Natural Product Reports

ROYAL SOCIETY OF CHEMISTRY

REVIEW

View Article Online
View Journal | View Issue

Cite this: *Nat. Prod. Rep.*, 2022, **39**, 2175

On the evolution of coenzyme biosynthesis†

Andreas Kirschning

Covering: up to 2022

The report provides a broad approach to deciphering the evolution of coenzyme biosynthetic pathways. Here, these various pathways are analyzed with respect to the coenzymes required for this purpose. Coenzymes whose biosynthesis relies on a large number of coenzyme-mediated reactions probably appeared on the scene at a later stage of biological evolution, whereas the biosyntheses of pyridoxal phosphate (PLP) and nicotinamide (NAD^+) require little additional coenzymatic support and are therefore most likely very ancient biosynthetic pathways.

Received 25th May 2022

DOI: 10.1039/d2np00037g

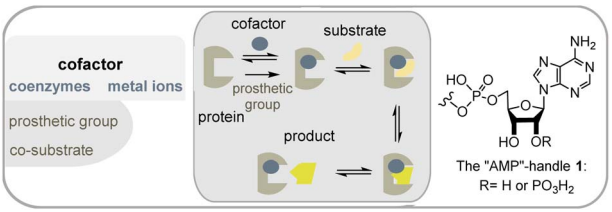
rsc.li/npr

1. Introduction

Metabolism is one of the cornerstones of life and is characterized by an elaborate network of interconnected biosynthetic pathways and their regulation.[1] It is responsible for the synthesis (anabolism) and degradation (catabolism) of biomolecules. In addition to the catalytically active proteins or enzymes, other small chemical entities, called cofactors, play an important role in the catalytic abilities of enzymes. Cofactors are often divided into metal ions and small organic molecules, so-called coenzymes (Fig. 1). However, the term "cofactor" is

Institute of Organic Chemistry, Leibniz University Hannover, Schneiderberg 1B, D-30167 Hannover, Germany. E-mail: andreas.kirschning@oci.uni-hannover.de; Fax: +49-(0)511-762 3011; Tel: +49-(0)511-762 4614

† Dedicated to Heinz G. Floss (Univ. Washington, Seattle, USA).



Scheme 1 Cofactors in enzyme catalysis and "AMP"-handle **1**.

sometimes associated exclusively with metal cations. Coenzymes can be differentiated according to the binding in the active pocket of the enzyme. The first is called a prosthetic group, which is made up of a coenzyme that is tightly, possibly covalently, and permanently bound to a protein whereas reversibly bound coenzymes are sometimes termed co-substrates. In this report, I will generally use the term coenzyme, despite the terminology just outlined.

From an evolutionary point of view, cofactors and also the organic representatives must be very old and give us a clue to the origin of life, since their purpose and role have remained unchanged since the presumed starting point of life.[2] It is likely that most of the examples described below already existed about 4 billion years ago.

Cofactors promote chemical reactions that the protein template cannot.[3] These include (a) redox chemistry including halogenations, (b) activation of functional groups such as alcohols and carboxylates, and (c) group transfer reactions such as methylations. Coenzymes are embedded in the active site of the protein template, where they take up substrates and convert them into products (Scheme 1). Polar or ionic elements in the coenzyme, as well as functional groups of the protein, ensure the common association and alignment of the substrate in the active site. Coenzymes that mediate redox and group transfer reactions require regeneration mechanisms.

Many coenzymes are based on heterocyclic cores, often structurally modified with nucleic acid elements, as is particularly manifested in the "AMP handle" **1**. In addition, elements of nucleotides are hidden in and biosynthetically derived from the heterocyclic core structures of some coenzymes, most notably guanosine triphosphate (GTP). Fig. 1 lists the major coenzymes and metal-dependent coenzymes **2**–**20**. These are subdivided according to their chemical properties and their role in metabolism. The recently discovered nickel pincer nucleotide cofactor (NPN, **2c**) is derived from nicotinamides and is a limiting case as it catalyzes racemizations of α-hydroxycarboxylates, however, this is a redox process. Since it is a metal complex, this cofactor will be discussed in section 2.2. Most of them are distributed across all phylogenetic kingdoms. 4-Methylideneimidazole-5-one (MIO, **20**) is also found in the list, although it is like pyrroloquinoline quinone (PQQ, **5**) a polypeptide modification.[4] The common biosynthetic precursor uroporphyrinogen III **8** is shown for the macrocyclic ligands heme **9** and cobalamin **10**, which are present in many metalloenzymes.

Methanogens rely on several coenzymes commonly not found in other organisms (Fig. 2).[5] These prokaryotes belong to the domain of archaea and are hydrogen-dependent autotrophs that produce methane and have been proposed as good candidates for the physiological primordial state.[5c] As such, they are limited to carbon dioxide, formate, methanol, methylamines, and acetate as possible carbon sources.

Such unique coenzymes are the 5-deazaflavins, coenzymes F_0 and F_{420} (**21a** and **21b**), which are structurally related to FMN **3a** and FAD **3b**, coenzyme M (**22**), 7-mercaptoheptanoylthreonine phosphate (coenzyme B, **23**), tetrahydromethanopterin (THMPT, **24**), methanofuran (**25**), and cofactor F_{430} (**26**).[6]

Coenzymes found today are formed enzymatically *via* biosynthetic cascades and are the result of an evolutionary process. Remarkably, in some cases nature has found distinctly different biosynthetic solutions for the same coenzyme (see below). The analysis of such different biosynthetic pathways can be seen as a way to shed light on the evolutionary development of modern coenzyme biosynthesis as was reviewed by Warren *et al.* before.[7] Consistent with these basic considerations, this review analyzes the known biosynthetic pathways to coenzymes, specifically focusing on other coenzymes that are required for their formation. As a result, (fragmented) proposals for the evolution of coenzyme biosynthetic pathways are presented. They are not based on genetically analyzed protein-coding genes and protein clusters, but choose the perspective of a (bio) synthetic chemist and metabolism. On the basis of these evaluations, with regard to known hypothetical approaches, an additional perspective on the possible origin of an early metabolism becomes available (Table 1).[2]

Andreas Kirschning studied chemistry at the University of Hamburg and at Southampton University (UK). In Hamburg, he joined the group of Prof. Ernst Schaumann and received his PhD in 1989. After a postdoctoral stay at the University of Washington (Seattle, USA) with Prof. Heinz G. Floss, he moved to the Technical University of Clausthal in 1991. In 2000, he was appointed full professor at Leibniz Universität Hannover. His research interests include all aspects of natural products including total synthesis, mutasynthesis and the use of terpene synthases. Another important aspect of his research has been the development of synthesis technologies, including flow chemistry combined with inductive heating techniques. More recently, his interest in natural products and enzymology has led to the exploration of theoretical aspects of molecular evolution, with a focus on coenzymes and cofactors.

2. Biosyntheses of coenzymes

In this section, the biosyntheses of most coenzymes are summarized, and all coenzymes required for their biosynthesis

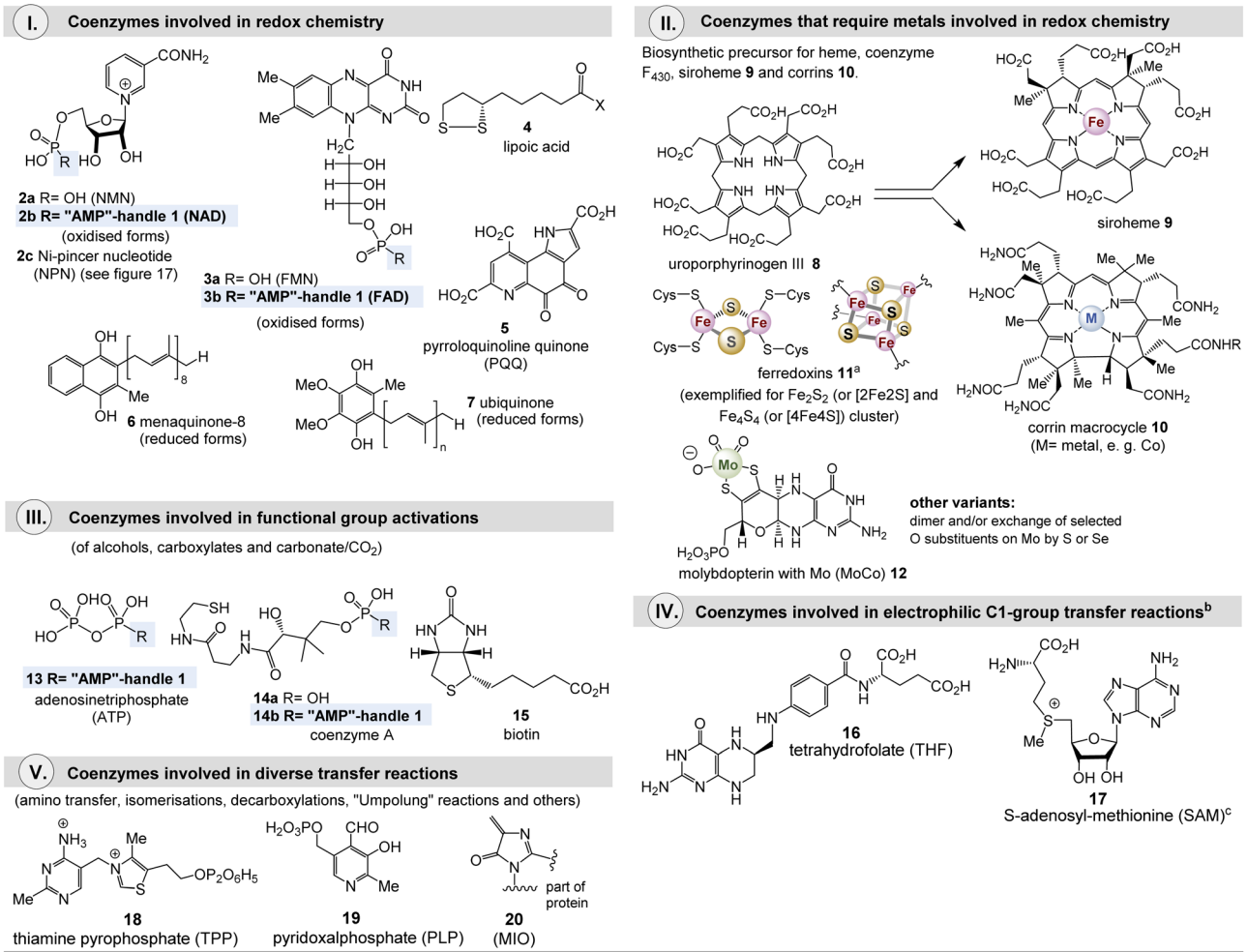


Fig. 1 Structures and chemical role of (metal-dependent) coenzymes **2**–**20** (phosphates and carboxylates are depicted in fully protonated form throughout the text, although physiologically the salt form would be correct) and polypeptide MIO **20** (numbers I–V are linked to sections 2.1–2.5). [a] Other iron/metal sulfur clusters are covered in section 2.2.2; [b] further details of chemical transformations promoted by coenzymes are found in section 2.2; [c] SAM is also involved in S-ylide chemistry and in combination with FeS-clusters also responsible for radical-triggered biotransformation.

in each case are listed in abbreviated form at the bottom of each graphical scheme. The classification by sections is made with reference to the chemical role of the individual coenzymes, *e.g.* for redox reactions. The author is aware that other classifications would have been equally possible. At the end of this review, when evolutionary considerations will be dealt with, the subdivision made will then resolve itself within the new context.

