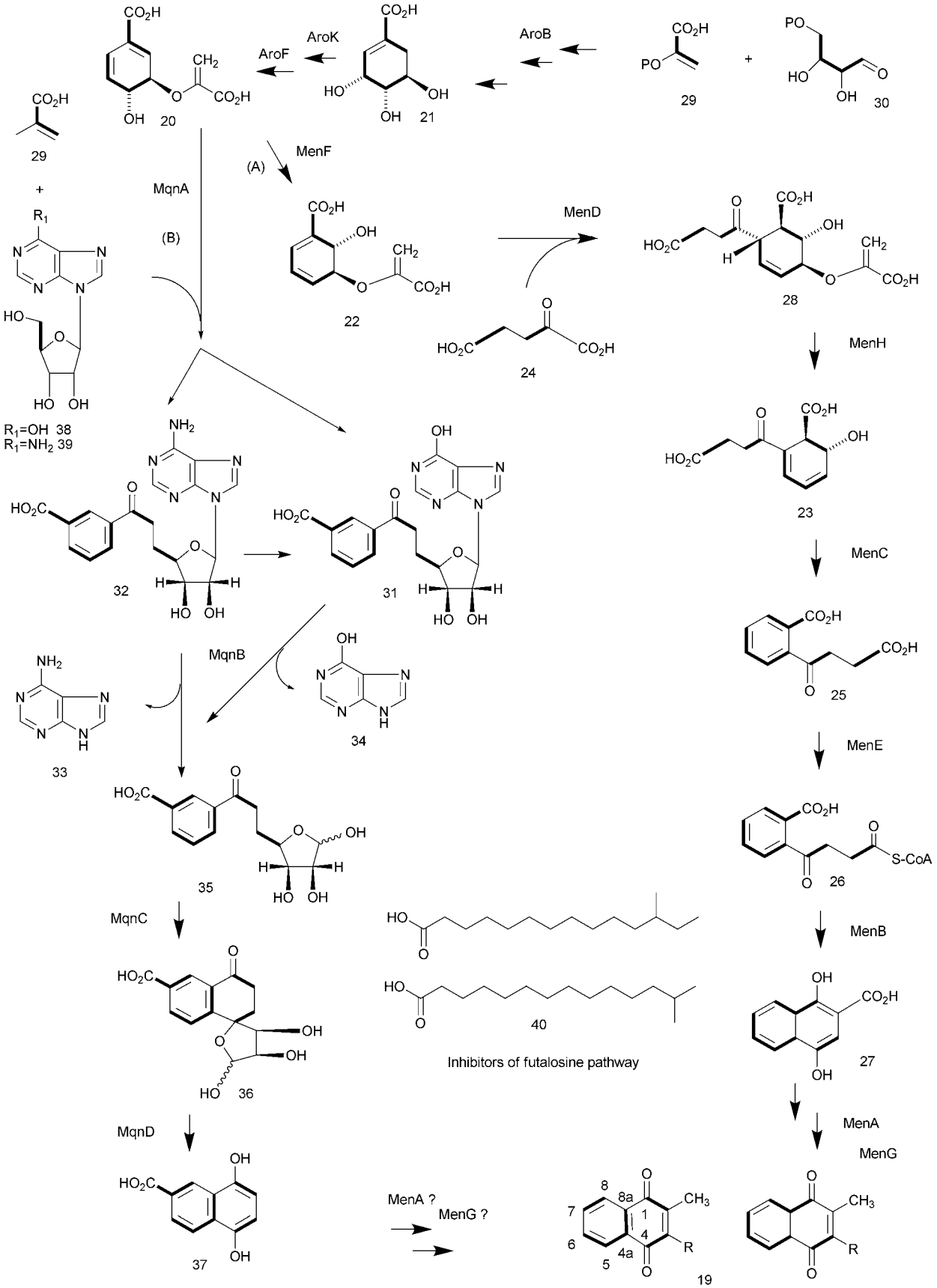

Scheme 3 MK biosynthetic pathways. Classical pathway (A) and futalosine pathway (B).

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Epsilon and Delta categories of proteobacteria, including *Helicobacter pylori*[100] and *Campylobacter jejuni*,[101] which are known to cause gastric carcinoma and diarrhoea, respectively, also lacked the *men* gene orthologues. These facts strongly suggested that the naphthoquinone moiety of **19** is synthesized *via* an alternative pathway in these bacteria.

To investigate whether an alternative pathway was operating in these bacteria, a tracer experiment was performed.[102] The ^{13}C-NMR spectrum of the A-ring of MK purified from *S. coelicolor* A3(2), to which [U-$^{13}C_6$]glucose was fed, showed a labelling pattern that could be explained by the incorporation of [U-$^{13}C_6$]glucose into MK through the shikimate pathway by the condensation of phosphoenolpyruvate (**29**) and erythrose-4-phosphate (**30**) (Scheme 3). These two compounds used for A-ring formation were the same as those in the classical pathway, except that the incorporated positions (**29** into C-7 and -8, and **30** into C-8a, -4a, -5, and -6) differed. Further experiments using glucose labeled with ^{13}C at different positions clearly showed that specific carbons of [3-^{13}C], [4-^{13}C], [5-^{13}C], and [6-^{13}C]glucose were incorporated into C-8a, -4a, -5, and -6 of MK, respectively. The enrichment of C-7 and C-8 by [5-^{13}C]glucose and [6-^{13}C]glucose, respectively, supported the incorporation of these precursors, *via* **29**, through the shikimate pathway. As for the B-ring, no specific incorporation of [1,2-$^{13}C_2$]acetate was observed in contrast to the classical pathway. These results revealed that an alternative pathway for the formation of MK was operating, at least in *S. coelicolor* A3(2).

3.3.2 Genes that participate in the alternative pathway. A bioinformatic strategy was employed to search for genes participating in the alternative pathway.[103] To date, total genome sequencing of over 900 microorganisms have been completed. Among them, *H. pylori*,[100] *C. jejuni*,[101] *Thermus thermophilus*,[104] and *S. coelicolor*[83] may possess the alternative pathway. In contrast, *E. coli*,[105] *B. subtilis*,[106] *Corynebacterium glutamicum*,[107] and *Mycobacterium tuberculosis*[108] use the classical pathway. First, common orthologues in each of the groups were investigated by reciprocal best-hit pairs using the blastp program. Next, candidate genes specifically present in the former group, but absent in the latter group, were selected. Consequently, four genes, *SCO4326*, *SCO4327*, *SCO4506*, and *SCO4550* in *S. coelicolor*, were selected as the final candidates, all of which were annotated as hypothetical proteins.

To examine whether the candidate genes indeed participated in the alternative pathway, these genes were disrupted by homologous recombination.[103] Each of the candidate genes was replaced with an antibiotic resistance gene using double-crossover homologous recombination. The disruptants were obtained only when selected on agar plates containing MK, showing that the four genes were essential for the biosynthesis of **19**.

Besides the bioinformatics approach, an attempt to isolate **19** auxotrophicmutants of *S. coelicolor*, followed bya shotgun cloning experiment with these mutants as hosts was also employed.[103] Mutants that required MK for growth were obtained using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagenesis. Most of the mutants were complemented by plasmids carrying the genes identified in the bioinformatic screening. However, a few mutants were not complemented and these were complemented by a plasmid carrying the *SCO1494*, *SCO1495*, and *SCO1496* genes, which encode 3-dehydroquinate synthase (aroB), shikimate kinase I (aroK), and chorismate synthase (aroF), respectively. The mutant was also shown to grow in the presence of **20**, suggesting that it lacked a functional chorismate synthase. Since the biosynthetic reaction catalysed by chorismate synthase is irreversible,[109] the alternative pathway appeared to be branched at **20** in a similar manner to the known pathway before diverging.

3.3.3 Intermediates in the alternative pathway[103]. The *SCO4326*-, *SCO4327*-, *SCO4506*-, and *SCO4550*-disruptants perhaps accumulated intermediates because each of the specific biosynthetic steps was disrupted in these mutants. An assay method was therefore developed in which the mutant was cultivated in the presence of MK. After the removal of MK by solvent extraction, the concentrated aqueous layer was added to an agar plate, and the growth of the other mutants on this medium was examined. Consequently, at least one disruptant was able to grow on the plates containing the extracts from the other disruptants. The extract from the *SCO4506*-disruptant did not confer growth to any other disruptants, suggesting that the *SCO4506* product may participate in the most upstream step in the pathway. Based on these results, the relative blocked points of the mutants in **19** biosynthesis were estimated to be in the following order: *SCO4506*, *SCO4327*, *SCO4550*, and *SCO4326*.

An intermediate that accumulated in the culture broth of the *SCO4327*-disruptant was purified and its structure was confirmed to be futalosine (**31**) (Scheme 3), which had previously been isolated from the culture broth of a *Streptomyces* strain.[110] Recently, aminodeoxyfutalosine (**32**), which has an adenine (**33**) residue instead of hypoxanthine (**34**) in futalosine, was characterized as an alternative biosynthetic intermediate in *Helicobacter pylori* (see 3.3.4).[111]

To identify the intermediate after futalosine, recombinant SCO4327 (MqnB) was incubated with futalosine and two reaction products were identified. One was confirmed to be hypoxanthine (**34**) and the other was dehypoxanthinyl futalosine (DHFL, **35**). Recombinant TTHA0556, which is an orthologue of MqnB in *T. thermophilus* HB8, also showed the same activity as that of SCO4327. Very interestingly, the *H. pylori* MqnB orthologue converted only **32** into **35** (see 3.3.4).[111]

The reaction after SCO4327 was judged to be catalysed by SCO4550, as described above. Hence an *in vitro* enzyme assay with the recombinant enzyme was performed. Although both recombinant SCO4550 and recombinant TTHA1092, an orthologue of *SCO4550* in *T. thermophilus* HB8, were incubated with **35** under various conditions, no specific products were detected by HPLC analysis. Then, the intermediate was purified from the culture broth of the *SCO4326*-disruptant, because the reaction product generated by SCO4550 would be the same as the intermediate accumulated in the culture broth of the *SCO4326*-disruptant. The intermediate was purified and its structure was determined to be cyclic DHFL (**36**), as shown in Scheme 3.[103]

Next, recombinant SCO4326 (MqnD) was incubated with **36**. A specific product was detected by HPLC analysis, and its structure was determined to be 1,4-dihydroxy 6-naphthoic acid (**37**). Although the structure of this compound is very similar to **27**, an intermediate of the classical pathway, the carbon to which the carboxyl group was attached is different.[102,103] Recently, the

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structural analysis of TTHA1568 (an orthologue of MqnD in *T. thermophilus* HB8) without the substrate has been reported.[112]

3.3.4 Biosynthesis in the early steps (MqnA). As described above, the alternative pathway appeared to branch at **20**, and the SCO4506 enzyme participated in the most upstream step. Based on the structures of **31** and **32**, the nucleoside moiety would be derived from inosine (**38**) or adenosine (**39**) derivatives. Furthermore, a C2 unit, such as **12** or **29**, was previously implicated as a building block between **20** and **38/39** (C6′ and C7′ positions of **31** and **32**) by tracer experiments. Recombinant SCO4506 was incubated with **20**, **12/29**, and **38/39** in the presence of various co-factors. However, no formation of **31** and **32** was detected by HPLC analysis, but 3-(1-carboxyvinyloxy)benzoate and *m*-hydroxybenzoate were formed from **20** in the absence and presence of flavin-mononucleotide (FMN), respectively.[103] Unidentified enzyme(s) might be essential for the formation of **31** and **32**.

Very recently, three routes to the formation of **35** in the futalosine pathway were suggested (Scheme 3).[111] **31** may have been directly formed by MqnA in *T. thermophilus* and converted into **35**. In *Acidothermus cellulolyticus*, *S. coelicolor*, and *H. pylori*, **32** was formed by MqnA. In the case of the former two strains, **32** was converted to **31** by deaminases, then to **35**. In contrast, **32** was directly converted to **35** in *H. pylori*.

3.3.5 Distribution of the futalosine pathway. Judging from the genome databases, the futalosine pathway would be distributed at least in the following microorganisms: Epsilon category (*Helicobacter*, *Wolinella*, *Thiomicrospira*, *Campylobacter*, *Arcobacter*, *Nitratiruptor*, *Sulfurovum*); Delta category (*Geobacter*, *Pelobacter*, *Desulfovibrio*, *Desulfococcus*, *Anaeromyxobacter*, *Syntrophobacter*); Acidobacteria (*Acidobacteria*, *Solibacter*); Synergistetes (*Syntrophomonas*); Bacillales of Firmicutes (*Bacillus halodurans Bacillus clausii*); Lactobacillus category of Firmicutes (*Symbiobacterium*); Clostridia category of Firmicutes (*Carboxydothermus*, *Desulfotomaculum*, *Pelotomaculum*, *Desulforudis*, *Heliobacterium*, *Moorella*); Actinobacteria (*Streptomyces*, *Frankia*, *Acidothermus*, *Salinispora*); Planctomyces (*Rhodopirellula*); Chlamydia (*Chlamydia*, *Chlamydophila*); Spirochete (*Leptospira*); Green nonsulfur bacteria (*Herpetosiphon*); Deinococcus-Thermus (Gram positive) (*Deinococcus*, *Thermus*); Hyperthermophilic bacteria (*Aquifex*); Euryarchaeota (*Archaeoglobus*, *Thermoplasma*); Crenarchaeota (*Ignicoccus*, *Pyrobaculum*, *Caldivirga*, *Thermoproteus*, *Nitrosopumilus*).

As described above, *E. coli* possess both the classical MK pathway and ubiquinone pathway. However, there are no microorganisms equipped with both the futalosine MK pathway and ubiqionone pathway. Moreover, no microorganism possesses both the futalosine pathway and the classical pathway to MK.

3.3.6 Inhibitors of the futalosine pathway. Since humans and some useful intestinal bacteria, such as lactobacilli, possess the classical pathway, and **19** biosynthesis is essential for survival of microorganisms,[103] the futalosine pathway is an attractive target for the development of specific anti-*H. pylori* drugs. Especially, MqnB in *H. pylori*, which specifically uses **32** as a substrate, is an attractive antibiotic target. Such inhibitors were screened for in metabolites produced by actinomycetes and fungi, and 12-methyltetradecanoic acid and 13-methyltetradecanoic acid (**40**) were shown to inhibit the futalosine pathway with a minimum inhibitory concentration of 32 μg mL^{-1}.[113] Their inhibition mechanism is presently not known.