2.1 Biosynthesis of redox coenzymes

2.1.1 NAD(P)$^+$ (2) and the biosynthetic precursor niacin (31). In recent years, the classical coenzyme NAD$^+$ has received renewed academic attention.[8] Nicotinamides have been found to function as signaling molecules in a variety of cellular processes in addition to their known role in redox biochemistry and energy metabolism.[8d,e] NAD(P)$^+$ also serves as a substrate in mono- and poly-ADP-ribosylation reactions leading to covalent modification of proteins.[8e]

Currently, two different NAD(P)$^+$ biosyntheses are known, with quinolinic acid (**30**) and niacin (**31**) being the key intermediates for both routes (Fig. 3). In bacteria, niacin is formed from dihydroxyacetone phosphate (DHA-3-P, **28**) and L-aspartate (**29a**) (Fig. 3, left),[9] whereas in plants, L-tryptophan (**33**) acts as a precursor (Fig. 3, right).[10]

In bacteria and archaea, aspartic acid (**29a**) is first oxidized by L-aspartate oxidase to the corresponding imine **29b**, using FAD **3** as a coenzyme in which oxygen and hence FADHOOH serve as oxidant. Alternatively, oxaloacetate **29c**, an intermediate of the TCA cycle, could also serve as a building block for intermediate **29b**, so that FAD-mediated oxidation is then not required at all. Subsequently, condensation with DHA-3-P **28** takes place, which is catalyzed by quinolinate synthase, whereby, remarkably, a [4Fe–4S] cluster serves as Lewis acid and is not employed for electron transfer.[11] Noteworthy, the imine **29b** can also be regarded as a formal condensation adduct of oxaloacetate **29c** and ammonia.[12]

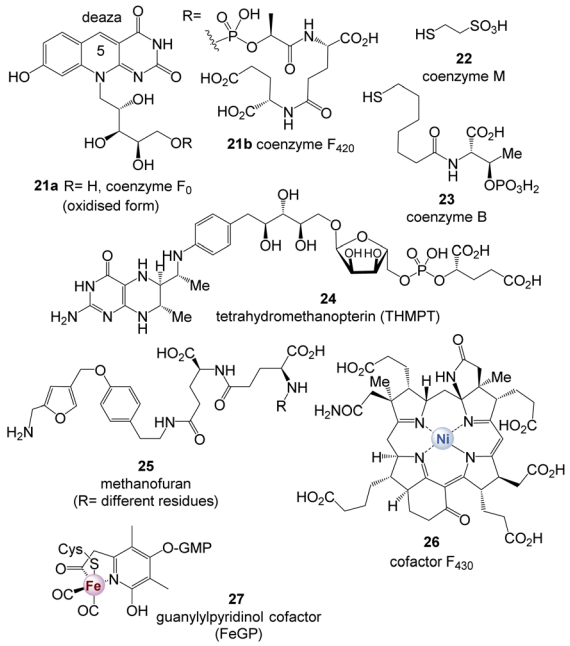


Fig. 2 Coenzymes 21–27 found in methanogens (note: CoF_{420} has been found in all methanogens but also in numerous actinomycetes) (GMP = guanosine monophosphate).

In plants and some bacteria, L-tryptophan (**33**) provides all the atoms for the construction of the niacin backbone with quinolinic acid as an intermediate.[12] Starting from niacin, the final steps involve the quaternization of the pyridine nitrogen atom with 5-phospho-D-ribose-1-diphosphate (PRPP, **32**) as the alkylating building block, which provides NAD^+ and from there ATP-mediated phosphorylation furnishes $NADP^+$.

The aspartate pathway is clearly the simpler of the two pathways, and indeed Cleaves and Miller suggested that this pathway should in principle be feasible by chemical means under presumably prebiotic conditions.[13] Another argument for why the tryptophan pathway should be more recent centers on the large number of coenzymes, including NADPH **2**, required for this multistep biosynthesis and the use of O_2 in association with heme. Finally, the kynurenine pathway relies on tryptophan as a starting building block, one of the rarest amino acids, which is built up *via* one of the most complex biosyntheses of any proteinogenic amino acid overall.[12] The kynurenine pathway seems somewhat meaningless so what might its significance be? It has been shown that some organisms such as yeast (*S. cerevisiae*) use this pathway during aerobic growth and the *de novo* pathway during anaerobic growth.[14]

2.1.2 Riboflavins 3, deazaflavins 21 and prenylated riboflavin 36. Flavonoids such as FMN **3a** and FAD **3b** are coenzymes essential for various redox reactions, including hydrogen and electron transfer reactions, electrophilic hydroxylations, and halogenations.[15] They are also involved in light sensitization processes, bioluminescence, circadian timing, and DNA repair.[16] Inherent redox reactivity is localized in the so called pterin ring system **34** and specifically in its extended version, the isoalloxazine core **35**. In their reduced form (*e.g.*, $FMNH_2$ and $FADH_2$), they are responsible for hydrogen transfer reactions through single electron transfer steps. In combination with oxygen, peroxyflavin species are formed that can provide a single atom of molecular oxygen or halogen.[15] Related to riboflavins are 6-hydroxy-7,8-dimethyl-isoalloxazine (6-hydroxy-FAD), 7-methyl-8-hydroxy-isoalloxazine, and prenylated flavin mononucleotide (prFMN) **36**.

Riboflavins are biosynthesized in plants, fungi, bacteria, and archaea by a remarkable pathway (Fig. 4).[15] The eastern part is derived from guanosine triphosphate (GTP, **38**), losing a C atom at C8 in the form of formate, and hydrolysis yields the 5,6-diaminopyrimidine dione derivative **39**. All other carbon and nitrogen atoms remain in the coenzyme. The dimethyl-substituted benzene ring is built up sequentially from two molecules of ribulose-5-phosphate (Ru-5-P, **37**) by a unique mechanism.

Recently, prenylated flavin (prFMN) **36** was discovered that was shown to be important in the ubiquitous microbial UbiDX system.[17] UbiX acts as a flavin prenyltransferase (Fig. 5)[18] while UbiD is a prFMN-dependent reversible (de)carboxylase, *e.g.* it promotes the decarboxylation of cinnamic acid derivatives. Although knowledge of prFMN-driven biochemistry is still in its

Table 1 Chemical reactions performed by coenzymes 21–27 found in methanogens (archaea)

$CO_2 + 4H_2 \rightarrow CH_4 + H_2O$ ($\Delta G_0' = -131$ KJ mol^{-1})

Coenzyme	Role in methanogenesis
Coenzyme F_0 **21a**	Biosynthetic precursor of coenzyme F_{420}
Coenzyme F_{420} **21b**	$F_{420}H_2$ reduces different C1-loaded THMPT intermediates; delivers electrons to hydrogenases
Coenzyme M **22**	Accepts methyl from methyl-H4MPT
Coenzyme B **23**	Forms disulfide with S-methylated coenzyme M yielding methane
THMPT **24**	Binds C1-units of different oxidation states
Methanofuran **25**	Accepts CO_2 to form *N*-carboxymethanofuran (1st step of methanogenesis)
Cofactor F_{430} **26**	Prosthetic group in methyl coenzyme M reductase: catalyses release of methane in the final step of methanogenesis. Catalysis the first step of methane oxidation
FeGP cofactor **27**	[Fe]-hydrogenase: reversible dehydrogenation of methylene-THMPT

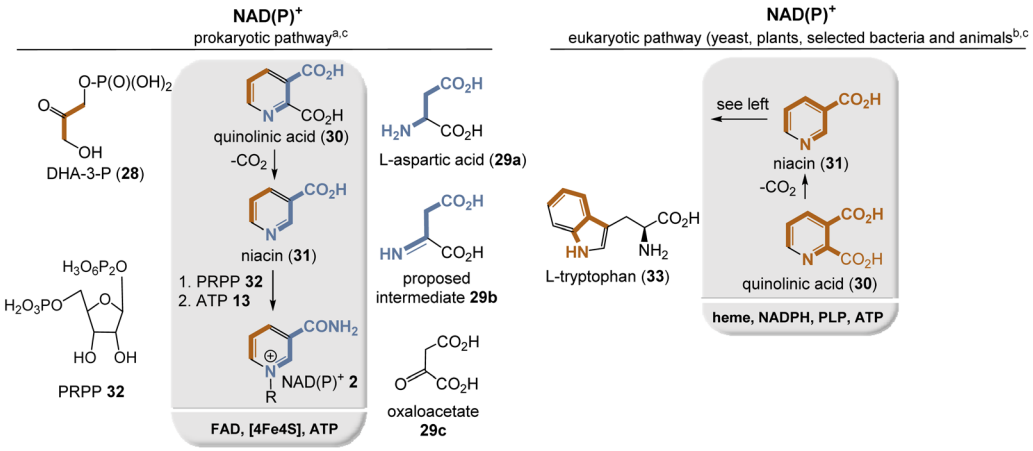


Fig. 3 Summary of NAD^+ biosyntheses in prokaryotes and plants: building blocks are marked in sienna and blue including positions where they end up in quinolic acid (**30**), niacin (**31**) and finally in $NAD(P)^+$ **2**. [a] Coenzyme-dependent enzymes (bacteria, archaea): FAD: L-aspartate oxidase (NadB); Fe_4S_4: quinolinate synthetase (NadA). [b] Coenzyme-dependent enzymes (eukaryotes): heme: tryptophan 2,3-dioxygenase (TDO); NADPH: kynurenine 3-monooxygenase (KMO); PLP: kynureninase (KYN); Fe^{II}: 3-hydroxyanthranilate-3,4-dioxygenase (HAD). [c] Both pathways: ATP for transformation of NAD^+ to $NAD(P)^+$.

infancy, it can be noted that it should not be able to perform classical N5-based flavin chemistry. Instead, it is capable of forming cycloadducts with dipolarophiles as well as with long-lived C4a-based radical species. In addition, photochemically driven isomerization chemistry was described.