Enzymatic properties of recombinant MqnB have also been investigated. **34** showed an inhibitory effect with a calculated K_i value of 1.1 mM, suggesting that compounds, such as the derivatives of **34** may have selective *H. pylori* antibiotic activity.[114]

3.4 Summary

As described, two successive biosynthetic steps in the classical pathway to MK were recently revised. However, it remains unclear whether the conversion of **26** into **27** is enzyme-driven or a purely chemical process. Recently, an enzyme catalysing this reaction was identified in cyanobacteria, which utilizes phylloquinone, a structurally related compound to MK,[115] suggesting that this step might be catalysed by such types of enzymes in all microorganisms possessing the classical pathway.

As for the futalosine pathway, an outline was successfully clarified. However, details of the first reaction catalysed by MqnA involving the conversion of **20** into **31** or **32** are presently unknown. An *in vitro* study of MqnC, which catalyses the conversion of **35** into **36**, is also essential. In the case of MqnD, the structure of a C_3 product, a counterpart product of **37** from **36**, has to be determined.

4 Lysine biosynthesis

4.1 Introduction

L-Lysine (**41**) is a proteinogenic amino acid required for protein synthesis, and is important as an essential amino acid for mammals, which lack lysine synthetic enzymes. L-Lysine synthesis is unique because two different pathways have evolved.[116] In bacteria and plants, **41** is synthesised *via* the diaminopimelate (DAP) pathway starting from L-aspartate (**42**) and ATP. This pathway is necessary for most bacteria because it provides DAP that serves as a component of cell wall peptidoglycan.[117] In lower eukaryotes belonging to Ascomycetes and Basidiomycetes, L-lysine is synthesised from 2-oxoglutarate (**43**) and acetyl-CoA through L-α-aminoadipate (**44**, L-2-aminoadipate, AAA) as a biosynthetic intermediate.[118] Between the two pathways there are no common biosynthetic intermediates. In addition to fungal L-lysine biosynthesis through AAA, a variant L-lysine biosynthesis through AAA was first found in *T. thermophilus*[119] and is now known to be widely distributed in microorganisms.[120] In this section, we introduce the classical DAP pathway briefly, and focus on the AAA pathway, especially the AAA pathway recently discovered in prokaryotes.

4.2 Classical DAP pathway

The DAP pathway starts by the phosphorylation of **42** using ATP by aspartate kinase (Scheme 4), which is regulated through feedback inhibition by end products.[121] L-4-Aspartyl phosphate (**45**) formed by the first reaction is reduced to L-aspartate 4-semialdehyde (**46**) by aspartate semialdehyde dehydrogenase.

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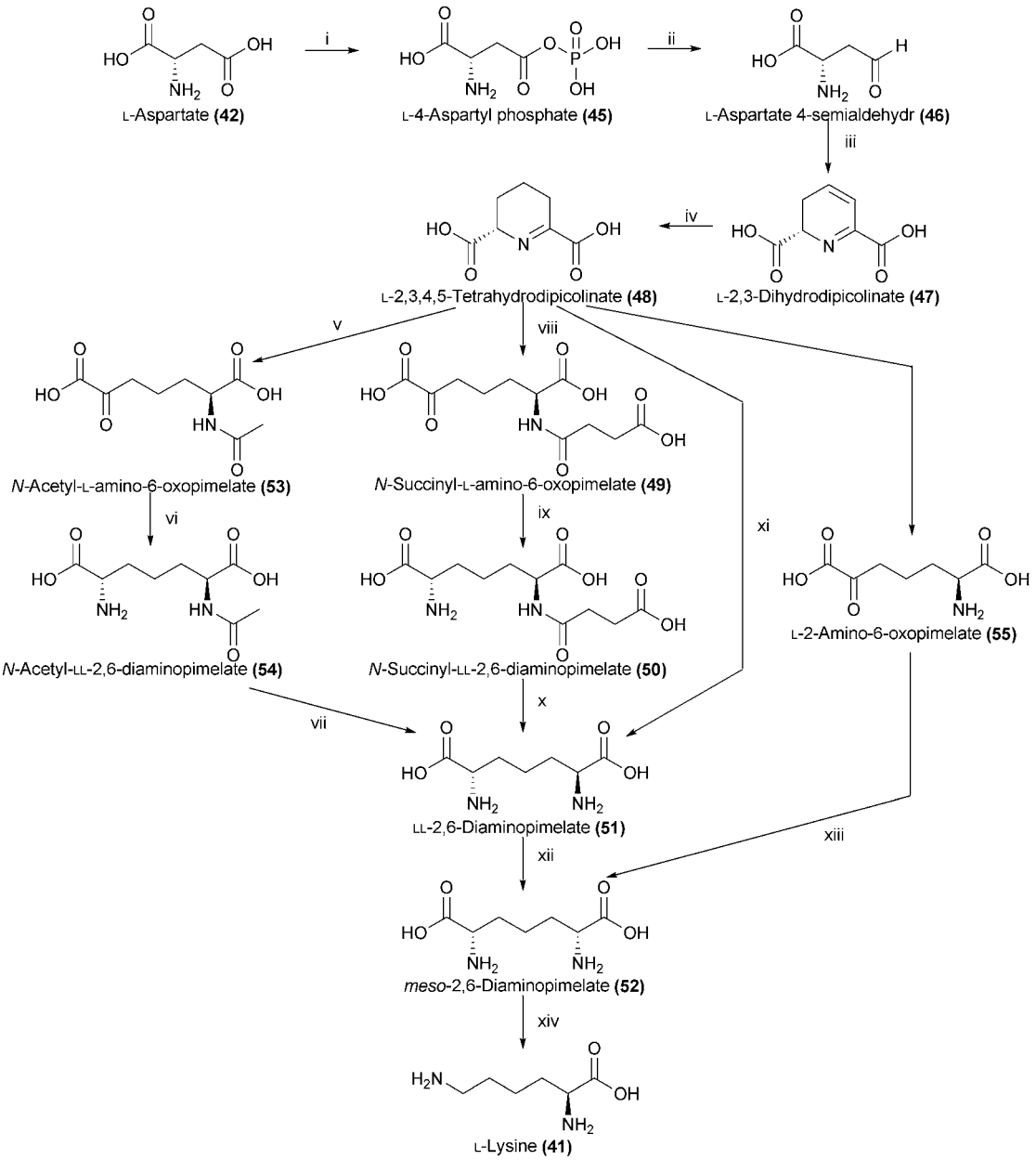
This is a branching point toward L-threonine and L-methionine biosynthesis because **46** is also used for L-threonine and L-methionine biosynthesis. **46** is converted to L-2,3-dihydrodipicolinate (**47**) by dihydrodipicolinate synthase and successively to L-2,3,4,5-tetrahydrodipicolinate (**48**) by dihydrodipicolinate reductase. Four variants of L-lysine biosynthesis through DAP are known. All the pathways proceed in the same way until L-2,3,4,5-tetrahydrodipicolinate synthesis. The first pathway to have been discovered uses *N*-succinylated intermediates, and this variant is the most widely distributed. In the succinylated pathway, **48** is succinylated by tetrahydrodipicolinate succinyltransferase to form *N*-succinyl-L-2-amino-6-oxopimelate (**49**).[122,123] The 2-oxo group of **49** is converted to an amino group by succinyldiaminopimelate aminotransferase to form *N*-succinyl-LL-2,6-diaminopimelate (**50**). The *N*-succinyl group of **50** is removed by succinyl-diaminopimelate desuccinylase to form LL-2,6-diaminopimelate (**51**). **51** is converted to *meso*-2,6-diaminopimelate (**52**) by diaminopimelate isomerase. In the last step, **52** is decarboxylated to form **41** by diaminopimelate decarboxylase. Thus, in most cases **41** is synthesised from L-aspartate in nine steps. The second pathway proceeds in a manner mechanistically similar to the succinylated variant pathway, but differs in that the pathway uses an acetyl group and not a succinyl group for the amino group modification of **48** through *N*-acetyl-L-2-amino-6-oxopimelate (**53**) and *N*-aetyl-LL-2,6-diaminopimelate (**54**).[124] This



Scheme 4 The DAP pathway. Aspartate kinase; ii, Aspartate semialdehyde dehydrogenase; iii, Dihydrodipicolinate synthase; iv, Dihydrodipicolinate reductase; v, Tetrahydrodipicolinate acetyltransferase; vi, Acetyldiaminopimelate aminotransferase; vii, Acetyl-diaminopimelate deacetylase; viii, Tetrahydrodipicolinate succinyltransferase; ix, Succinyldiaminopimelate aminotransferase; x, Succinyl-diaminopimelate desuccinylase; xi, LL-diaminopimelate aminotransferase; xii, Diaminopimelate epimerase; xiii, Diaminopimelate dehydrogenase; xiv, Diaminopimelate decarboxylase.

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pathway is limited to *Bacillus* species. In the third pathway, diaminopimelate dehydrogenase directly converts **48**, actually L-2-amino-6-oxopimelate (**55**) that is formed by natural decyclisation of **48**, to **52** using NADPH and ammonia.[125,126] This variant pathway bypasses the use of acyl intermediates and the epimerase reaction, and converts **52** to **41** by diaminopimelate decarboxylase in the last step. This pathway has only been found in some *Pseudomonas*, *Bacilli*, and *Corynebacteria*. Recently, a novel variant of the DAP pathway was found in *Arabidopsis thaliana* and cyanobacteria, where **48** is converted to **51** by LL-diaminopimelate aminotransferase.[127] In this variant pathway, **51** is converted to **41** through **52** in the same way as in the succinylated pathway.

4.3 AAA pathway

4.3.1 Fungal pathway. In fungi belonging to Ascomycetes and Basidiomycetes, **41** is synthesised in a pathway totally different from the DAP pathway. The fungal L-lysine biosynthetic pathway starts by the synthesis of homocitrate (**56**) from **43** and acetyl-CoA by homocitrate synthase (Scheme 5).[118] The reaction catalysed by homocitrate synthase is the first committed step of the fungal L-lysine biosynthetic pathway. **41** is known to inhibit homocitrate synthase.[128] *Candida utilis*, *Schizosaccharomyces pombe*, *Neurospora crassa*, and *Penicillium chrysogenum* carry a single gene encoding homocitrate synthase, while *S. cerevisiae* possesses two isozymes, both of which are localised in the nucleus.[129] Recently, crystal structures were determined for the apo- and L-lysine-bound forms of *S. pombe* homocitrate synthase.[130,131] Homocitrate synthase is a homologue of 2-isopropylmalate synthase used in L-leucine biosynthesis. Homocitrate is then converted to homoisocitrate (**57**). The reaction is analogous to that catalysed by aconitase in the tricarboxylic acid cycle. The aconitase reaction is divided into two steps: first, citrate is dehydrated to *cis*-aconitate, and then *cis*-aconitate is hydrated to isocitrate. By analogy, homoaconitase is thought to conduct two sequential reactions, dehydration and hydration, with *cis*-homoaconitate (**58**) as an intermediate, although the first reaction has not been demonstrated experimentally. Homoaconitase, encoded by *LYS4* in *S. cerevisiae*, belongs to the aconitase superfamily that includes isopropylmalate isomerase involved in L-leucine biosynthesis, which contains a Fe_4–S_4 cluster for catalysis.[132] In the following reaction, **57** is converted to 2-oxoadipate (**59**) accompanied by oxidative decarboxylation, which is catalysed by homoisocitrate dehydrogenase encoded by *LYS12* in *S. cerevisiae.* Lys12 is a paralogue of 3-isopropylmalate dehydrogenase in L-leucine biosynthesis and of isocitrate synthase in the tricarboxylic acid cycle. The 2-oxo group of **59** is converted to an amino group to form **44**. L-α-Aminoadipate aminotransferase activity has been identified in *S. cerevisiae* and *Torulopsis utilis*.[133,134] In *S. cerevisiae*, two isozymes were isolated.[135,136] Isozyme I is localised in the mitochondria, while isozyme II is localised in cytosol. A kynurenine aminotransferase isolated from *Hansenula schneggii* exhibited L-α-aminoadipate aminotransferase activity.[137] Aromatic aminotransferases exhibited activity not only against aromatic amino acids but also against **44** and branched chain amino acids in *S. cerevisiae*.[138,139] Such aminotransferases with broad substrate specificity might conduct multiple functions including this reaction. Consistent with this, an L-α-aminoadipate aminotransferase gene solely involved in L-lysine biosynthesis has not yet been isolated, even in *S. cerevisiae*.