Comparing FMN/FAD biosynthesis with that of prFMN **36**, it is evident that the latter must be an evolutionary downstream metabolite that appeared on the scene only after riboflavin-based coenzyme biosynthesis was established.

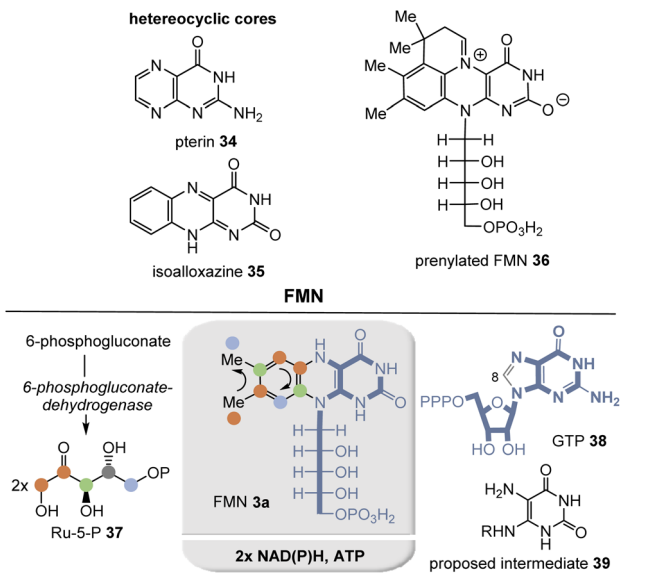


Fig. 4 Structures of pterin **34**, isoalloxazine **35** and prFMN **36** (top). Summary of flavin biosynthesis with building blocks marked in sienna and blue including positions where they end up (bottom). Coenzyme-dependent enzymes: NAD(P)H: 6-phosphogluconate-dehydrogenase and 5-amino-6-(5-phosphoribosylamino)uracil reductase (*ribD*); ATP: riboflavin kinase (*ribF*); biosyntheses in fungi and bacteria only differ in timing of individual steps.

The group of 5-deazaflavins includes coenzymes F_0 and F_{420} **21a** and **21b**, which are thought to be truly ancient redox factors.[19] Functionally, 5-deazaflavin F_{420} **21b** facilitates various two-electron redox reactions in methanogenic, sulfate-reducing, and probably methanotrophic archaea. In addition to its role in methanogenesis, coenzyme F_{420} is also involved in antibiotic biosynthesis and DNA repair.[20] Both structurally and chemically, coenzymes F_0 and F_{420} **21a** and **21b** are more closely related to nicotinamide than to flavins, which is why they have occasionally been referred to as "nicotinamide in a flavin's clothing".[21]

The biosynthesis of the deazaflavin chromophore of F_{420} is supposed to start from tyrosine (**41**) and GTP **38**, respectively[22] and is closely related to the biosynthesis of riboflavins **3** (Fig. 6) Again, diaminopyrimidine-dione **39** serves as one key intermediate. Interestingly L-tyrosine (**41**) is first oxidized to the phenol radical which spontaneously fragments to *p*-methide quinone **42**. It was suggested that Michael addition of C-5 in **39** occurs onto the δ-position in **42** and later a second Michael addition of

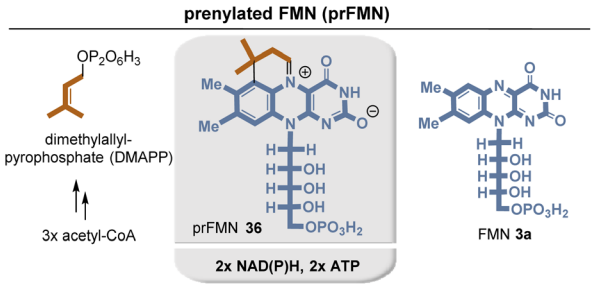


Fig. 5 Summary of prenylated flavin mononucleotide biosynthesis with building blocks marked in sienna and blue including positions where they end up in prenylated flavin mononucleotide (prFMN) **36**. Coenzyme-dependent enzymes (refer to the synthesis of DMAPP by the mevalonate pathway): NAD(P)H: HMG-CoA reductase; ATP: mevalonate kinase and phosphomevalonate kinase.

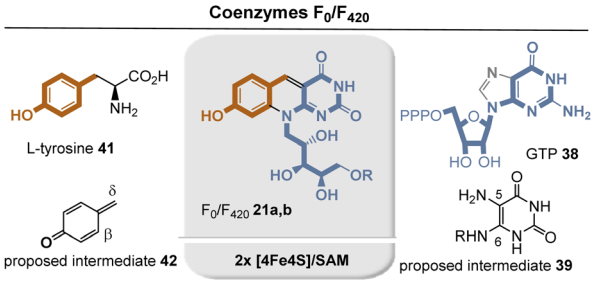


Fig. 6 Summary of coenzymes F_0/F_{420} biosynthesis: building blocks are marked in sienna and blue including positions where they end up in coenzymes F_0/F_{420} **21a** and **21b**. Coenzyme-dependent enzymes: Fe_4S_4/SAM: F_0 synthase (sequentially generates the adenosyl radical at two separate sites).

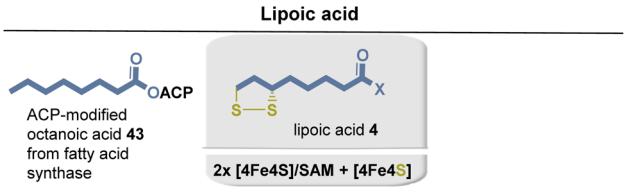


Fig. 7 Summary of lipoic acid biosynthesis: carbon atoms marked in blue and positions where they end up in lipoic acid **4** (for fatty acid biosynthesis, NADH and $FADH_2$ are required as coenzymes, as well as ATP and CoA for acyl activation; if malonyl-CoA acts as an extender, biotin would be required as a third coenzyme). Coenzyme-dependent enzymes: Fe_4S_4/SAM/F_4S_4: lipoate synthase (LipA).

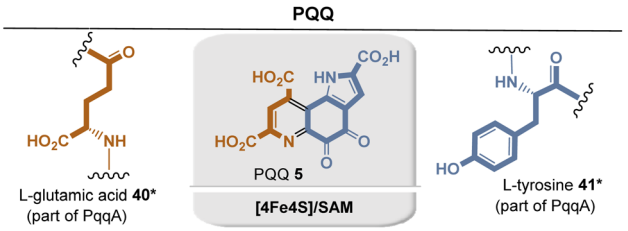


Fig. 8 Summary of PQQ biosynthesis: building blocks are marked in sienna and blue including positions where they end up in PQQ **5**. Coenzyme-dependent enzymes: Fe_4S_4/SAM: PqqE.

the amino nitrogen at C-6 onto the β-position of **42** sets up the tricyclic system. In between a second H radical abstraction at the benzylic position initiates removal of the amino group at C-5 through elimination. The two radical abstraction steps are promoted by radical SAM/Fe_4S_4.[22a]

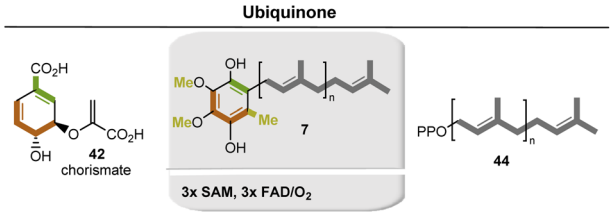


Fig. 10 Formation of ubiquinone (**7**). Coenzyme-dependent enzymes: SAM: methyl transferase; FAD: monooxygenases UbiB, UbiH, and UbiF.

There is a direct link to thiamine pyrophosphate (TPP, **14**, *vide supra*)[23] and the [FeFe]-hydrogenase maturase HydG, the latter being responsible for the synthesis of the CO and CN– ligands present in [FeFe]-hydrogenase H cluster which is generated from tyrosine-derived dehydroglycine (NH=$CHCO_2H$) (see also section 2.2.2).[24] Both enzymes yield cresol as a byproduct resulting from reduction of **42**.

2.1.3 Lipoic acid (4). Lipoic acid (**4**), a metabolite derived from the fatty acid octanoic acid **43** operates almost universally in aerobic metabolism.[25] In the first step, ACP-activated octanoic acid bound to fatty acid synthase is transferred to LipB (Fig. 7). Then, incorporation of the disulfide moiety by the enzyme LipA is initiated by radical SAM chemistry using the 5′-deoxyribosyladenosyl 5′-radical as a key mediator. A second iron–sulfur cluster has been identified as the source of the sulfur atoms.[26] The incorporation occurs in a manner similar to S-transfer in biotin biosynthesis (*vide supra*).[27]

2.1.4 Pyrroloquinoline quinone (5). Pyrroloquinoline quinone (PQQ, **5**) is a redox coenzyme found in many prokaryotes and is produced from a ribosomal and posttranslationally modified peptide PqqA.[28] Here, the glutamic acid (**40***) and tyrosine (**41***) residues represent the critical amino acids of the precursor peptide (Fig. 8).