44 is converted to saccharopine through adenylation and subsequent reduction to form L-α-aminoadipate 6-semialdehyde (**60**).[140] The reaction is catalysed by L-α-aminoadipate reductase (L-α-aminoadipate 6-semialdehyde dehydrogenase). L-α-Aminoadipate reductase is a heterodimer composed of Lys2 and Lys5 in *S. cerevisiae*. Lys2 is a multidomain protein containing an adenylation domain, a peptidyl carrier protein domain, with a 4-phosphopantetheine-binding consensus sequence and an active site serine residue, and reductase domain. The reaction starts by the phosphopantetheinylation of the serine of Lys2 by Lys5 using coenzyme. The activated reductase adenylates the 6-carboxyl group of **44** to form 5-adenylyl-L-α-aminoadipate (**61**) in the adenylation domain, followed by an acyl transfer of the amino adipoyl moiety to the thiol group of the phosphopantetheine. The thioester intermediate is reduced by NADH to generate reduced phosphopantetheine and **60** in the reductase domain. In the final step, the reduction of the thioester yields a thiohemiacetal intermediate that decomposes to release the semialdehyde. The L-α-aminoadipate reductase system is similar to the nonribosomal peptide synthetases.[141] **60** formed by AAA reductase is condensed with L-glutamate in the presence of NADH to yield L-saccharopine (**62**). Saccharopine reductase [saccharopine dehydrogenase ($NADP^+$, L-glutamate forming)] that catalyses this reaction is encoded by *LYS9* in *S. cerevisiae*. The final step of the fungal L-lysine biosynthetic pathway is the cleavage of **62** into **43** and **44**. This reaction is catalysed in the presence of NAD^+ by saccharopine dehydrogenase (L-lysine forming), which is encoded by *LYS1* in *S. cerevisiae*. Lys1 from *S. cerevisiae* exhibits strict substrate specificity.[142,143] For more details on L-lysine biosynthesis and metabolism in fungi, see these well-written reviews.[132,144]

4.3.2 Variant AAA pathway found in thermophilic bacteria

4.3.2.1 Discovery. It was believed that all bacteria synthesise **41** through the DAP pathway. An extremely thermophilic bacterium, *Thermus thermophilus*, possesses a single aspartate kinase that is regulated by L-threonine.[145] In general, there are two types of bacteria: one carrying several aspartate kinases, each of which is regulated in a different way by **41** and L-threonine, and the other having a single aspartate kinase that is regulated by both **41** and L-threonine. Thus, roughly speaking, total cellular aspartate kinase activity is controlled by concentrations of both **41** and L-threonine. The above observation for the *T. thermophilus* aspartate kinase suggested the possibility that this bacterium biosynthesises **41** in a pathway different to the DAP pathway. To this end, Kobashi and co-workers isolated a DNA fragment that complemented the growth of a *T. thermophilus* mutant with a L-lysine-auxotrophic phenotype.[146] DNA sequencing revealed that the DNA fragment carried open reading frames (ORFs) homologous to genes encoding putative large and small subunits of homoaconitase. Furthermore, a knockout mutant of the chromosomal copy for the large subunit of homoaconitase exhibited an L-lysine-auxotrophic phenotype, which was restored by the addition of **44**. These observations revealed that this bacterium biosynthesises **41** through **44**. L-Lysine biosynthesis through **44** was also found in the same bacterium by Kosuge and co-workers.[147]

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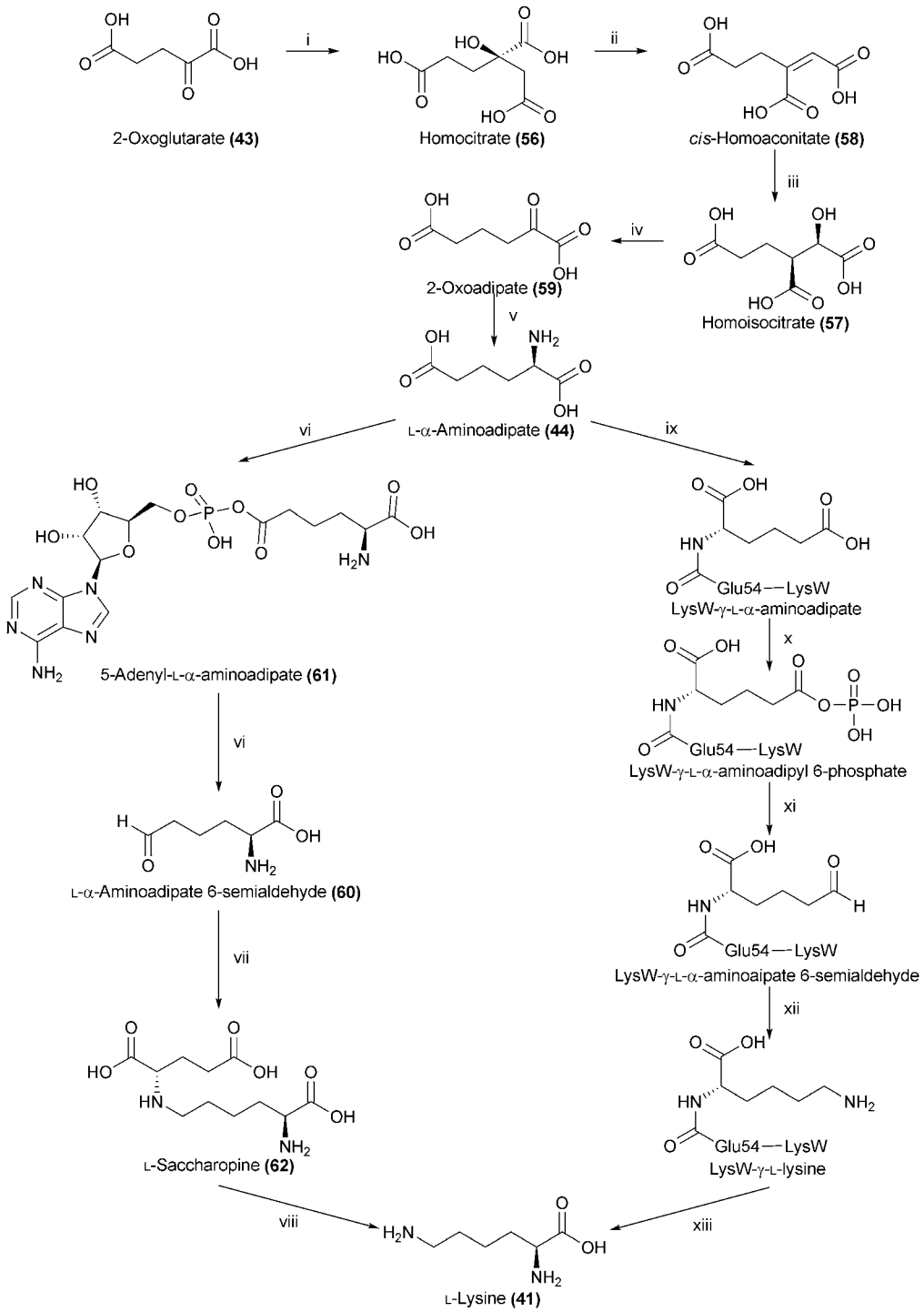

Scheme 5 The AAA pathway.i, Homocitrate synthase; ii, Homoaconitase, aconitase; iii, Homoaconitase; iv, Homoisocitrate dehydrogenase; v, α-Aminoadipate aminotransferase; vi, α-Aminoadipate semialdehyde dehydrogenase; vii, Saccharopine reductase [Saccharopine dehydrogenase (NADP+, L-glutamate forming)]; viii, Saccharopine dehydrogenase (L-lysine forming); ix, LysX; x, LysZ; xi, LysY; xii, LysJ; xiii, LysK.

4.3.2.2 The first half of L-lysine biosynthesis (from 2-oxoglutarate to AAA). The first half of the AAA pathway found in *T. thermophilus* proceeds mostly in a manner similar to that in the fungal AAA pathway described above.[119] The pathway starts with the synthesis of **56** from **43** and acetyl-CoA by homocitrate synthase, which is then converted to **57**. **57** is oxidised to **59** accompanied by decarboxylation, which is catalysed by homoisocitrate dehydrogenase. The 2-oxo group of **59** is converted to an amino group to form **44**.

The first enzyme in the bacterial L-lysine biosynthetic pathway through **44** is homocitrate synthase. A gene encoding a putative homocitrate synthase gene is present upstream of the genes encoding large and small subunits of homoaconitase. A knockout of the putative homocitrate synthase gene caused

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T. thermophilus cells to exhibit an L-lysine-auxotrophic phenotype that was restored by the addition of **44**, suggesting that the gene encodes homocitrate synthase. The activity measurements of the recombinant protein verified that the gene encodes homocitrate synthase in this bacterium. L-Lysine biosynthesis through **44** is controlled by feedback inhibition of homocitrate synthase through **41**. Two models have been proposed for the inhibitory mechanism of **41**. Biochemical studies on homocitrate synthases from *T. thermophilus* and *P. chrysogenum* suggest that **41** is a competitive inhibitor of **43**, indicating that **41** is bound to the active site of homocitrate synthase.[148,149] The other proposed mechanism is the allosteric model, hypothesising that **41** is bound to an effector-binding site and not the active site.[128,150,151] Crystal structures have been determined for homocitrate synthase from *T. thermophilus* in three forms: a **43**-bound form, a **56**-bound form, and a **41**-bound form.[152] Homocitrate synthase from *T. thermophilus* is a homodimer, each forming a TIM barrel structure with a C-terminal extension. These crystal graphic analyses have revealed, not only the substrate binding residues, but also the catalytic mechanism, including the role of a His residue in the C-terminal extension from another subunit of the dimer in the proton abstraction from the reactive methyl group of acetyl-CoA. Based on the structures, it was also elucidated that **41** directly competes with **43** for binding to the active site. Crystal structures of homocitrate synthase from *S. pombe* in an apo-form, a substrate-bound form, and a **41**-bound form have been determined, and lead to the same conclusion of competitive inhibition by **41**.[130,131] Homocitrate synthase from *T. thermophilus* recognises **41** by changing the side chain orientations of the amino acid residues around the active site without causing conformational change of the TIM barrel structure. Binding of **41** to the active site causes a gross conformational change in the C-terminal region that carries the catalytic His residue (Fig. 4).

Microorganisms that synthesise **41** through **44** contain homoaconitase. By analogy to the reaction by aconitase, a homologue of homoaconitase, homoaconitase is thought to catalyse the conversion of **56** to **57** *via* **58** by two sub-reactions: first, **56** is dehydrated to **58**, and then **58** is hydrated to **57**. Jia and co-workers characterised a putative homoaconitase from *T. thermophilus* and detected it was able to reversibly convert **58** to **57**; however, it was not able to convert **56** to **58** and vice versa.[153] Interestingly, they found that porcine aconitase had the ability to reversibly convert **56** to **58**. They also revealed that a coupled assay containing homocitrate synthase, homoaconitase, and homoisocitrate dehydrogenase from *T. thermophilus*, and porcine aconitase caused the reduction of NAD^+, but no reduction was observed in the absence of homoaconitase or aconitase. While porcine aconitase catalyses the reversible conversion of **56** to **58**, it cannot convert **58** to **57**. These results suggest that the dehydration of **56** to **58** is catalysed by aconitase present in *T. thermophilus* cells. Aconitase from *T. thermophilus* has not yet been characterised. The involvement of aconitase in both L-lysine biosynthesis and the tricarboxylic acid cycle in *T. thermophilus* remains to be solved experimentally. Similar to *T. thermophilus* homoaconitase, the enzymes from *S. cerevisiae* and *Aspergillus nidulans* catalyse only the second half of the reaction to isomerise **56**.[154,155] To date, homoaconitase from *Methanocaldococcus jannaschii*, which is also involved in the full isomerisations of $homo_2$-citrate and $homo_3$-citrate in the chain elongations of coenzyme B synthesis,[156] is only the enzyme known to catalyse the full conversion of **56** to **57**.[157]

Homoisocitrate dehydrogenase, which converts **57** to **59** using oxidative decarboxylation, is a paralogue of 3-isopropylmalate dehydrogenase, which is involved in L-leucine biosynthesis, and isocitrate dehydrogenase, a member of the tricarboxylic acid cycle. One of the paralogues, which shows the highest similarity to the homoisocitrate dehydrogenase gene from *S. cerevisiae* and the putative homoisocitrate dehydrogenase gene from *Deinococcus radiodurans*, was cloned from *T. thermophilus*. An auxotrophic test of a knockout mutant of the paralogue, and its enzymatic characterisation, revealed that the cloned gene encodes a homoisocitrate dehydrogenase.[158] Homoisocitrate dehydrogenase from *T. thermophilus* is a promiscuous enzyme that recognises isocitrate as a substrate better than homoisocitrate, which contrasts with homoisocitrate dehydrogenase from *S. cerevisiae*, which is specific to homoisocitrate. *T. thermophilus* homoisocitrate dehydrogenase was easily changed to an enzyme specific to **57** by the exchange of only seven amino acid residues, without any loss of specific activity and stability, suggesting that the promiscuity is maintained in this enzyme for some reason. Replacement of an Arg residue caused the homoisocitrate dehydrogenase to exhibit 3-isopropylmalate dehydrogenase activity, which was further enhanced by eight other amino acid replacements, among which five replacements are found far from the catalytic residues, by means of directed evolution.[159] The crystal structure of homoisocitrate dehydrogenase from *T. thermophilus* revealed that the tetramer formation was one of the reasons for the elevated thermal stability of this enzyme.[160]

The last reaction in the first half of L-lysine biosynthesis through AAA is catalysed by L-α-aminoadipate aminotransferase. As described above, a L-α-aminoadipate aminotransferase that is solely involved in lysine biosynthesis has not yet been isolated, even from *S. cerevisiae*. Rat kynurenine aminotransferase II (KAT II), which is involved in the metabolism of tryptophan, has been shown to exhibit L-α-aminoadipate aminotransferase activity. Miyazaki and co-workers cloned the gene homologous to KAT II gene from *T. thermophilus* and showed that the gene product actually exhibited L-α-aminoadipate aminotransferase activity.[161] A knockout mutant of the gene exhibited a leaky L-lysine-auxotrophic phenotype: very slow growth in minimal medium, which was accelerated by the addition of **44** or **41**. These observations suggest that the rat kynurenine aminotransferase II homologue makes a major contribution to conversion of **59** to **44** in L-lysine biosynthesis and an unknown enzyme, or enzymes, supports the conversion of **59** to **44**. Interestingly, the addition of **44** or **41** did not completely restore the growth rate of the knockout mutant of *T. thermophilus* to that of the wild-type strain, suggesting that the aminotransferase might also function in a cellular event, or events, other than L-lysine biosynthesis. In fact, L-α-aminoadipate aminotransferase catalyses transamination of various substrates, including **44**, L-glutamate, L-leucine, and aromatic L-amino acids. The crystal structure of L-α-aminoadipate aminotransferase from *T. thermophilus* has been determined for several forms: with pyridoxal 5′-phosphate (PLP), with PLP and L-leucine, with *N*-phosphopyridoxyl-L-leucine, and with *N*-phosphopyridoxyl-L-α-aminoadipate. Comparison of these structures revealed that L-α-aminoadipate aminotransferase can

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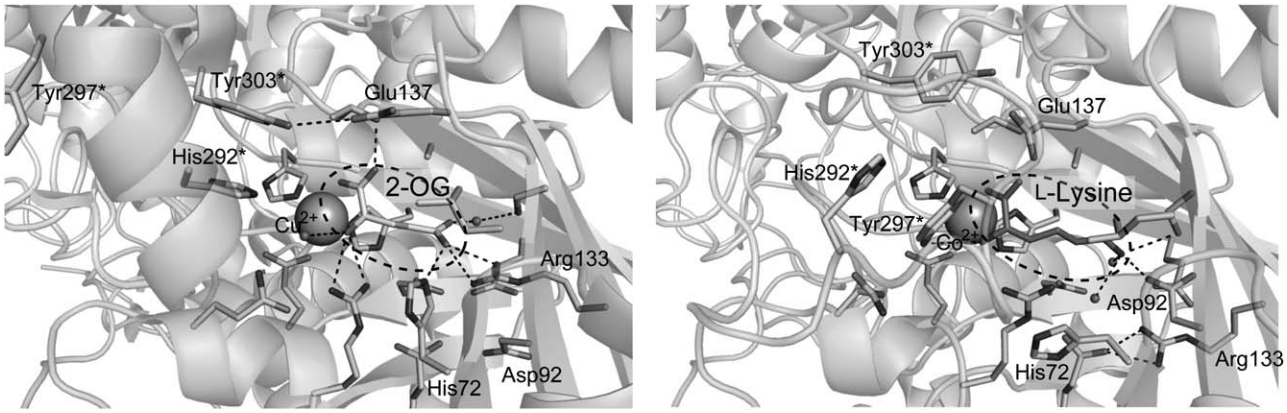

Fig. 4 The conformational change of homocitrate synthase on L-lysine binding. Left, Active site structure of homocitrate synthase binding the substrate, 2-oxoglutarate indicated by 2-OG. Right, Active site structure of homocitrate synthase binding the inhibitor, L -lysine. The α-helix carrying Tyr292* and the catalytic His292* from another subunit is deformed to displace these residues by binding L-lysine. His72, Asp92, and Arg133 change their side chain orientation on L-lysine binding. Bound 2-oxoglutarate and L-lysine are enclosed by dotted oval lines.

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recognise various kinds of substrates using the mobile α2 helix.[162] Comparison of crystal structures of L-α-aminoadipate aminotransferase and KAT II indicates that domain swapping occurs in KAT II but not in L-α-aminoadipate aminotransferase. In L-α-aminoadipate aminotransferase, the α2 helix is oriented to cover the active site of the same subunit; while in Kat II, the corresponding helix interacts with another subunit of the dimer and has the same role in covering the active site cleft.

4.3.2.3 The second half of L-lysine biosynthesis (from AAA to L-lysine). In the downstream region of *lysTU* encoding the large and small subunits of homoaconitase, several ORFs homologous to genes coding for RimK, ArgB, and ArgC are present. RimK from *E. coli* adds two to four glutamate molecules to the C-terminal end of ribosomal protein S6 in *E. coli*. ArgB and ArgC are involved in L-arginine biosynthesis: the former catalyses phosphorylation of *N*-acetyl-L-glutamate to produce *N*-acetyl-L-glutamate-4-phosphate and the latter catalyses reduction of *N*-acetyl-L-glutamate 5-phosphate to form *N*-acetyl-L-glutamate 5-semialdehyde. To elucidate whether the ORFs were involved in L-lysine biosynthesis, chromosomal copies of these ORFs were disrupted by the insertion of a kanamycin nucleotidyl transferase gene and auxotrophy of the mutants was examined. Unexpectedly, all mutants with knockouts of *rimK*, *argB*, and *argC* homologues showed a lysine-auxotrophic phenotype, which was not complemented by the addition of **44**.[119] These results indicated that these genes were involved in the process of conversion of **44** to **41**. A hyperthermophilic archaeon, *Pyrococcus horikoshii*, carries a gene cluster similar to that in *T. thermophilus*. The cluster of *P. horikoshii* contains two additional genes homologous to *argD* and *argE* at the 3′-flanking region. The *argD* gene encodes N^2-acetylornithine 5-aminotransferase, which converts *N*-acetyl-L-glutamate 5-semialdehyde to N^2-acetyl-L-ornithine, and the *argE* gene encodes N^2-acetyl-L-ornithine deacetylase, which converts N^2-acetyl-L-ornithine to L-ornithine, in L-arginine biosynthesis. These observations suggest that *P. horikoshii* biosynthesises **41** by the AAA pathway and the second half of L-lysine synthesis proceeds in a manner similar to that in L-arginine biosynthesis. **44** is a compound structurally similar to L-glutamate, both carrying α-amino and α-carboxyl groups and carboxyl side chains, differing by only a single methylene unit. When **44** is converted by a series of reactions similar to those of L-arginine biosynthesis, the compound with one methylene unit longer than L-ornithine must be produced. This compound is **41**. After finding the novel L-lysine biosynthesis pathway through AAA, genomic sequences have been determined for a lot of microorganisms. Based on the genome sequences, many archaea and thermophilic bacteria are presumed to synthesise **41** through **44** by a pathway similar to that found in *T. thermophilus*. L-Lysine biosynthetic genes homologous to *rimK*, *argB*, and *argC* are named *lysX*, *lysZ*, and *lysY*, respectively. Homologues of *argD* and *argE*, which are involved in L-lysine biosynthesis, are named *lysJ* and *lysK*, respectively.[163,164]

4.3.2.4 Carrier protein-mediated amino group modification of AAA. In L-arginine biosynthesis, the amino group of L-glutamate is protected by an acetyl group by the transacetylation activity of ArgA and/or ArgJ. This protection prevents the amino group from intramolecular cyclisation with the semialdehyde group formed during the biosynthesis, which assures the efficient synthesis of L-ornithine and L-arginine. As described above, the latter part of prokaryotic L-lysine biosynthesis through AAA, the conversion of **44** to **41**, proceeds in a manner similar to the conversion of L-glutamate to L-ornithine during L-arginine biosynthesis. Therefore, it was presumed that the amino group of **44** is protected by some compound before the modification of the **44** side-chain in L-lysine biosynthesis. However, enzymes homologous to ArgA and ArgJ are absent from the *Thermus* L-lysine pathway. Their absence suggests that the pathway includes an alternative amino group-modification system.

LysX is a member of the ATP-dependent carboxylate-amine/thiol ligase superfamily and it has significant amino acid sequence identity (28%) to RimK from *E. coli*. RimK is known to add two to four L-glutamate molecules to the C-terminal glutamate residue of ribosomal protein S6 in *E. coli*. Knockout of the *lysX* gene in *T. thermophilus* resulted in the requirement of lysine for growth, indicating involvement of LysX in the L-lysine biosynthetic pathway of *T. thermophilus*. Based on these observations, it was speculated that LysX used a C-terminal glutamate

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group of a small protein for the amino group of **44**. The only likely candidate for such a small protein was LysW, a 54-amino-acid acidic peptide with a conserved C-terminal sequence, EDWGE, encoded upstream of the *lysX* gene, as the amino acceptor of **44**. All microorganisms that are thought to biosynthesise **41** through a pathway similar to that in *T. thermophilus* also contain the *lysW* homologue in the putative lysine biosynthetic gene cluster. Consistent with this, the *lysW*-knockout mutant shows L-lysine auxotrophy. Horie and co-workers reconstituted the conversion of **44** to **41** *in vitro* and found that LysX modifies the amino group of **44** by attaching it to the γ-carboxyl group of the C-terminal Glu54 of LysW.[120] LysX discriminates **44** from L-glutamate specifically and attaches LysW to the amino group of **44**. The crystal structural analysis revealed that LysX takes an ATP-Grasp fold like glutathione synthetase.[165]

The remaining reactions, starting with the phosphorylation of LysW-fused AAA (LysW-γ-AAA) and ending with the release of **41**, are catalysed by LysZ, LysY, LysJ, and LysK in that order, which are homologues of ArgB, ArgC, ArgD, and ArgE in L-arginine biosynthesis, respectively. In L-lysine biosynthesis, the side chain of **44** is converted to the lysyl side chain while it is still attached to LysW, and **41** is subsequently liberated from the LysW/L-lysine fusion after a series of reactions of these enzymes.

LysW is a small and acidic protein with a calculated pI of 3.7; whereas, all the enzymes involved in the second half of L-lysine biosynthesis possess catalytic residues surrounded by many positively charged residues. The conflicting charge distribution of LysW and biosynthetic enzymes suggest that their interaction is stabilised electrostatically, which was verified experimentally using LysK.[120] The fact that biosynthetic enzymes recognise the acidic globular domain of LysW indicates that LysW acts as a carrier protein, or protein scaffold, for the biosynthetic enzymes.

4.3.3 Promiscuous activities of the L-lysine biosynthetic enzymes. In addition to L-α-aminoadipate aminotransferase and homoisocitrate dehydrogenase, L-lysine biosynthetic enzymes recognise compounds that are not involved in L-lysine biosynthesis. LysJ recognises the δ-amino group of N^2-acetyl-L-ornithine.[163] In L-lysine biosynthesis, LysK catalyses the last step, hydrolysis of LysW-γ-L-lysine to LysW and **41**, but it cannot cleave LysW-α-L-lysine, thus showing γ-linkage specificity.[120] On the other hand, LysK can deacetylate N^2-acetyl-L-ornithine *in vitro*.[164] N^2-Acetyl-L-ornithine is a biosynthetic intermediate in L-arginine biosynthesis. Distinct functions of LysJ and LysK in L-arginine biosynthesis may suggest roles of the enzymes in both L-lysine and L-arginine biosyntheses.

4.4 Summary

As described above, L-lysine biosynthesis in *T. thermophilus* differs from L-lysine biosynthesis through DAP found in most other bacteria. Although L-lysine biosynthesis through AAA was previously reported in *Euglenoid* and *Thermoproteus neutrophilus*,[116,166] it had not yet been analysed in detail. As described above, L-lysine biosynthesis through AAA is now known to be widely present in microorganisms. It should be noted that L-lysine biosynthesis through AAA is widely found in archaea that are clustered close to the root in the phylogenetic tree of life based on 16S ribosomal DNA sequence. It is suggested that the earliest Eukarya originated from permanent whole-cell fusion between a *Thermoplasma*-like organism and a *Spirochaeta*-like organism.[167] Interestingly, *Picrophilus torridus*, a member of *Thermoplasmatales*, possesses genes for L-lysine biosynthesis through AAA[168] and is likely to synthesise **41** through **44** in a manner similar to *T. thermophilus*. In any case, the wide distribution of L-lysine biosynthesis through AAA in archaea suggests that the prokaryotic L-lysine biosynthetic pathway through AAA is an origin of L-lysine biosynthesis.

The evolutionary relationship between fungal AAA L-lysine biosynthesis, the tricarboxylic acid cycle and L-leucine biosynthesis was shown by Irvin and Bhattacharjee.[169] Later, Velasco and co-workers suggested that DAP L-lysine biosynthesis is evolutionarily related to L-arginine biosynthesis.[170] Recently, Fondi and co-workers analyzed the evolutionary interconnection between L-leucine, L-arginine, and L-lysine biosynthesis, and suggested that primordial cells synthesised L-leucine, L-arginine, and **41** through a single ancestral and common metabolic pathway that was composed of enzymes able to react with a wide range of substrates,[171] according to the patchwork hypothesis raised by Jensen.[172] In addition, the crystal structure of saccharopine reductase from *Magnaprthe grisea* revealed that the fungal AAA L-lysine biosynthetic enzyme contains structural elements similar to those of diaminopimelate dehydrogenase, dihydrodipicolinate reductase, and aspartate semialdehyde dehydrogenase.[173] The structural similarity may indicate an evolutionary relationship between these enzymes, and suggest these enzymes might have evolved from a common ancestor. Thus, L-lysine, L-arginine, and L-leucine biosynthesis provides an interesting study-model for the evolution of metabolic pathways.

5 Convergent strategies in aromatic polyketide biosynthesis

5.1 Introduction

Aromatic polyketides constitute a large group of secondary metabolites widely distributed in bacteria, fungi, lichen, and higher plants with remarkable structural diversity and biological activities. Biosynthetically, basic carbon skeletons of aromatic polyketides, substituted polyphenols, are constructed from acetate building units, such as acyl-CoA and malonyl-CoA, by successive Claisen condensation and cyclisation/aromatisation catalysed by polyketide synthases (PKSs).

PKSs, the key enzymes responsible for the carbon skeleton construction of all polyketides, are classified into three main types, type I, II, and III, based on their architectures.[174,175] Type I PKSs are very large multifunctional proteins with individual catalytic domains, including at least ketoacyl synthase (KS), acyl transferase (AT) and acyl carrier protein (ACP), as well as ketoreductase (KR), dehydratase (DH), enoyl reductase (ER) and thioesterase (TE).[176] Type II PKSs, which are common among actinomycete bacteria, are dissociable multi-enzyme complex systems composed largely of mono-functional proteins targeting aromatic polyketides. Each PKS contains at least KSα and KSβ (also referred as the chain-length factor) and ACP, as well as additional proteins including KR, cyclase, aromatase and

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oxygenase for modification and/or tailoring reactions.[177] In contrast to these multi-functional systems, type III PKSs are homodimeric proteins with condensing enzymes (KS enzyme) only. Type III PKSs catalyse the iterative condensation of acetate units derived from malonyl-CoA to a CoA-linked starter molecule. After chain extension, the polyketide intermediate is cyclised in the active site cavity. Plant enzymes, chalcone synthases (CHSs) and stilbene synthases (STSs), are the main members of this class of enzymes.[178] However, type III PKSs have recently been discovered in bacteria and fungi.[179]

Type I PKSs are further classified into 1) modular type I, and 2) iterative type I. Modular type I PKSs are responsible mainly for the biosynthesis of bacterial macrocyclic polyketides. On the other hand, iterative type I PKSs (iPKSs) are the single modular-type found in fungi and some bacteria. These are further subdivided into: 1) non-reducing iPKSs for aromatic polyketides (NR-PKSs); 2) partially reducing iPKSs for 6-methylsalicylic acid (**63**) (PR-PKS); and 3) highly reducing iPKSs for aliphatic polyketides (HR-PKSs).[180] However, recent phylogenetic analysis indicated some PR-PKSs, such as MSAS (PKS for **63**), originated from bacterial iPKS by horizontal gene transfer.[181,182]

Aromatic polyketides are classified based on their structures, that is, benzenoids (single aromatic ring polyketides), naphthalenoids (fused aromatic bicyclic polyketides), and anthraquinonoids (fused tricyclic aromatic polyketides), although there are lots of aromatic polyketides other than those classified above in nature. These basic aromatic structures could be formed convergently by divergent polyketide biosynthesis systems. Although recent reviews covered convergent biosynthetic strategies in anthraquinonoid biosynthesis revealed by feeding experiments,[183–185] here we deal with aromatic polyketides for which the information about the PKSs responsible is available on genetic and/or enzyme levels.