SAM-mediated H-radical abstraction at the γ-position of the glutamic acid side chain by the enzyme PqqE initiates the formation of a C–C bond, with the C atom *ortho* to the phenol group. This step requires the presence of the chaperone PqqD, which forms a complex with PqqA prior to oxidation. Further details of PQQ biosynthesis have not yet been fully elucidated. Only the final steps promoted by the PqqC enzyme have been studied *in vitro*. It promotes an aza-Michael ring closure and an

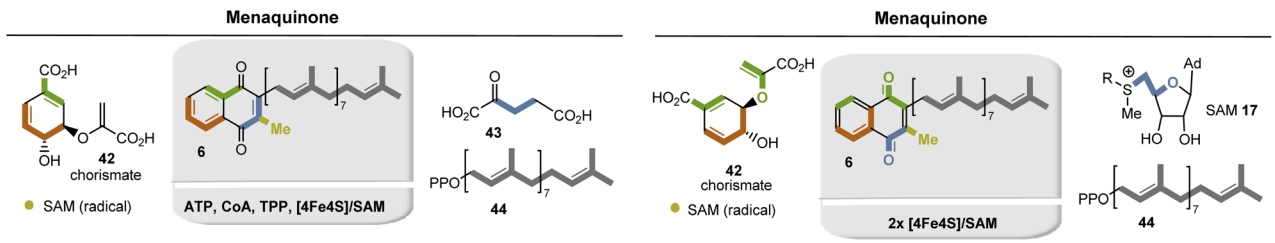


Fig. 9 Two biosynthetic routes towards menaquinone (**6**). Coenzyme-dependent enzymes (left): ATP/CoA: succinylbenzoate–CoA ligase (MenE); TPP: 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (MenD); radical SAM: (right): radical SAM: aminofutalosine synthase (MqnE); dehypoxanthinyl futalosine cyclase (MqnC); radical SAM: demethylmenaquinone methyltransferase (DmtH).

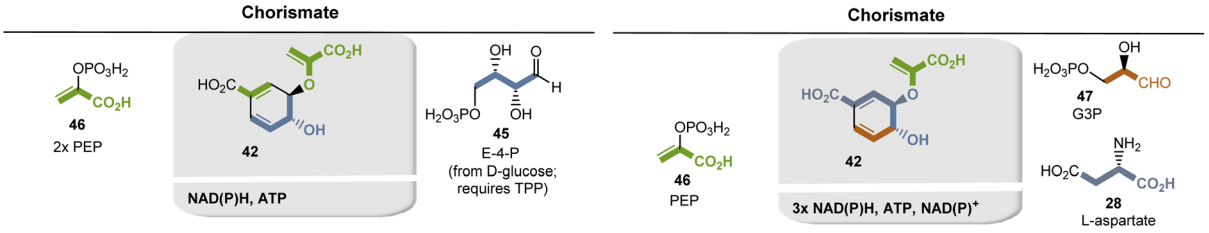


Fig. 11 Formation of chorismate (**42**). Coenzyme-dependent enzymes (left): NAD(P)H: shikimate dehydrogenase; ATP: shikimate kinase; (right): NAD(P)H: aspartate semialdehyde dehydrogenase and methylglyoxal dehydrogenase; ATP: aspartate kinase; NAD^+: responsible for an oxidative deamination.

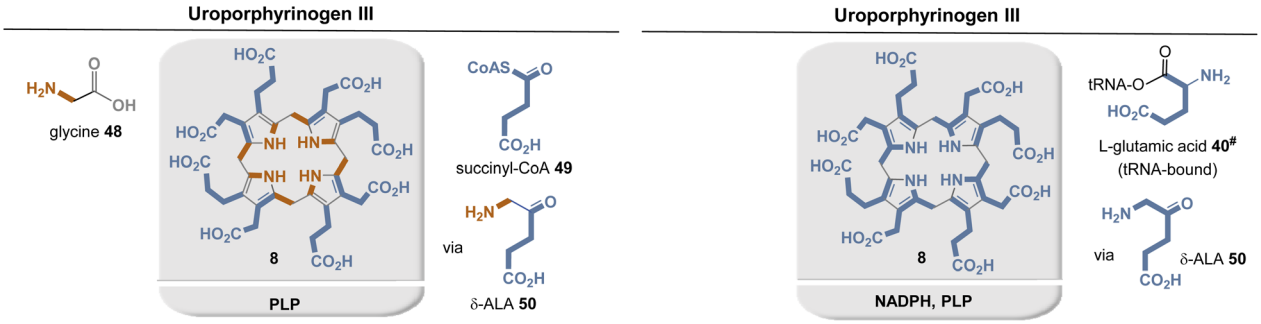


Fig. 12 Summary of uroporphyrinogen III biosynthesis: building blocks are labelled in sienna and blue at positions where they end up in uroporphyrinogen III (**8**). Coenzyme-dependent enzymes (left): PLP: ALA synthase (hemA). Coenzyme-dependent enzymes (right): NADPH: glutamyl-tRNA reductase (GluTR); PLP: glutamate-1-semialdehyde aminomutase (GSAM).

otherwise unknown eight-electron oxidation with O_2 as oxidant.[29]

2.1.5 Naphthoquinone coenzymes menaquinone (6) and ubiquinone (7). Menaquinone (**6**) is a coenzyme bearing a quinone unit that is involved in electron transfer between membrane-bound redox enzymes and is commonly found in the respiratory and photosynthetic systems of microorganisms and plants.[30] In addition, demethylmenaquinone is known that lacks the methyl group that is marked in yellow in Fig. 9, and both quinone derivatives are produced by essentially identical biosynthesis pathways (Fig. 9, left). In the biosynthesis of demethylmenaquinone the methyl transferase is missing. Isoprenoid side chains can vary in size depending on the number of monomeric C5 isoprene units, with $n = 7$ and 8 being the most common. Menaquinone biosynthesis commences from chorismate **42** in *Escherichia coli*.[31]

The formation of the second ring is initiated by TPP-mediated acylation with α-ketoglutarate **43** as building block and the other substituents, the methyl group and the isoprene side chains (oligoprenoid pyrophosphates **44**) are finally introduced by electrophilic substitutions.

Bioinformatic analyses of whole-genome sequences have suggested that some microorganisms, such as *Helicobacter pylori* and *Campylobacter jejuni* use another route to menaquinone (Fig. 9, right).[32] Both biosynthetic pathways diverge at chorismate **42** and re-converge again after the formation of naphthoquinol. Between these two points, the two pathways differ fundamentally. In contrast to the first *o*-succinylbenzoate pathway the futalosine pathway relies heavily on radical chemistry triggered by radical SAM.[33] SAM also provides two carbons that become part of the quinone ring.

Ubiquinone (**7**) is an important coenzyme of electron transfer chains in proteobacteria and eukaryotes. Recent results indicate a rather large diversity of biosynthetic pathways in bacteria (Fig. 10).[34] The first phase of the common pathway to ubiquinone is the shikimate pathway. Chorismate **42** is first converted to *p*-hydroxybenzoate, which undergoes prenylation, two monooxygenase-mediated hydroxylations, and a third after decarboxylation. In addition, two phenolic groups and the last free position on the aromatic ring are methylated. Accordingly, the coenzymes SAM **17** and FAD **3** are required repeatedly. Structurally related to ubiquinone are plastoquinone and rhodoquinone which are not covered here.[34]

The key building block chorismate **42** is a product of the shikimate pathway. This in turn serves to provide access to aromatic amino acids and various other aromatic natural substances.[35] An aldolase-mediated coupling of erythrose-4-phosphate (E4P, **45**, formed from D-glucose and requires TPP) and phosphoenolpyruvate (PEP, **46**), followed by an intramolecular aldol reaction producing 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), from which shikimic acid and finally chorismate are formed (Fig. 11, left).

An alternative, TPP-free entrance into the shikimate pathway was found in some archaea such as *Methanocaldococcus jannaschii* (Fig. 11, right). 6-Deoxy-5-ketofructose-1-phosphate is formed from glyceraldehyde-3-phosphate (G3P, **47**) and from

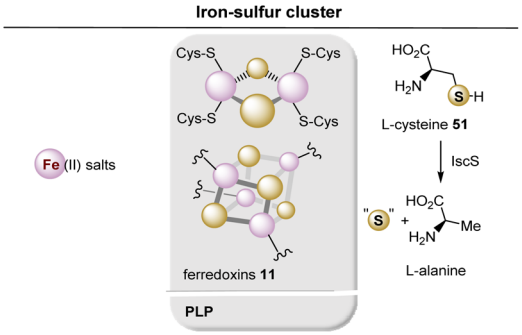


Fig. 13 Summary of ferredoxin (iron–sulfur cluster) **11** generation. Coenzyme-dependent enzymes: PLP: cysteine desulfurase (IscS).

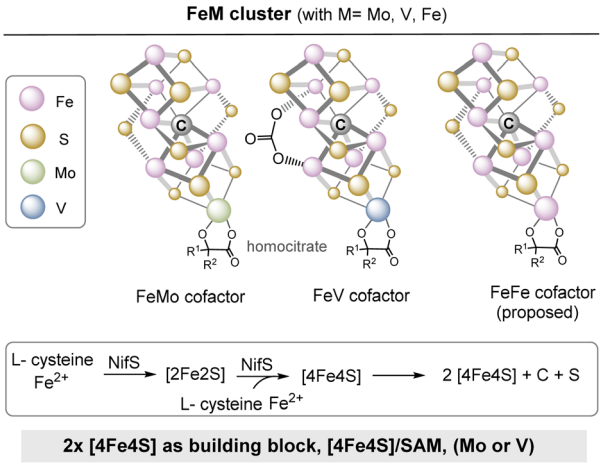


Fig. 14 Structures of FeM (M = Mo, V, Fe) cofactors found in different nitrogenases and the coenzymes involved in their synthesis (starting from the [4Fe–4S] cluster). Coenzyme-dependent enzymes: Fe_4S_4/SAM, NifB (linking two [4Fe–4S] subcubanes *via* sulfur and a carbon).