63 was the first compound of a polyketide nature that was determined experimentally.[186] Along with orsellinic acid (**64**), these compounds are typical benzenoid aromatic polyketides. MSAS was the first iPKS cloned from the fungus *Penicillium patulum*.[187] As shown in Scheme 6, MSAS catalyses the formation of a tetraketide intermediate and its aldol-type cyclisation, nucleophilic attack from C-2 methylene to C-7 carbonyl carbon. Due to this cyclisation mechanism, **63** is considered to be an aldol cyclisation-type benzenoid. In contrast, cyclisation from the opposite direction, from C-6 methylene nucleophile to C-1 thioester carbonyl carbon, Claisen-type cyclisation, could occur in some other benzenoid formations, such as forming acyl phloroglucinol compounds. Therefore, benzenoid aromatic polyketides could be classified into two types based on this cyclisation mechanism: 1) aldol-type cyclisation PKS products; and 2) Claisen-type cyclisation PKS products. This classification, based on the final cyclisation mechanism, is considered to be applicable to categorise PKSs and their products. Therefore, in this section, we describe the aldol-type cyclization PKS pathways first, and then the Claisen-type PKS pathways, for benzenoids, naphthalenoids, and anthraquinonoids.

5.2 Aldol-type cyclisation PKS pathway

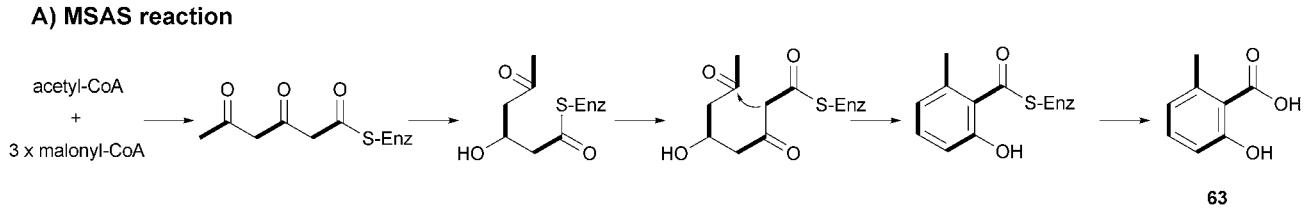
5.2.1 Resorcinol/resorcylic acid-type aldol benzenoids. Aldol-type cyclisation in benzenoid PKS reaction occurs by nucleophilic attack from the C-2 methylene of thioester carbonyl (ACP or CoA) on the C-7 carbonyl of the poly-β-ketoacyl intermediate. Dehydration and aromatisation leads to the formation of a benzene ring. Thus, the aldol-type benzenoid ring is constructed from two intact acetate units and an acyl (starter) C-1 carbonyl carbon and a C-2 methylene carbon of a malonyl extender unit (Scheme 6).

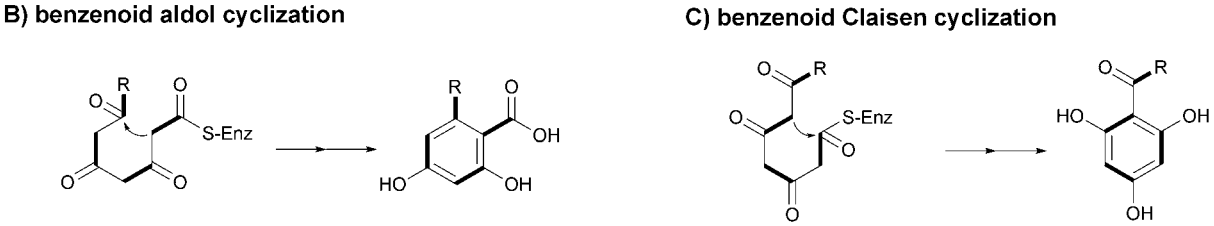
MSAS consists of KS, AT, DH, KR, and ACP domains. The DH domain in MSAS was recently identified to be a thioester hydrolase (TH) responsible for product release.[188] Because of the presence of a KR domain, MSAS has been classified as a PR-PKS.[180] However, PKSs with similar domain architecture were found in actinomycete bacteria. Some of them possess a KR domain for synthesis of **63**, but some lack a KR domain and function to synthesise **64**. Mutation in the KR domain Y1450F in ChlB1 MSAS for chlorothricin biosynthesis converted its function to be a PKS for **64** (OAS).[189] The presence of a DH domain in OAS has long been puzzling. Now, its function is considered to be a thioester hydrolase responsible for product release, as is that in MSAS.[188] Therefore, these MSAS and OAS could be PKSs of the same class, with basically the same architecture regardless of the loss or gain of a KR domain, which was supported by the recent phylogenetic analysis of PKSs indicating that the fungal MSAS was horizontally transferred from bacteria.[181,182] Thus these are classified as bacterial iPKS.

Although MSASs have been found in fungi, the bacterial type OAS gene has not been found in fungal genome database. Instead, NR-PKS genes for OAS were recently identified in fungi,[190,191] of which the domain architecture is SAT–KS–AT–PT–ACP–TE, where SAT is a domain for a starter acyl transferase[192] and PT is a product template domain,[193] as proposed by Townsend and co-workers. Both bacterial and fungal OASs functionally catalyse the same reaction to form an orsellinate thioester on ACPs. However, bacterial OAS uses the TH domain located in the middle of protein, whereas, fungal OAS uses the TE domain at the C-terminus, to release the product. Similarly, a fungal NR-PKS for methylorcinaldehyde (**65**) (MOS) was reported in which the C-terminus TE domain is replaced with a reductive R domain to release the product as an aldehyde (Fig. 5).[194]

Orsellinic acid moieties are also found in the resorcylic acid lactone (RALs) family of fungal polyketides, such as radicicol (**66**), zearalenone (**67**), hypothemycin (**68**) and pochonins (**69**).[195] Recent genetic and biochemical analysis of RAL biosynthesis[196–199] revealed that HR-PKS serves as the aliphatic starter unit synthase, which is transacylated to NR-PKS with the aid of its SAT domain. The addition of three malonyl-CoA molecules and aldol cyclisation leads to the resorcylic acid moiety. The final lactonisation is carried out by the C-terminal TE domain of NR-PKS.[200] Basically, the same RAL biosynthesis strategies have been found in **66**, **67**, and **68** biosynthesis. PKS13, which is involved in biosynthesis of **67**, was expressed in *E. coli* and proved to catalyse three malonyl-CoA elongations primed with versatile fatty acyl-CoAs and its cyclisation/aromatisation to form the resorcylic acid moiety (Fig.6).[197]

In addition to aldol benzenoids produced by type I PKSs, some type III PKSs have been known to catalyse aldol benzenoid biosynthesis. In the genome of *Neurospora crassa*, a single type III PKS gene was found. This gene was expressed in *E. coli* and





Scheme 6 Benzenoid cyclisation.

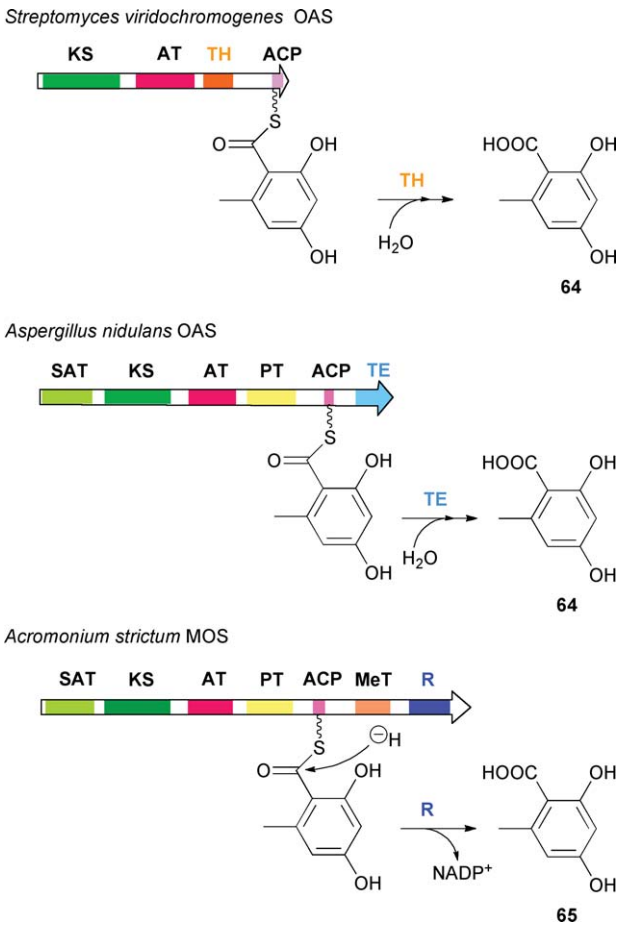


Fig. 5 Product releasing mechanism in NR-PKSs.

its function was characterised to be a 2′-oxoalkylresorcylic acid synthase (ORAS) that forms pentaketide alkylresorcylic acid and prefers stearoyl-CoA as a starter.[201] The *Aspergillus oryzae* genome project revealed the presence of four type III PKS genes named *csyA–D*.[202] Expression of CysA under an α-amylase promoter in a fungal host revealed its main product to be 3,5-dihydroxybenzoic acid, which could be derived from a orsellinic acid derivative and modified by host enzymes. Thus, CsyA is proposed to be a tetraketide alkyl-resorcinol/resorcylic acid synthase (Scheme 7).[203]

Similarly, aldol-type benzenoid biosynthesis by type III PKS was reported in bacteria. In the biosynthesis of 3,5-dihydroxyphenylglycine during glycopeptide antibiotic biosynthesis, a type III PKS was determined to catalyse the condensation of four malonyl-CoAs followed by decarboxylative C-8 to C-3 aldol cyclisation to form 3,5-dihydroxypheylacetyl-CoA (**70**) in *Amycolatopsis* bacteria.[204–206] A type III PKS of the same function was identified in the biosynthesis of kendomycin (Scheme 8).[207]

In contrast to the divergent PKS strategies for the formation of aromatic polyketides in microbes, plants are, to date, known to use only type III PKSs for this purpose. CHSs are well-known type III PKSs involved in flavonoid biosynthesis and STSs are close relatives of CHSs. They use the opposite mechanisms for the cyclisation of benzene rings: STS uses aldol cyclisation by nucleophilic attack from C-2 to C-7 and CHS uses Claisen cyclisation from C-6 to C-1 as described in 5.3.1. The results of crystallographic and mutation analyses show that a subtle difference between CHS and STS, so-called aldol switches, dramatically changes the cyclisation mode of the tetraketide intermediate.[208]

5.2.2 Aldol-type naphthoic acids. Aldol-type cyclisation in naphthalenoid PKS reaction is involved in the formation of the naphthalenoid scaffold by bacterial iPKS naphthoic acid synthase (NPAS). Naphthoate moieties are included in enediyne antibiotics chromophores in neocarzinostatin,[209] kedarcidin,[210] N1999A2,[211] and azinomycins.[212] Some structural modifications, such as *O*-methylation, chlorination and hydroxylation, occur in naphthoate moieties. NPAS was first identified in the gene cluster for neocarzinostatin biosynthesis.[213] Of the two PKS genes in the cluster, *ncsB* codes for bacterial iPKS. Thus it was considered to be a PKS for the naphthoic acid moiety of neocarzinostatin. To identify its function, *ncsB* was placed under a modified version of the *ermE** promoter to construct an expression plasmid, which

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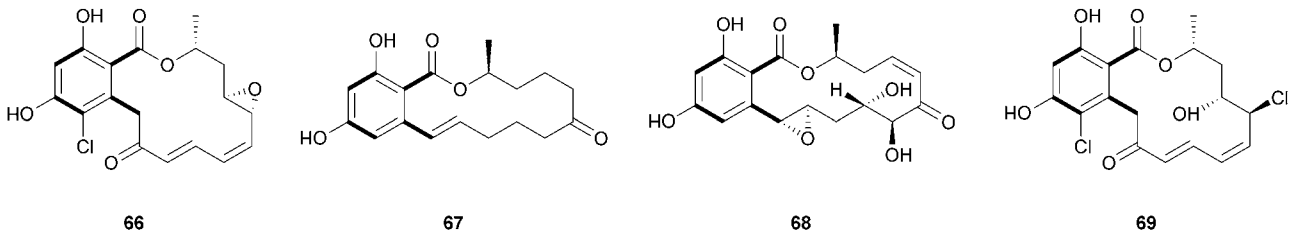

Fig. 6 Resorcylic acid lactone compounds.

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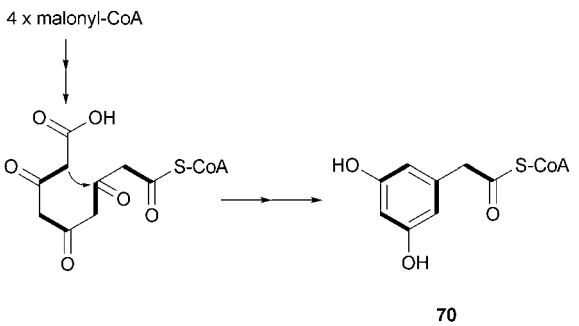
was introduced into the streptomycete host bacterium *Streptomyces lividans*.[209] The transformant produced two naphthoic acid compounds, 2-hydroxy-5-methylnaphthoic acid (**71**) and 2-hydroxy-5-hydroxymethylnaphthoic acid. Production of the latter compound is considered to be due to the oxidation of the 5-methyl group by the host bacterium. Thus, the *ncsB* gene product was named neocarzinostatin naphthoate synthase (NNS).

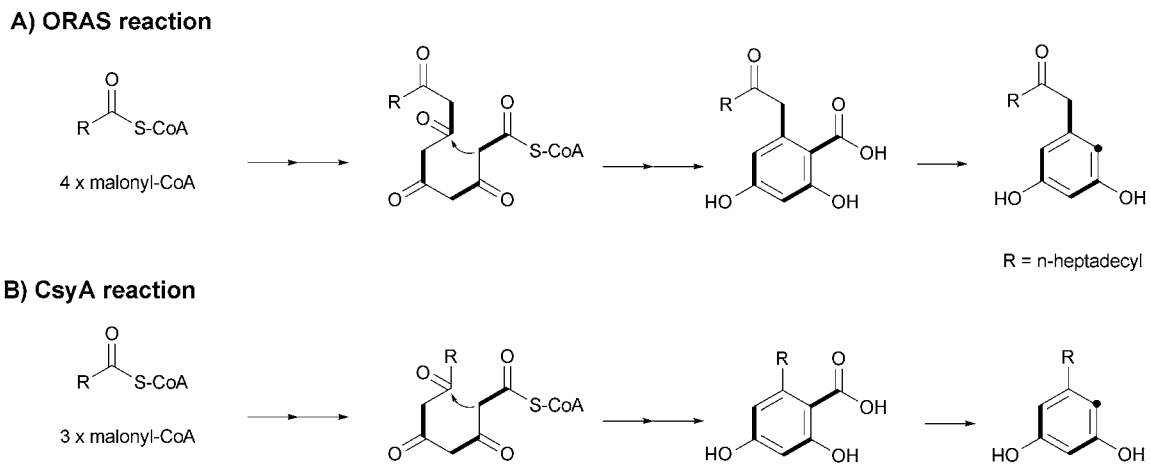
Another NPAS gene was identified in the biosynthetic gene cluster of azinomycin B.[212] Because it has a 3-methoxy-5-methylnaphthoic acid moiety, the bacterial iPKS gene *aziB* was considered to encode a PKS for this naphthoic acid. AziB was also expressed in the streptomycete host *Streptomyces albs* under the *ermE** promoter. The naphthoate compound produced by the transformant was identified to be 5-methylnaphthoic acid (**72**), which is different to **71**, a product of NNS from *S. carzinostaticus*. The domain architecture of NcsB, AziB, and MSAS is identical, though NPAS and MSAS are different in chain-length control, forming the hexaketide and tetraketide. Furthermore, NcsB and AziB are different in reduction regioselectivity. The former reduces the C-9 keto group, and the latter reduces the C-3 and C-9 keto groups, of the hexaketide intermediate. The mechanisms of these regioselective reduction controls are still unknown (Scheme 9).

5.2.3 Aldol-type anthraquinonoids. Aldol-type cyclisation in anthraquinonoid PKS reaction is found in an NR-PKS for atrachrysone (**73**), a precursor of anthraquinone emodin (**74**). Atrachrysone synthase (ACAS) consists of SAT–KS–AT–PT–ACP domains but lacks a terminal TE/CLC (Claisen cyclase) domain. Expression of ACAS in *A. oryzae* was carried out by Horinouchi and co-workers.[214] However, no product was observed when ACAS was expressed alone. The presence of a β-lactamase type hydrolase (ACTE) gene next to the ACAS gene in *Aspergillus terreus* prompted them to co-express ACAS and ACTE, which resulted in the production of **73** and related anthraquinonoids, including **74**. Thus, ACAS was confirmed to be a synthase for **73** and ACTE to be a hydrolase that releases the ACAS-bound polyketide product. Decarboxylation and dehydration of **73** leads to the formation of emodin anthrone (**75**) and its oxidation results in **74**. In this ACAS reaction, all three ring closures proceed by aldol cyclisation (Fig. 7). A similar PKS and β-lactamase hydrolase system was found in the monodictyphenone biosynthetic gene cluster of *A. nidulans*.[215] However, an NR-PKS with a C-terminus domain just for thioester hydrolysis, as found in fungal OAS, has not been found for aldol cyclisation-type anthraquinonoid biosynthesis.

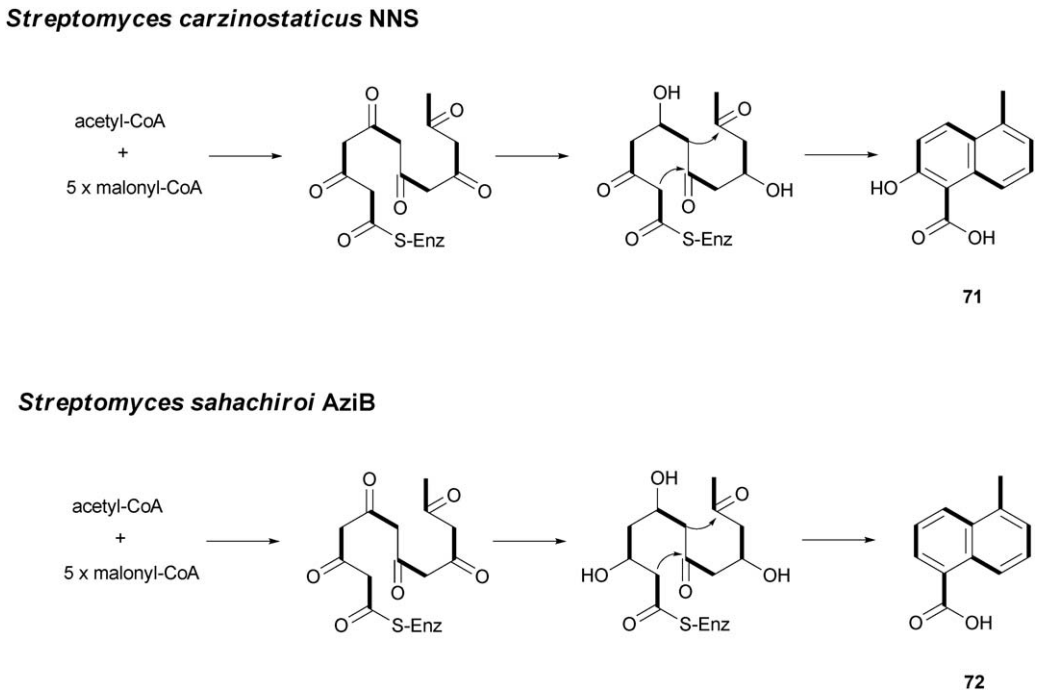
Although anthraquinones are known as pigments in plants and fungi, some bacteria also produce anthraquinones. Most



Scheme 8 The biosynthesis of 3,5-dihydroxypheyl- acetyl-CoA.



Scheme 7 Alkyl-resorcinol/resorcylic acid synthase reactions.



Scheme 9 Naphthoic acid synthase reactions.

bacteria utilise type II PKS systems for the formation of aromatic polyketides. Below are two examples of bacterial type II PKS systems involved in aldol-type cyclisation in anthraquinonoid biosynthesis.

R1128s are anthraquinones with alkyl side chains produced by *Streptomyces* sp. R1128. Because of its potent oestrogen receptor antagonist activity, its biosynthetic gene cluster was identified to include 14 genes covering a 17-kb region. It is a typical type II PKS biosynthetic gene cluster and it contains KSα, KSβ, ACP, and tailoring enzyme genes. Two cyclases, ZhuI and ZhuJ, are probably involved in the first and second ring cyclisation and aromatisation. The third cyclisation may proceed spontaneously to form the anthraquinone structure of R1128 (**76**) (Scheme 10).[216,217]

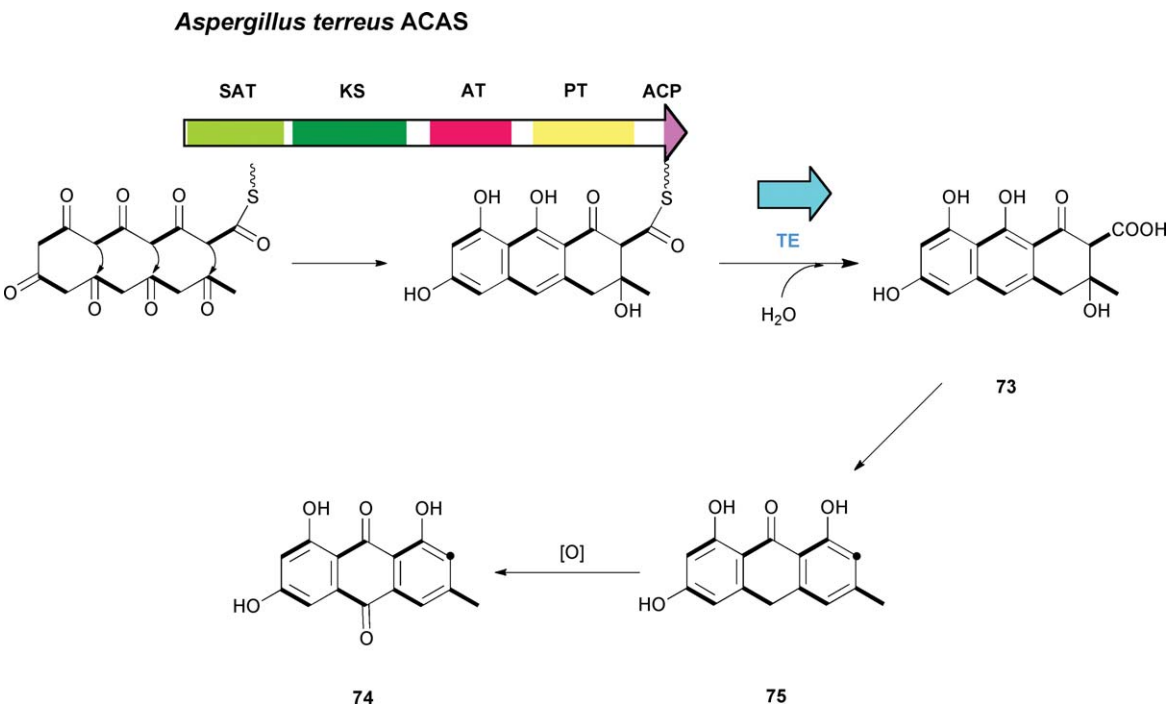


Fig. 7 Atrachrysone synthase reaction.

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In addition to Gram-positive actinomycetes, type II PKS systems were found in Gram-negative bacteria. A type II PKS gene cluster for anthraquinone was identified in the entomopathogenic bacterium *Photorhabdus luminescens*. *P. luminescens* TT01 produces mainly AQ-256 (**77**) and AQ-270a, which were confirmed to be heptaketide anthraquinones by feeding experiments. Analysis of the genome sequence revealed a gene cluster encoding a type II PKS system. This gene cluster is quite similar to the typical actinomycete type II PKS gene clusters. It encodes not only the minimal PKS, KSα, KSβ and ACP, but also KR and two cyclases (AntH and AntC) together with other genes. Because the disruption of AntH cyclase/aromatase resulted in the accumulation of mutactin and dehydromutaction, as reported for *actIV* deletion, similar biosynthetic reactions are involved in anthraquinone formation in *P. luminescens*. However, the third cyclisation proceeds by aldol cyclisation catalysed probably by AntC and/or AntH. Acetyl-ACP cleavage from three ring intermediate results in the formation of heptaketide anthraquinone (Scheme 11).[218]

5.3 Claisen-type cyclisation PKS pathway

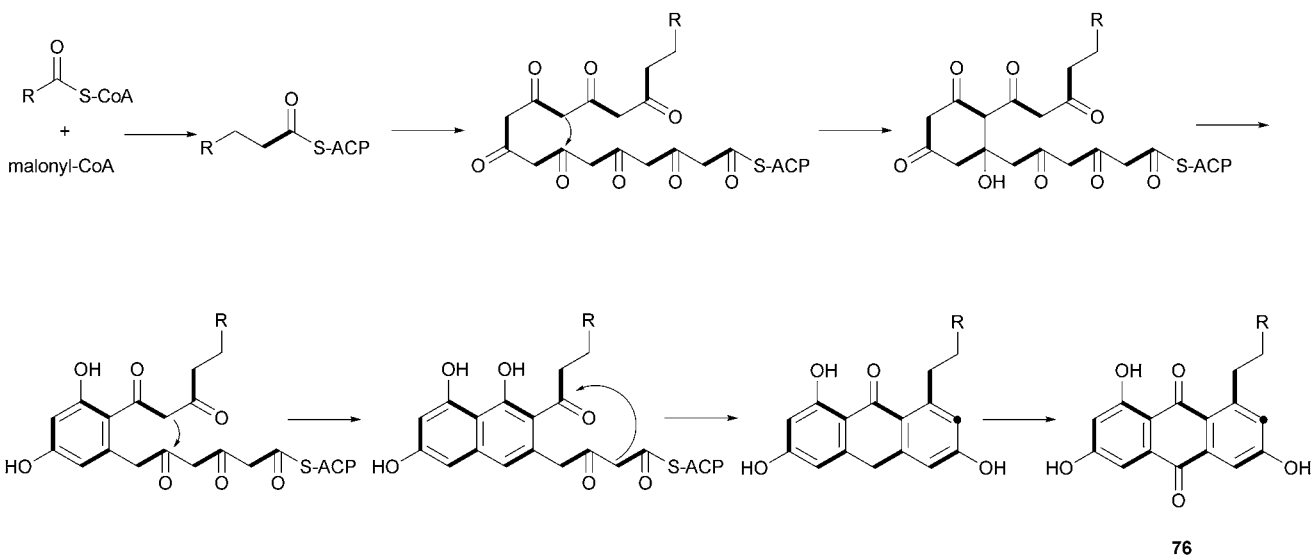
5.3.1 Claisen cyclisation to benzenoids. Claisen-type cyclisation reactions result in the formation of some benzenoids, such as in acyl phloroglucinol compounds. In phloroglucinol-type benzenoids, Claisen cyclisation of the poly-β-keto intermediate occurs by nucleophilic attack from C-6 to the C-1 carbonyl of the thioester of ACP or CoA followed by thioester cleavage and aromatisation to give the benzene ring. Plant CHSs use this cyclisation strategy for their flavonoid A-ring formation. Based on the crystal structure of CHS, it was proposed that the intermediate itself provides the driving force for carbanion formation and protonation of the carbonyl oxygen because there is no base capable of proton abstraction from the tetraketide intermediate. Thus, intramolecular Claisen cyclisation of the intermediate occurs spontaneously, possibly through an internal proton transfer upon proper elongation and folding within the active site.[219,220] A similar Claisen cyclisation is used by other plant type III PKSs, such as benzophenone synthase,[221] phlorisovalerophenone synthase,[222] although recognition of starter acyl moieties are different.