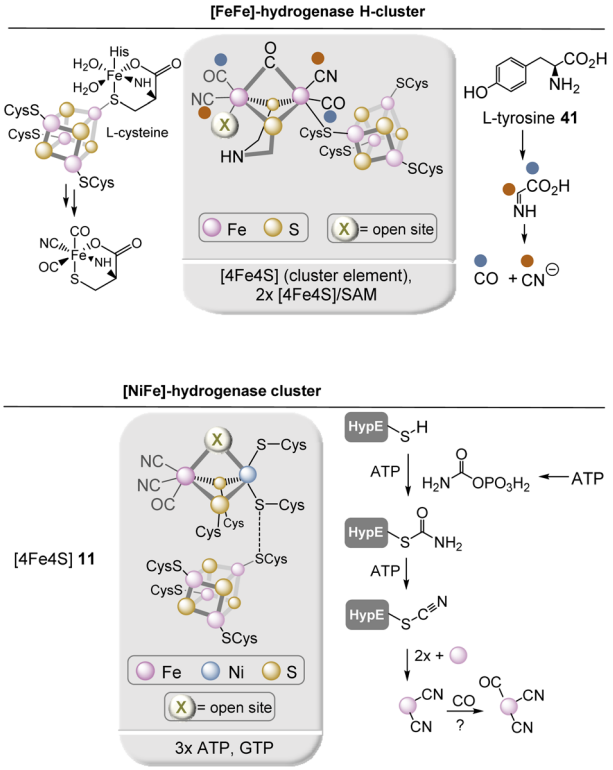


Fig. 15 (a) Structure of the [FeFe]-hydrogenase H-cluster and the coenzymes involved in their biosynthesis (hypothesis). Coenzyme-dependent enzymes: [4Fe–4S]/SAM = Fe–S maturase composed of HydG, HydE, HydF. (b) Structure of the [NiFe]-hydrogenase cluster and the coenzymes involved in their biosynthesis (hypothesis). Coenzyme-dependent enzymes: ATP = HypF and carbamoyl phosphate synthase; GTP = HypA/HypE (Ni-insertase).

aspartate **29a**, both of which are first reduced to the corresponding aldehydes.[36] Oxidative deamination leads to 4,5-dihydroxy-2,6-dioxoheptanoic acid which is converted to 3-dehydroquinic acid and hence to chorismate **42**.

2.2. Biosynthesis of transition metal dependent coenzymes

2.2.1 Uroporphyrinogen III (8). Uroporphyrinogen III (**8**) is an important biosynthetic precursor for heme, cofactor F_{430}, cobalamins, and siroheme (**9**) and the last common biosynthetic precursor for all tetrapyrroles.[37,38] Porphyrin-containing proteins are ubiquitously distributed in all kingdoms of life, and among them, heme and chlorophylls are the most important members.[39]

Siroheme (**9**) is derived from **8** and is part of the active sites of enzymes responsible for the six-electron reduction of sulfur and nitrogen. Structurally, corrin macrocycle **10** is related to uroporphyrinogen III, but the ring size is reduced by one carbon atom compared to **8**. The corrin macrocycle is found in cobalamin and frequently forms complexes with cobalt(I) (see also section 2.4.1). From an evolutionary perspective, it seems reasonable to assume that transition metals such as Fe, Ni, Co, Mo, W, and others dominated redox chemistry during the transitional phase from protometabolism to the biotic world.[40,41]

The two known biosynthetic pathways for uroporphyrinogen III (**8**), found in all kingdoms of life including archaea,[38,42] utilize 5-aminolevulinic acid (δ-ALA, **50**) as a linear precursor (Fig. 12, left). δ-ALA is biosynthesized either from glycine (**48**) and succinyl-CoA (**49**) or from glutamyl-tRNA (**40**#) in a two-step enzymatic process. In the first case, ALA synthase catalyzes the decarboxylative coupling of glycine to succinyl-CoA and this is catalyzed by PLP **19**. In the second pathway, NADPH **2b** and PLP **19** participate as coenzymes in the biosynthesis of δ-ALA **50** (Fig. 12, right). Subsequently, eight molecules of δ-ALA are condensed, which finally form macrocycle **8**.

2.2.2 Ferredoxins 11 and other metal-sulfur clusters. Ferredoxins **11** are proteins that contain [Fe–S] clusters and these represent a versatile and modular system that is widely distributed in nature. While the [2Fe–2S] and [3Fe–4S] clusters are mainly used for one-electron transfer reactions, the chemistry of the [4Fe–4S] clusters is much more diverse. In addition to electron transfer and initiation of radical chemistry, they also play a role in the coupling of proton and electron transfer, substrate binding and activation. They also regulate enzyme

activity and gene expression, recognize reactive species, and donate sulfur.[43]

Although [Fe–S] centers in proteins can assemble spontaneously, they require iron and sulfide in concentrations that far exceed those found in cells. Such concentrations, however, would be highly toxic, and indeed a complex mechanism has evolved for their biosynthesis.[44] Thus, three major pathways have been identified: the Isc system (iron–sulfur cluster), the Suf system (sulfur formation), and the Nif system (nitrogen fixation). Without going into too much detail within the scope of this report, one pathway involves the donation of sulfur catalyzed by cysteine desulfurase (IscS) in which L-cysteine (**51**) serves as sulfur donor (by transforming a cysteine residue in IscS into active R-S-S-H), while iron is provided by an iron chaperone (such as CyaY) or by direct iron capture at IscU (Fig. 13).[45]

The central iron–sulfur cluster motif is replicated in several other variants that can be regarded to be "follow up" clusters. The FeMo cofactor has the stoichiometry Fe_7MoS_9C and is the most important cofactor of the nitrogenase.[46] This is the key enzyme in nitrogen fixation, in which molecular nitrogen (N_2) is reduced to ammonia (NH_3).[47] The cluster consists of an Fe_4S_3 sub-cluster and a $MoFe_3S_3$ sub-cluster and the two sub-clusters are connected by three sulfide bridges (Fig. 14). Structurally closely related are VFeco and FeFeco, in which the replacement of molybdenum by vanadium and iron, respectively, is the most important new feature.[46,48] It has to be noted that the MoFe protein is an $\alpha_2\beta_2$ heterotetramer, while VFe and FeFe proteins are $\alpha_2\beta_2\gamma_2$ heterohexamers.

Three proteins (NifH, NifEN, NifB) serve for the biosynthesis of the FeMo cofactor.[48] NifB is responsible for the assembly of the Fe–S core, in which two [4Fe–4S] clusters are stitched together. It relies on the coenzyme SAM **17** and [4Fe–4S] to provide the carbide-like carbon atom located in the center of the iron–sulfur cluster. Thus, [4Fe–4S] has two roles here: (a) a building block and (b) cofactor to merge two [4Fe–4S] cubanes. Noteworthy, it is speculated that the FeMo cofactor, and thus the nitrogenases need the extra carbon atom as part of the metal sulfur cluster architecture to acquire catalytic activity, since the carbon atom keeps the structure rigid.

In the context of Fe- and S-containing metalloclusters, it is sensible to include hydrogenases in the discussion.

Hydrogenases are enzymes that catalyze the utilization and production of H_2. They are particularly essential for the anaerobic bacteria as well as sulfate-reducing bacteria of the genus *Desulfovibrio*. The best known hydrogenases are [NiFe] hydrogenases found in bacteria and archaea as well as [FeFe] hydrogenases found in bacteria and eukaryotes. Key elements of these hydrogenases are [FeFe]-hydrogenase H-cluster and [NiFe]-hydrogenase cluster (Fig. 15).[49] In addition to the dinuclear metal center, both hydrogenases have at least one [4Fe–4S] cluster positioned nearby. Independent of these hydrogenases, there is a third type of hydrogenase, the [Fe] hydrogenase that bears a mononuclear iron center. It will be covered in section 2.6. The biosynthesis of the [2Fe] H-subcluster[50] requires several maturases that rely on or require iron–sulfur clusters. A bifunctional radical S-adenosylmethionine (SAM) enzyme

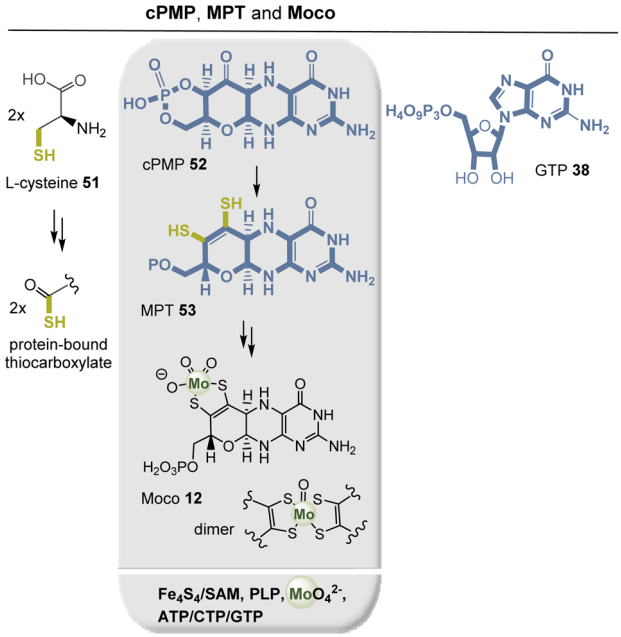


Fig. 16 Summary of molybdenum cofactor biosynthesis: building blocks are marked in sienna and blue at positions where they end up in cPMP **52** and MPT **53**, precursor of molybdenum cofactor Moco **12**. Coenzyme-dependent enzymes: Fe_4S_4/SAM: molybdenum cofactor biosynthesis protein MoaA; PLP: cysteine desulfurase (IscS); ATP/CTP/GTP: late stage modification of Moco **12**.

(HydG) first cleaves tyrosine **41** to finally generate CO and cyanide. The resulting organometallic precursor that contains an $Fe(CO)_2(CN)$ moiety is eventually incorporated into the H-cluster. So far it is only known that these two reactions are carried out by two Fe–S clusters in HydG but many details have not been elucidated so far.

The biosynthesis of the $Fe(CN)_2CO$ complex occurs in a protein cluster composed of HypC, HypD, and HypE. Here, cyanide originates from enzyme-bound thiocarbamoylate and requires ATP for activation before cyanide is transferred to iron. Interestingly, enzyme-bound carbamothioate has been proposed as intermediate.

Other important iron–sulfur-containing clusters are associated with C1 fixation, specifically in the Wood–Ljungdahl pathway. Of particular note here are carbon monoxide dehydrogenase (CODH) and acetyl-CoA synthase (ACS), which are formed from the [4Fe–4S] cluster and are mixed with nickel atoms. However, they will not be discussed in this report.

2.2.3 Cyclic pyranopterin monophosphate (cPMP, 52), molybdopterin (MPT, 53) and molybdenum cofactor (Moco, 12). Molybdenum is an essential micronutrient found in all kingdoms of life. This metal exhibits very rich coordination and redox chemistry, and it is the only member of the second transition series with important biological functions.[51] Without being embedded in a ligand sphere, it is biologically unimportant. Moco **12** is such a molybdenum complex containing one or two dithiolate ligands. It is based on a tricyclic pterin moiety commonly referred to as molybdopterin (MPT, **53**).

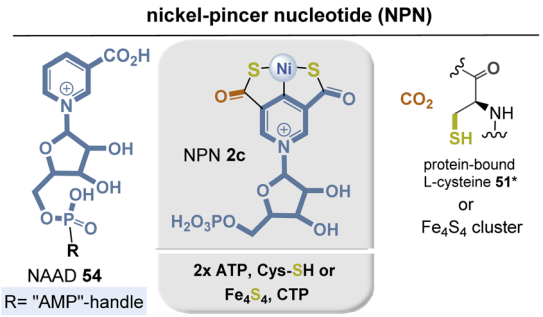


Fig. 17 Summary of nickel–pincer nucleotide (NPN) (**2c**) biosynthesis: building blocks are marked in sienna, blue and yellow at positions where they end up in NPN. Coenzyme-dependent enzymes: ATP/cystidyl or Fe_4S_4: sulfur insertase (LarE), Ni/CTP: nickel insertase (LarcC).

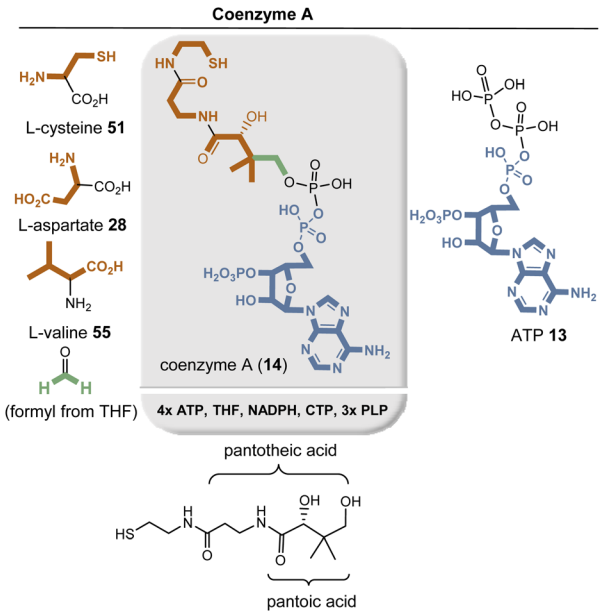


Fig. 18 Summary of coenzyme A (**14**) biosynthesis: building blocks are marked in sienna, blue and green at positions where they end up in coenzyme A. Coenzyme-dependent enzymes: ATP: PanA, CoaC, CoaD, CoaE; CTP: CoaB; THF: ketopantoate hydroxymethyltransferase (PanB); NADPH: ketopantoate reductase (PanE); PLP: aspartate 1-decarboxylase (PanD), 4′-phosphopantothenoyl cysteine decarboxylase (CoaC) and valine-pyruvate transaminase.

The molybdenum cofactor (Moco, **12**) and its dimer (Fig. 16) are part of redox enzymes, and the pterin ligand controls the redox behavior of the metal. Moco and its dimer are capable of transferring an oxygen atom that is ultimately extracted from or incorporated into water. Typical Moco-dependent enzymes include formate dehydrogenase, sulfite oxidase, nitrate reductase, and glyceraldehyde-3-phosphate ferredoxin oxidoreductase.[51,52] Moco biosynthesis is evolutionarily conserved, occurring in eukaryotes as well as eubacteria and archaea. In bacteria, the biosynthesis of cyclic pyranopterin monophosphate (cPMP, **52**) and MPT **53**, and thus of Moco **12**, can be divided into the following main steps: (a) formation of cPMP **52**, (b) formation of MPT **53**, (c) insertion of molybdenum into molybdopterin to form Moco, and (d) the additional modification of Moco by addition of GMP or CMP to the phosphate group in MPT **53**.[53]

GTP **38** serves as the starting point for the biosynthesis of all pterin-containing enzymes. Mediated by SAM, the cyclic pyranopterin monophosphate (cPMP, **52**) is formed in a radical cascade. The dithiolene function in MPT is generated by PLP-mediated desulfurization of two protein-bound terminal thiocarboxylates that are generated from a persulfide made *via* free L-cysteine. This protein-mediated sulfur transfer is similar to the S-transfer process also found in TPP biosynthesis (see section 2.5.2). The two thiol groups in MPT **53** finally serve as ligands to trap inorganic molybdate (MoO_4^{2-}). Remarkably, tungsten appears to be the physiologically active metal for MPT **53** in hyperthermophilic archaea. Tungsten plays a role in aldehyde ferredoxin oxidoreductase (AOR), formaldehyde ferredoxin oxidoreductase (FOR), and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) in archaea.

2.2.4 Nickel–pincer nucleotide (NPN). The structurally unique nickel–pincer nucleotide cofactor (NPN) **2c** was recently discovered in studies on lactate racemase (LarA) from *Lactobacillus plantarum*.[54] There, this so-called nickel–pincer nucleotide (NPN) functions as a transient hydride acceptor.

Apparently, it is recruited from $NAD(P)^+$ biosynthesis with the intermediate nicotinic acid adenine dinucleotide (NAAD) **54** serving as precursor.[55] An initial protein-bound cysteine-mediated activation of the pyridinium ring allows the introduction of the second carboxylate at C5 with CO_2 as C1 source. Carboxylation is accompanied by hydrolysis of the diphosphate moiety and cleavage of AMP. Next, sulfur transfer occurs from cysteine (in *L. plantarum*) to form dehydroalanine or from a [4Fe–4S] cluster (in *Thermotoga maritima*) that repeatedly can act as a sulfur source in the presence of cysteine desulfurase (IscS) and free L-cysteine (Fig. 17). In this process, the thiocarbonyl functions are formed after ATP-mediated activation of the carboxylic acids. Finally, nickel is introduced by a CTP-dependent nickel insertase. Bioinformatics studies show that the biosynthetic genes are widely distributed in microorganisms, so the full potential of NPN has not yet been fully explored.

2.3. Coenzymes in functional group activations

2.3.1 Adenosine triphosphate (13). The nucleotide adenosine triphosphate (**13**) is the most abundant chemical energy currency in the biotic world. It is usually formed, catalyzed by ATP synthase, from adenosine diphosphate (ADP) and inorganic phosphate (P_i). The process is energetically unfavorable, which is why this step is usually coupled to an electrochemical gradient with cellular respiration. In bacteria, this gradient results from the difference in proton concentration at the plasma membrane.

The evolution of ATP synthase has been extensively studied and is thought to have begun with the merging of two functionally independent subunits, a presumably early step in evolution, since the structure and activity of ATP synthases are found in all phylogenetic trees.[56]

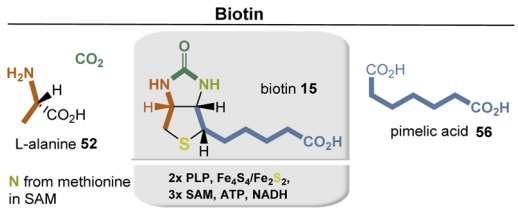


Fig. 19 Summary of biotin (**15**) biosynthesis: building blocks are marked in sienna, blue, yellow and green at positions where they end up in biotin (**15**). Coenzyme-dependent enzymes: SAM: *O*-methyltransferase (BioC); SAM/Fe_4S_4/Fe_2S_2: biotin synthase (BioB); PLP: 8-amino-7-oxononanoate synthase (BioF), PLP/SAM: 7,8-diaminononanoate synthase (DANS, BioA); ATP: dethiobiotin synthase (DTBS, BioD); NADH: fatty acid synthase (FabI and FabG).

2.3.2 Coenzyme A (14). Coenzyme A (**14**) serves to chemically activate carboxylic acids as corresponding thioesters. It consists of cysteamine, β-alanine, pantoic acid, and adenosine diphosphate (ADP) (Fig. 18).[57] In its acetyl form, coenzyme A (**14**) is widely found and performs metabolic functions in both anabolic and catabolic pathways.

Several amino acids are required for the biosynthesis of coenzyme A (**14**), namely L-cysteine (**51**), L-aspartate (**29a**), and L-valine (**55**), as well as the nucleotide ATP **13**.[58] PLP-mediated decarboxylations of aspartate and cysteine, the latter already bound to pantothenic acid, give rise to the β-alanine and cysteamine units in coenzyme A (**14**). Biosynthesis of pantoic acid begins with L-valine (**55**), which is transaminated by PLP to 3-methyl-2-oxobutanoic acid and formylated with formaldehyde derived from the coenzyme THF **16**. The keto group is then reduced to the secondary alcohol of pantoic acid with the assistance of NADPH, and subsequently the individual building blocks are linked together with the aid of ATP and CTP.