The simplest Claisen benzenoid polyketide is phloroglucinol (**78**) formed by the type III PKS PhlD from three malonyl-CoAs. The *Phl* gene cluster found in *Pseudomonas fluorescens* is responsible for the biosynthesis of 2,4-diacetylphloroglucinol.[223] Heterologous expression experiments of genes in the cluster identified that PhlD forms **78** by decarboxylative Claisen cyclisation of C-6 to C-1 of the triketide intermediate.[224,225]

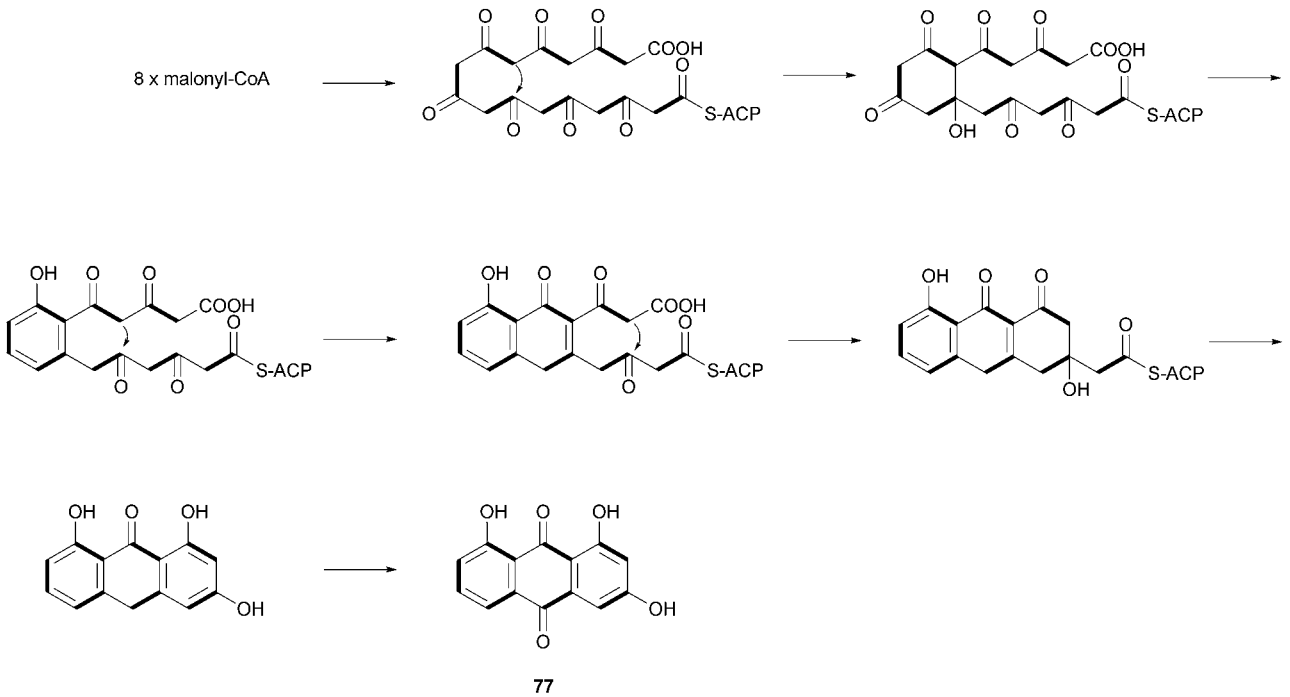
Biosynthetic studies of the differentiation-inducing factors (DIFs) in *Dictyostelium discoideum* amoeba revealed the presence of two hybrid type I fatty acid synthases (FASs) – type III PKS genes named "Steely 1 and 2" possibly involved in acylphloroglucinol scaffold synthesis. The type I FAS part could catalyse the formation of a hexanoyl starter unit, which is then intramolecularly transacylated to the type III PKS domain. The type III PKS then catalyses three rounds of malonyl elongation followed by a Claisen cyclisation. The recombinant type III PKS of Steely 2 showed the expected catalytic activity as it formed phlorocaprophenone (**79**) from hexanoyl-CoA and malonyl-CoAs, but the Steely 1 type III PKS gave triketide and tetraketide pyrones. The Steely 2 C-terminal type III PKS shares ~30% sequence identity with CHS and Steely 1 (Scheme 12).[226]

As described above, all Claisen cyclisation-type PKSs involved in the formation of benzenoid polyketides are type III and no Claisen cyclisation-type benzenoid NR-PKS have been identified.

5.3.2 Claisen cyclization to naphthalenoids. 1,3,6,8-Tetrahydroxynaphthalene (T4HN) (**80**) is a typical naphthalenoid polyketide in which all four hydroxyl groups are derived from carbonyls of acetate units. In fungi, the biosynthesis of 1,8-dihydroxynaphthalene (DHN)-melanin derived from **80** has been intensively studied because DHN-melanin has an important role in phytopathogenicity and self-defence mechanisms.[227–229] The T4HN scaffold is also present in other fungal metabolites, such as naphthopyrones, which are also known as spore pigment



Scheme 10 The biosynthesis of R1128 (**84**).

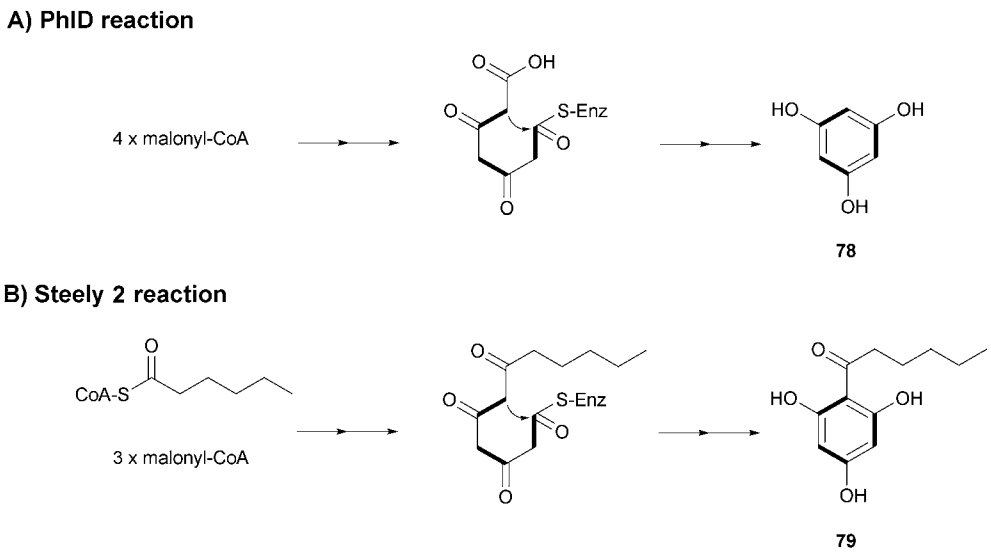


Scheme 11 The biosynthesis of AQ-256 (**77**).

compounds in fungi.[230–233] In actinomycete bacteria, naphthoquinone moieties are found in meroterpenoids, such as naphterpin,[234] furaquinocin A[235] and related compounds.[236–239]

Two distinct cyclisation patterns in aromatic polyketides from fungi and streptomycetes have been identified by Thomas.[240] In the mode-S folding in *Streptomyces* type II PKS, the first ring is cyclised using aldol cyclisation from an odd number carbon nucleophile to +5 even number carbonyl, such as C-7 to C-12 or C-9 to C-14. In the mode-F folding in fungi, the first ring cyclisation proceeds by an aldol cyclisation from an even number carbon nucleophile to +5 odd number carbonyl, such as from C-2 to C-7 or C-4 to C-9. Thus, the acetate labelling patterns in S-fold and F-fold rings are different. Since **80** is a symmetric compound, it is impossible to differentiate these labelling patterns in **80**. However, two types of intermediates with a cyclised ring could be possible, as shown in Scheme 13.

80 is a common precursor of DHN-melanin in black fungi, including *Verticillium dahliae*,[241] *Magnaporthe grisea*,[227,242] *Colletotrichum lagenarium*.[243] **80** is sequentially converted to scytalone, 1,3,8-trihydroxynaphthalene, vermelone and DHN, which is considered to be a substrate of oxidative polymerisation to form black melanin. These sequential biosynthetic reactions, reduction and dehydration, have been well studied at the enzymic level in *M. grisea*[244] and *C. lagenarium*.[245] The PKS responsible for this symmetrical compound was first identified in the phytopathogenic black fungus *C. lagenarium*, causing



Scheme 12 The biosynthesis of Claisen benzenoid polyketides.

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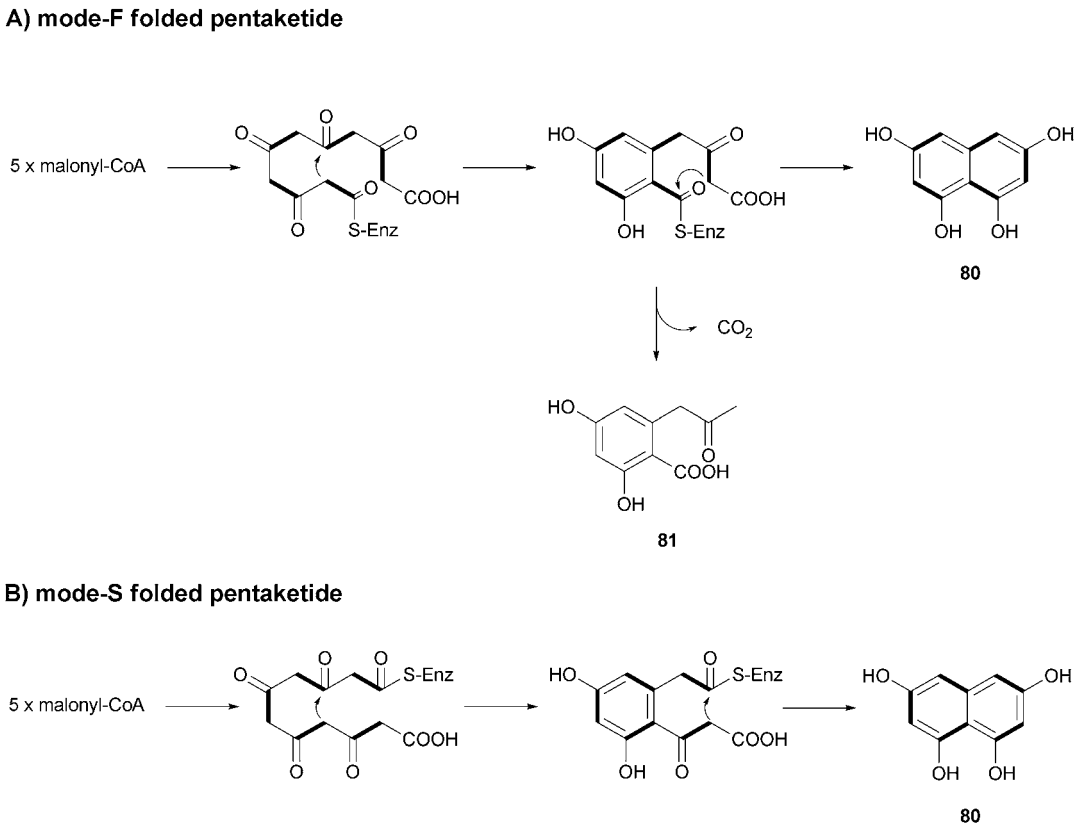

Scheme 13 Two cyclization patterns in T4HN formation.

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anthracnose in cucumber.[246,247] This PKS, named PKS1, is a typical NR-PKS with SAT, KS, AT, PT, ACP, and TE/CLC domains. Its architecture is identical to that of the above mentioned fungal OAS. Although detailed enzyme analysis has not been carried out, *in vitro* analysis proved that PKS1 uses five malonyl-CoAs as the sole substrate to form pentaketide **80**.[248] Isolation of the monocyclic by-product α-acetylorsellinic acid (**81**)[247] indicated its F-mode cyclisation and decarboxylation-assisted Claisen cyclisation of the second ring to form **80**, as shown in Scheme 13.

In contrast to black fungi, Aspergilli fungi show greenish spore pigmentation.[249] Analysis of *Aspergillus nidulans* WA PKS identified the precursor compound to be naphthopyrone YWA1 (**82**).[250,251] Its heterologous expression in *A. oryzae* caused the production of this yellow pigment, which is considered to be a substrate of oxidative polymerisation to give greenish spore pigmentation.[252,253] **82** is a hemiketal form of acetoacetyl-T4HN and its equilibrium favours the formation of naphthopyrone hemiketal. Feeding experiments confirmed its F-mode cyclisation pattern.[254] WA PKS is an NR-PKS with SAT, KS, AT, PT, ACP, and TE/CLC domains. Deletion and mutation analysis of its C-terminus TE domain revealed it was not just a thioesterase domain to hydrolyze the thioester intermediate to release product **82**, but also a catalytic domain to form a second aromatic ring by Claisen cyclisation and concomitant release of the product.[254] Thus, this C-terminus domain was named a Claisen cyclase (CLC) domain. Similar to the isolation of **81** from the *C. lagenarium* PKS1 transformant, WA mutants with either CLC deletion or CLC mutations produced citreoisocoumarin (**83**) (Fig. 8).

Aspergillus fumigatus is a human pathogenic fungus causing allergy, noninvasive colonisation, or life-threatening invasive pulmonary aspergillosis. *A. fumigatus* shows bluish-green spore pigmentation, neither greenish nor black. This bluish-green spore formation is also critical for the fungus to invade human organs, such as the lungs. The responsible gene cluster was cloned from a clinical isolate strain and found to include six genes.[255] Most genes show homology with known genes involved in DHN-melanin biosynthesis. The *alb1* gene was considered to encode an NR-PKS for **80**. However, Alb1p shows higher similarity to WA PKS1 for **82** than *C. lagenarium* PKS1 for **80**. Actually, heterologous expression of Alb1p PKS confirmed its ability to produce naphthopyrone **82**, not **80**.[256]

The involvement of heptaketide **82** in the pentaketide DHN-melanin pathway in *A. fumigatus* was solved by the functional analysis of Ayg1p.[257,258] *In vitro* experiments using recombinant Ayg1p confirmed that Ayg1p catalyses the conversion of **82** to **80** by hydrolytic side-chain C–C bond cleavage (Scheme 14). Thus, the resultant **80** could be converted to DHN and DHN-melanin as in black fungi. Since *A. fumigatus* possesses two phenol oxidase genes *abr1* and *abr2*, one may catalyse DHN black melanin formation and the other may catalyse green pigment formation from naphthopyrone. The resultant mixed pigmentation appears as the bluish-green spore pigment. In addition to the above mentioned pathways for **80**, a third was found in *Wangiella dermatidis*, which is also a human and animal

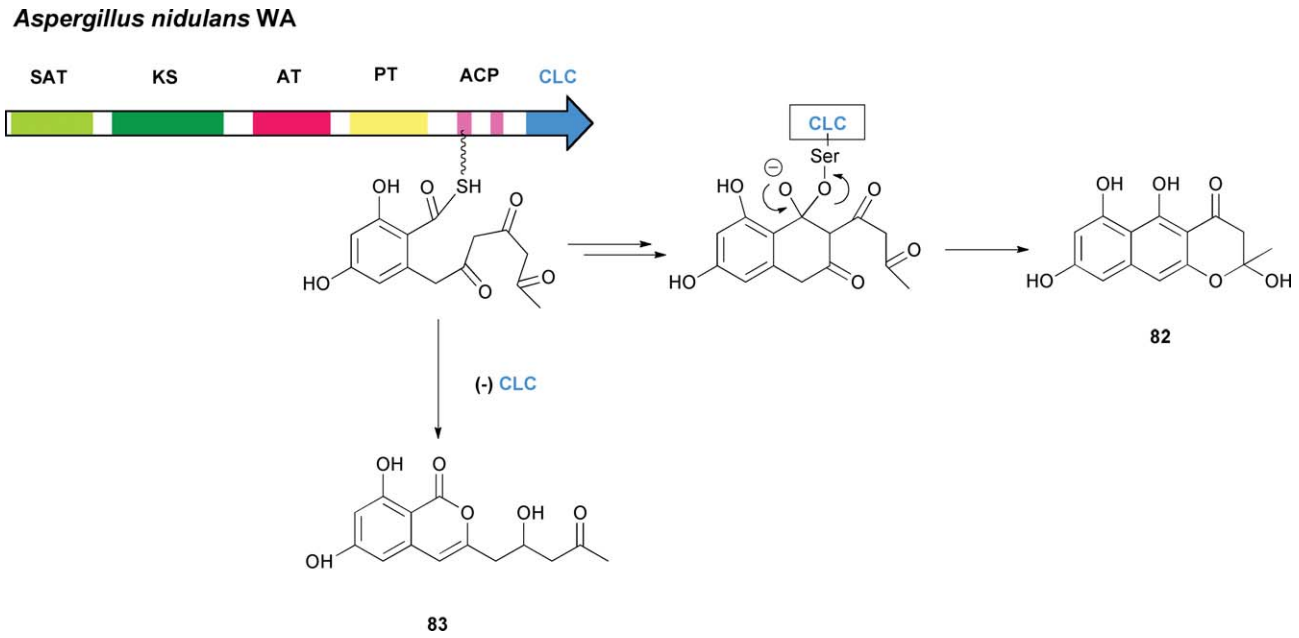

Fig. 8 The Claisen cyclase domain in WA PKS.