2.3.3 Biotin (15). The coenzyme biotin (**15**) serves as a carboxyl group carrier agent, shuttling carbon dioxide equivalents onto nucleophilic organic substrates. This chemical property is localized in the ureido ring at N8 of the bicyclic system, which is additionally annulated with a tetrathiophene ring.[59] The reaction with carboxyphosphate, which is formed by ATP activation of bicarbonate, produces the intermediate *N*-carboxybiotin. This intermediate is stabilized by the particular geometry of the bicyclic ring system, which prevents sp^3 hybridization of the N8 nitrogen atom.

Biotin (**15**) is biosynthesized from L-alanine (**52**) and pimelic acid (**56**), with a variety of coenzymes involved in each enzymatic step (Fig. 19). Pimelic acid (**56**) is biosynthesized by a fatty acid synthase that uses malonyl-CoA methyl ester as one building block. Subsequently, several reduction steps are involved that require NADH as a coenzyme. PLP-dependent transamination, in which SAM serves as an amino donor, results in the formation of 7-keto-8-aminopelargonic acid (KAPA). This is probably the only known example of the use of SAM as a source of an amino group. The formation of the urea group from carbonate is promoted by ATP. The sulfur atom is finally inserted from an iron–sulfur cluster (Fe_2S_2) by a radical SAM-promoted process (Fe_4S_4/SAM).

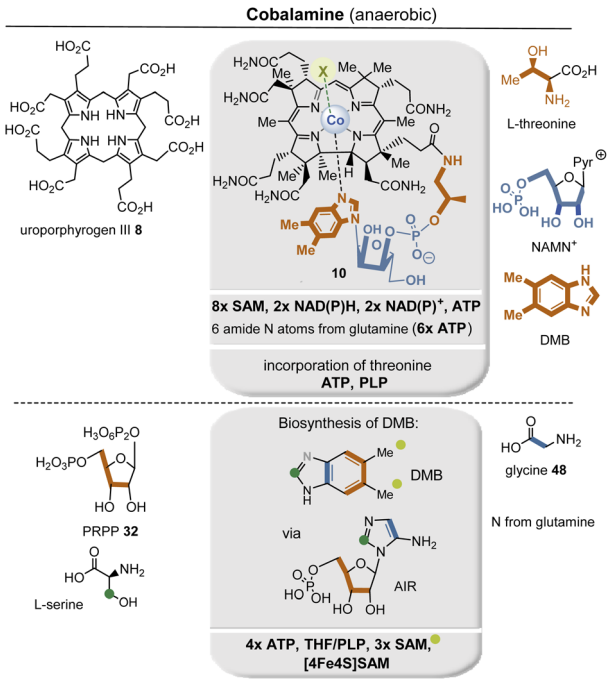


Fig. 20 Summary of cobalamin (**10**) biosynthesis: the building blocks are marked in sienna and blue at positions where they end up in cobalamine. In addition, the ligand **X** and the central metal are coloured. Coenzyme-dependent enzymes in anaerobic organisms (top): SAM: CysG, CbiL, CbiH, CbiF, CbiD, CbiE/T; NAD(P)H: CbiJ, CobR; NAD^+: CysG; ATP = CobA. Coenzyme-dependent enzymes to introduce threonine: ATP: L-threonine kinase; PLP: L-threonine-*O*-3-phosphate decarboxylase (CobD). Coenzyme-dependent enzymes in anaerobic organisms (bottom): 3× SAM: methyltransferases BzaC, BzaD, and BzaE; THF: phosphoribosylglycinamide formyltransferase, [4Fe–4S]SAM: hydroxybenzimidazole (HBI) synthase.

2.4. Coenzymes for C1-transfer reactions

2.4.1 Cobalamin (10). Cobalamin **10** plays a prominent role in radical-initiated rearrangements and transfers of methyl groups, as in methionine synthase and dehalogenation

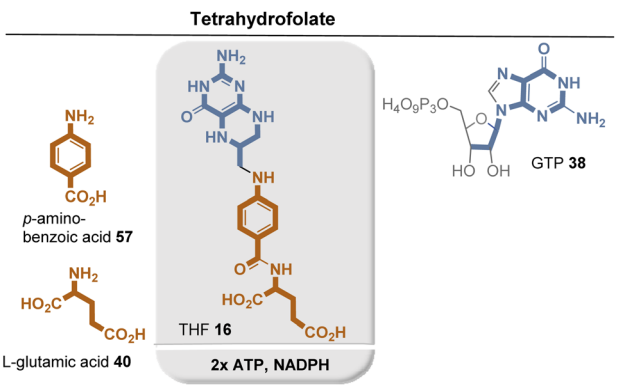


Fig. 21 Summary of tetrahydrofolate biosynthesis: building blocks are marked in sienna and blue at positions where they end up in THF **12**. Coenzyme-dependent enzymes: ATP: 7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase (HPPK), folylpolyglutamate synthase (FPGS), NAD(P)H: dihydrofolate reductase (DHFS).

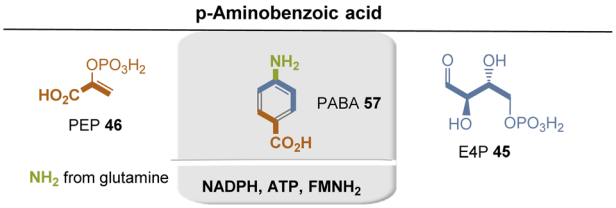


Fig. 22 Summary of *p*-aminobenzoic acid (**57**) biosynthesis: building blocks are marked in sienna, blue and green at positions where they end up in PABA. Coenzyme-dependent enzymes: NADPH: shikimate dehydrogenase (EC 1.1.1.25); ATP: shikimate kinase (EC 2.7.1.71); $FMNH_2$: chorismate synthase (EC 4.2.3.5).

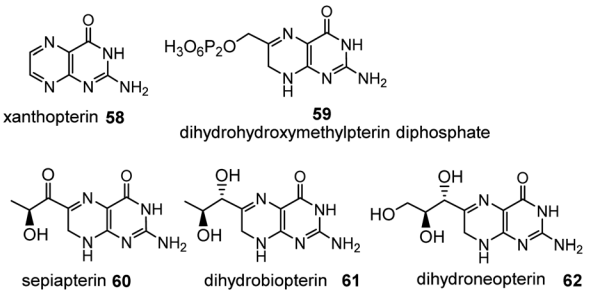


Fig. 24 Structures of natural pterins **58**–**62**.

reactions. In these methyl transfer reactions, cobalt(I) serves as a transition metal able to accept a methyl group.[60] Its biosynthesis is confined to a few bacteria and archaea and it starts with uroporphyrinogen III **8** and is characterized by eight SAM-mediated methylations and a ring contraction (Fig. 20). In addition, the so-called lower ligand consists of amino-2-propanol derived from L-threonine (*via* 2-amino-3-oxobutanoate), a ribosyl phosphate moiety derived from nicotinamide mononucleotide (NAMN), and the unusual dimethylbenzimidazole (DMB), whose central precursor is phosphoribosylaminoimidazole (AIR).[61]

2.4.2 Tetrahydrofolic acid (16) and THMPT (24). Tetrahydrofolic acid (**16**) is a coenzyme that serves as an electrophilic donor for a carbon atom in various oxidation states (methyl, methylene, and methine).[62] Tetrahydrofolate is charged with formaldehyde, which in turn may be derived from L-serine.[63] Folates are involved in the biosynthesis of purines and pyrimidines such as inosine monophosphate and 2′-deoxythymidine-5′-phosphate (dTMP) from 2′-deoxyuridine-5′-phosphate (dUMP), which is catalyzed by thymidylate synthase (Fig. 21).

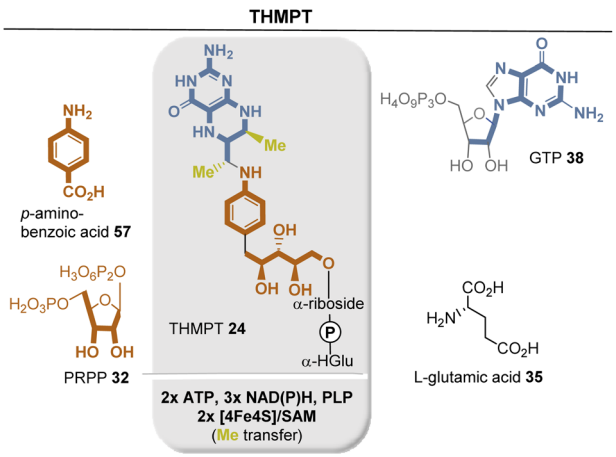


Fig. 23 Summary of tetrahydro-methanopterin biosynthesis: building blocks marked in sienna, blue and yellow and positions where they end up in THMPT **24** (α-HGlu = α-hydroxyglutarate). Coenzyme-dependent enzymes: The identification of enzymes involved in the biosynthesis has proved very difficult, and many of the enzymes in the later part of the pathway are still unknown so that only established enzymes are listed. ATP: 7,8-dihydro-6-hydroxy-methylpterin-pyrophospho-kinase (HPPK); folylpolyglutamate synthase (FPGS); NADPH: dihydrofolate reductase (DHFS).

THF **16** consists of a pterin core, *p*-aminobenzoic acid (PABA, **49**), and glutamic acid (**35**). It is biosynthesized in plants and fungi as well as in certain protozoa, bacteria, and archaea. Similar to the biosynthesis of riboflavins and deazaflavins and molybdenum cofactor, THF biosynthesis relies on GTP (**33**) as the starting point for the assembly of the pterin core.[64] Zn-promoted hydrolysis of GTP by GTP cyclohydrolase I (GTPCH1h) yields the bicyclic pterin core, while formic acid is formed as a byproduct. Dihydroneopterin aldolase (DHNA) removes two carbon atoms of the original D-ribose in GTP in the form of glycoaldehyde. Only the two amide-forming steps require the assistance of the external coenzyme ATP. Finally, the resulting dihydrofolate is reduced to THF **12** by the NAD(P)H-dependent dihydrofolate reductase.