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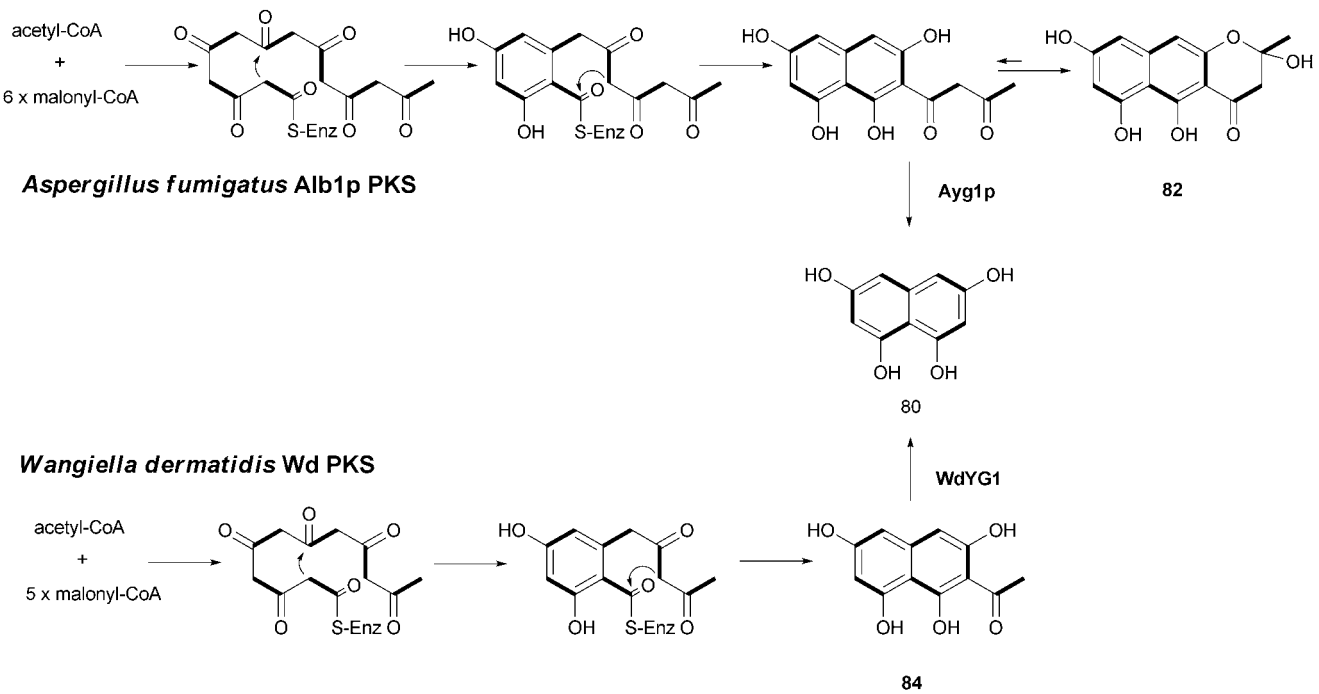
pathogenic black fungus.[259,260] DHN black melanin formation was expected to occur in this fungus as in *M. grisea* and *C. lagenarium*. The NR-PKS responsible, named Wd PKS, was cloned[261] and its expression was carried out in the heterologous host *A. oryzae*.[262] The phenotype of the Wd PKS transformant was quite different from that of *C. lagenarium* PKS1, in which the induction culture became black due to oxidation of **80**. On the other hand, the Wd PKS transformant appeared to be a yellow colour on induction. The Wd PKS product was identified to be hexaketide acetyl-T4HN (**84**). Apparently, **84** is not converted to **80** in *A. oryzae*, and the conversion requires the Ayg1p orthologue WdYG1.[263] Actually, the recombinant WdYG1 catalyses the hydrolytic cleavage of the acyl side-chain of **84**, though its specificity has not yet been characterised (Scheme 14).

In bacteria, it was accepted that aromatic polyketides are produced by type II PKS systems until the formation of **80** by a bacterial type III PKS was reported by Horinouchi and co-worker in 1999. This was the first example of a non-CHS, non-STS, microbial type III PKS.[264] The responsible *rppA* gene was cloned from *Streptomyces griseus*, which is involved in melanin-like spore pigmentation confirmed by a gene disruption experiment. The recombinant RppA expressed in *E. coli* catalysed the



Scheme 14 T4HN biosynthesis from hexaketide and heptaketide precursors.

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formation of **80** from five malonyl-CoAs. Because of its symmetric structure, it is impossible to differentiate whether there is an S-fold or an F-fold in **80**. However, no α-acetylorsellinic acid-type by-product was observed in the RppA *in vitro* assay, indicating that **80** formed by RppA is S-fold, as is assumed from its origin (Scheme 13). RppA accepts aliphatic acyl-CoAs with a carbon length of C-4 to C-8 as starter substrates and catalyses sequential condensation of malonyl-CoA to yield α-pyrones and phloroglucinols.[265]

Following the discovery of RppA, a number of type III PKS genes have been identified in bacteria and lower eukaryotic microbes. The genome project of model filamentous bacterium *S. coelicolor* revealed the presence of type III PKS genes.[83] One of them, SCO1206 was confirmed to be a synthase for **80** (THNS) by expression in *E. coli*.[266] Then its crystal structure was determined.[267] RppA, and related THNSs, catalyse dual intramolecular aldol and Claisen condensations of the linear pentaketide intermediate to produce the fused ring skeleton of **80**. As with other type III PKSs, THNS utilises a conserved Cys-His-Asn catalytic triad in an internal active site cavity. Involvement of an additional cysteine residue facilitates malonyl-primed polyketide chain extension. The crystal structure also revealed a novel cavity for extra polyketide extension when primed with a long acyl starter.[267]

In the genome sequencing projects of myxobacteria *Myxococcus xanthus* DK 1622 and *Sorangium cellulosum* So ce56 genes, putative type III PKS genes were identified. There are no known products of these microorganisms that correspond to these enzymes, indication that these genes are considered to be silent under the cultivation conditions. Thus heterologous expression was carried out to identify the biosynthetic product. First it was tried to express SoceCHS1 in *E. coli* but no expression was observed. Then, expression was attempted in *Pseudomonas putida*. The transformant turned red when the incubation time was prolonged. Since the production of flaviolin, the oxidation product of **80**, was verified by HPLC analysis, SoceCHS1 was identified to be myxobacterial type III THNS.[268]

5.3.3 Claisen-type anthraquinonoids. The most well characterised NR-PKS for an anthraquinonoid is PksA involved in biosynthesis of norsolonic acid (**85**), the precursor of aflatoxin. PksA is an NR-PKS consisting of SAT–KS–AT–PT–ACP–TE/CLC domains, and it combines a hexanoyl starter unit and seven malonyl-CoA extender units to synthesise norsolorinic acid anthrone (**86**). The N-terminus SAT domain of PksA was first identified to function as a starter unit:ACP transacylase.[192] In the PksA reaction, its SAT domain selects the hexanoyl starter unit from a pair of specialised FASs, and transfers it onto the PksA ACP to prime polyketide chain elongation. Following the hexanoyl starter octaketide formation, the PT domain catalyses the first and second aldol cyclisations.[269] The third Claisen cyclisation is catalysed by the C-terminus CLC domain.[270] Recent structural analysis of the PT and CLC domains were carried out by the Townsend and Tsai groups. The PT domain is now considered to bind a fully extended ACP-bound polyketomethylene intermediate into the cyclisation chamber, and catalyse the first and second aldol cyclisations.[269] The thus formed bicyclic intermediate is transferred from the PT domain to the CLC domain and transesterified from the ACP to the Ser residue of the CLC. Then, C–C bond forming Claisen cyclisation proceeds with the aid of a His residue and releases the anthrone product from the PksA enzyme.[270] As exemplified by PksA, most

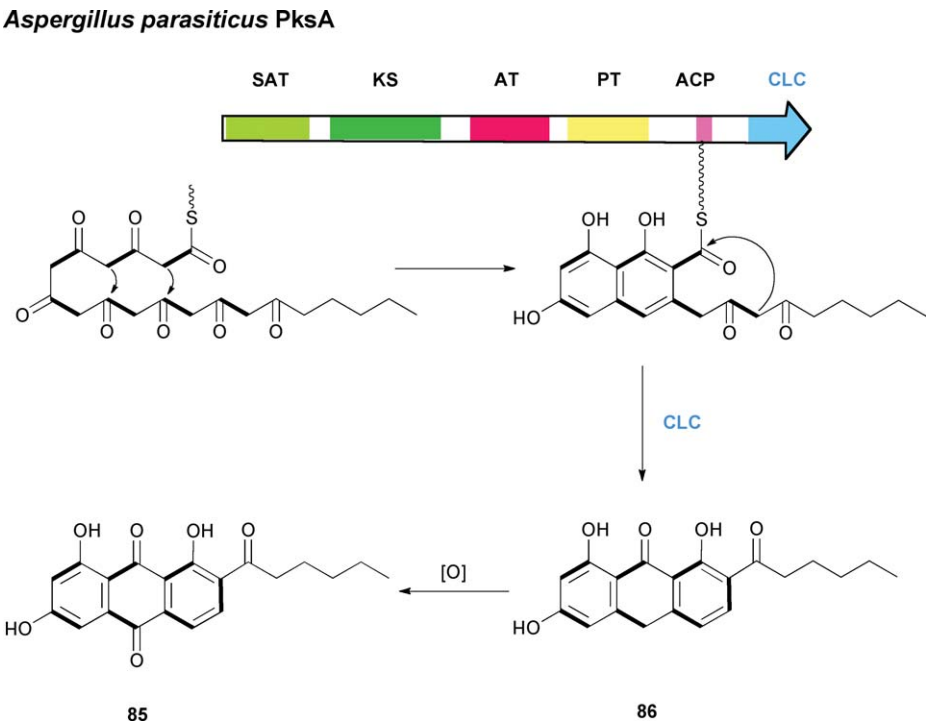

Fig. 9 *A. parasiticus* PksA reaction.

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of the Claisen cyclisation-type anthraquinones are considered to be formed by NR-PKSs with the same reaction mechanism (Fig.9).

5.4 Summary

Plants, bacteria, and fungi utilise different architecture types of PKSs for aromatic product biosynthesis. Regardless of their PKS types, aromatic polyketides are classified depending on the cyclisation mechanisms of the final rings by the PKSs, aldol cyclisation-type *versus* Claisen cyclisation-type, as summarised in this section. As typically exemplified in benzenoid cyclization, directions of nucleophilic attacks are opposite, which provides structural variety in addition to polyketide chain-length. In Claisen iterative type I NR-PKSs, specific domain for Claisen cyclisation, CLC domain was identified. However, mechanistic understanding of enzymatic aldol cyclisation by PKSs is still in its infancy. Recently, the PT domain of PksA has been reported to control the carbon chain-length and aldol cyclisation, thus determining the product scaffold.[269] In addition to the PksA PT domain structure, more structural information on other PT domains of NR-PKSs is required for further understanding the mechanism controlled by PT domains. In type III PKSs, no anthraquinonoid PKS has been identified although some have been reported to catalyse the formation of octaketide intermediates. With such type III PKSs for anthraquinones in hand, structural comparison with PhlD for benzenoid and RppA for naphthalenoid may reveal how type III PKSs for aromatic compounds control the carbon chain-lengths and cyclisations to form their products.

6 Conclusion

Microorganisms evolved convergent strategies in biosynthesis of small molecules as highlighted in this review. Although the reasons why microorganisms utilize the different strategies in convergent metabolism has not been addressed, microorganisms might attempt a solution of the problem through independent mechanisms in response to various environmental pressures and refine them after repeated trial and error so as not to waste energy during evolution. For example, the genome sizes of symbiotic bacteria, such as the genus *Mycoplasma*, are less than 1 megabase. They chose a strategy to survive depending on host organisms and decreased their genome DNA by deletion. In contrast, most microorganisms whose genome sizes are between 2 to 10 megabases increased their genome size through gene acquisition and gene duplication to provide enzymes for many pathways of primary and secondary metabolism for survival in various environments.

The known biosynthetic pathways of primary metabolism have been established with model microorganisms, such as *Escherichia coli*, which propagate in a special environment, guts in animals. However, a variety of microorganisms exist in various environments. Considering this fact and that the function of more than half of the genes/open reading frames (ORFs) estimated by whole genome sequencing remains unclear in many microorganisms, microorganisms are still expected to have a completely new pathway or a variation of the known pathway for biosynthesis of small compounds.

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