PABA **57** is formed *via* the shikimate metabolic pathway (see Fig. 22),[35,65] which has been identified in bacteria, archaea, fungi, algae, some protozoa, and also in plants. Therefore, its biosynthesis is also analyzed in terms of the coenzymes required for its biosynthesis. PEP **46** and E4P **45** are the starting point, and three coenzyme-dependent enzymes are involved on the way to the chorismate **42**, requiring NADPH, ATP, and $FMNH_2$, respectively. Remarkably, one of these enzymes, chorismate synthase, uses $FMNH_2$ in a redox-neutral elimination process of phosphate. Finally, the amino group is introduced at the chorismate level, with glutamine serving as the amino donor. Since chorismate **42** is an important intermediate in PABA biosynthesis, a second molecule of PEP **46** is required but removed in the final step once the aromatic system has been established.

Chemically, the methanogenic coenzyme THMPT **24** and THF **16** behave similarly, as both are carbon carriers. Methanopterin is also capable of uploading oxidation states between formyl and methyl, but there are differences between the two pterin-based coenzymes. As such ATP is consumed in the entry of carbon from CO_2 into the 5,6,7,8-tetrahydrofolate pathway, which is not the case for tetrahydromethanopterin.

Biosynthetically, the pterin ring is derived from GTP **38**. The guanine ring of GTP is cleaved to release formic acid, followed by a recycle involving the ribose moiety (Fig. 23). Another interesting and unique transformation is the condensation of 4-aminobenzoate with the purine precursor 5-phospho-D-ribosyl diphosphate (**32**). This reaction is unique among known PRPP transferases in that a benzoate is decarboxylated to yield a C-

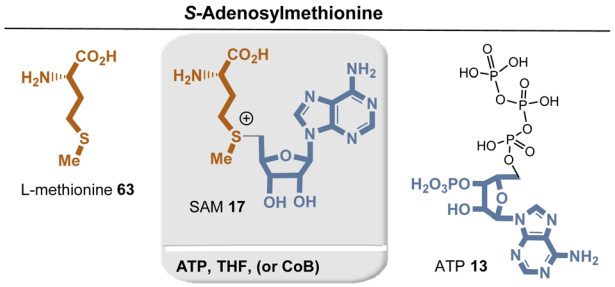


Fig. 25 Summary of *S*-adenosylmethionine (SAM) biosynthesis: building blocks are marked in sienna and blue at positions where they end up in SAM **17**. Coenzyme-dependent enzymes: ATP as building block. Regeneration of methionine occurs by THF- or cobalamin-dependent methionine synthases.

riboside. Finally, SAM and a [4Fe–4S] cluster serve for the radical methylation of C7 and C9 of the pterin system.[66]

2.4.3 Other pterins related to folic acid. The pteridine nucleus can undoubtedly be called a privileged heterobicyclic structure particularly suitable for redox chemistry. Other natural pterins **58–62** are found in various coenzymes with different chemical roles in nature (Fig. 24).

On the route to folic acid, biopterin **61** (dihydroform) and hydroxymethylpterin **59** (dehydroform) are formed, which show redox properties similar to riboflavins, with the 5,6,7,8-tetrahydro derivative being the reduced form.[67] The biopteridine redox system is of particular importance in the oxidation of aromatic rings. A distinctive feature of these oxidations is that they require the presence of molecular oxygen. They occur in all kingdoms of life, including archaea.[68]

Xanthopterin (**58**) occurs as a yellow pterin pigment in butterfly wings, for example. Sepiapterin (**60**) is a yellow pigment found in the eyes of Drosophila and is formed from D-*erythro*-dihydroneopterin triphosphate.

2.4.4 *S*-Adenosylmethionine (17). *S*-Adenosylmethionine (**17**) is a coenzyme involved in methyl group transfer reactions, with methyl transfer to nucleophilic C, N, O, and S centers being most common. In combination with single-electron transfer cofactors such as Fe_4S_4 clusters, it is involved in many radical processes.[69] SAM is biosynthesized by methionine adenosyltransferase from methionine (**63**) and adenosine triphosphate **13** (Fig. 25).

2.5 Other coenzymes for group transfer reactions

2.5.1 Pyridoxal phosphate (19). Pyridoxal phosphate (**19**) is a coenzyme that promotes a myriad of biotransformations mainly in amino acid metabolism. However, it is also very abundant in the biosynthesis of secondary metabolites such as polyketides, alkaloids and rare deoxyaminosaccharides. Typical reactions include transaminations in which the amino derivative pyridoxamine phosphate acts as a key intermediate, decarboxylations, racemizations, retro-aldol reactions, and Michael additions.[70] It also participates as a "partner" in radical-mediated reactions with radical SAM, as found in lysine 2,3-aminomutase.[71] Its unique chemical reactivity can be attributed to two structural elements. The aldehyde is capable of forming imines with amino groups, and protonation of the pyridine nitrogen atom removes electron density from the bound substrate, triggering various types of C–H and C–C bond cleavages. In this way, the N-heterocycle acts as an electron acceptor and donor system, which in turn provides several options for further transformations. In essence, pyridoxal phosphate serves as an equivalent for an enolizable carbonyl group equivalent that is only transiently bound to the substrate. PLP is also known to act as a singlet oxygen scavenger and to have protective effects against oxidative stress in fungi.

It is biosynthesized in microorganisms and plants, and to date two biosynthetic pathways have been described (Fig. 26).[72] The ribose-5-phosphate-dependent (or deoxy-xylulose-phosphate-independent) pathway, found for example in *Bacillus subtilis*, uses glyceraldehyde-3-phosphate **47** and ribose-5-phosphate (R5P, **64** in equilibrium with ribulose-5-phosphate (RuP)) as carbon sources, while the nitrogen atom is recruited from glutamine in the form of ammonia (Fig. 26, left).[73] This biosynthetic pathway has also been found to occur in archaea, fungi, and plants. PLP synthase, which carries an additional glutaminase site to provide ammonia, condenses **47** and **64** directly to PLP **19**. Remarkably, the entire metabolic pathway requires no other coenzyme except ATP to regenerate glutamine from glutamate.

The second metabolic pathway (Fig. 26, right) has been studied in detail in *E. coli*. It utilizes 3-hydroxy-aminoacetone phosphate (AHP, **65**) and 1-deoxy-xylulose 5-phosphate (DX5P, **66**), which are brought together by pyridoxine 5′-phosphate synthase to form the pyridine ring. AHP **65** is formed from erythrose 4-phosphate (**45**), which is oxidized to 4-

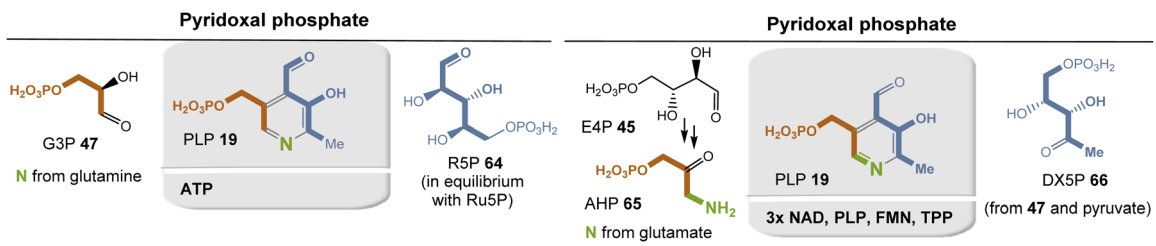


Fig. 26 Summary of pyridoxal phosphate biosynthesis: building blocks are marked in sienna, blue and green at positions where they end up in PLP **19**. Coenzyme-dependent enzymes (left): ATP: glutamine synthase. Coenzyme-dependent enzymes (right): NAD^+: erythrose-4-phosphate dehydrogenase; NAD^+: erythronate-4-phosphate dehydrogenase; NAD^+: 4-hydroxythreonine-4-phosphate dehydrogenase; PLP: phosphoserine aminotransferase; FMN: pyridoxine 5′-phosphate oxidase; TPP: 1-deoxyxylulose 5-phosphate synthase.

phosphoerythronate and further to 3-hydroxy-2-oxo-4-(phosphonatooxy)butanoate, 4-phosphohydroxy-L-threonine, and finally to AHP **65**. One coenzyme is required for each of the four steps (3× NAD^+ and 1× PLP). Finally, the resulting pyridoxine is oxidized to PLP **19**, for which FMN serves as a redox coenzyme. The biosynthesis of DX5P **66** begins with the TPP-dependent coupling of G3P **47** with pyruvate **67**. This route is clearly to be designated as a "straggler" pathway, precisely because PLP is needed here for its own formation.

2.5.2 Thiamine pyrophosphate (18). The coenzyme thiamine pyrophosphate (**18**) plays a key role in carbohydrate metabolism, where it is involved in the "Umpolung" of carbonyl groups and subsequently in formal acyl anion reactions.[74] Interestingly, the bacteria *Borrelia* and *Rickettsia* do not require TPP **18** for their metabolism.[75]

Nature has evolved three different biosynthetic pathways to TPP **18**, all of which are completed by linking hydroxymethylpyrimidine phosphate (HMP-P; **69**) with hydroxyethylthiazole phosphate (HET-P; **70**) in a substitution reaction followed by phosphorylation to the corresponding pyrophosphate (Fig. 27).

In bacteria, plant chloroplasts, and archaea, the enzyme ThiC plays a key role. In a mechanistically highly remarkable radical SAM-mediated cascade reaction, it converts AIR **68** to HMP-P **69** (Fig. 24, top left).[76] AIR **56** is an interesting intermediate in that it also serves as a general precursor for purine metabolism. The HET building block **70** is biosynthesized in most bacteria from DX5P (**66**) and glycine (**48**; in *E. coli*, glycine is replaced by L-tyrosine).[77] The sulfur atom derives from a protein-bound thiocarboxylate that ultimately originates from the free amino acid cysteine *via* a persulfide intermediate. Mechanistically, this process is similar to the S-transfer in the biosynthesis of molybdopterin **54** (see above). DX5P **66** is formed from pyruvate **67** and glyceraldehyde-3-phosphate (**47**), and TPP **18** is required for this enzymatic step.

The second biosynthetic pathway was found in yeasts, *e.g.*, *Saccharomyces cerevisiae* (Fig. 27, top right). HMP-P **69** is formed from L-histidine (**71**) and PLP **19**, for which a remarkable

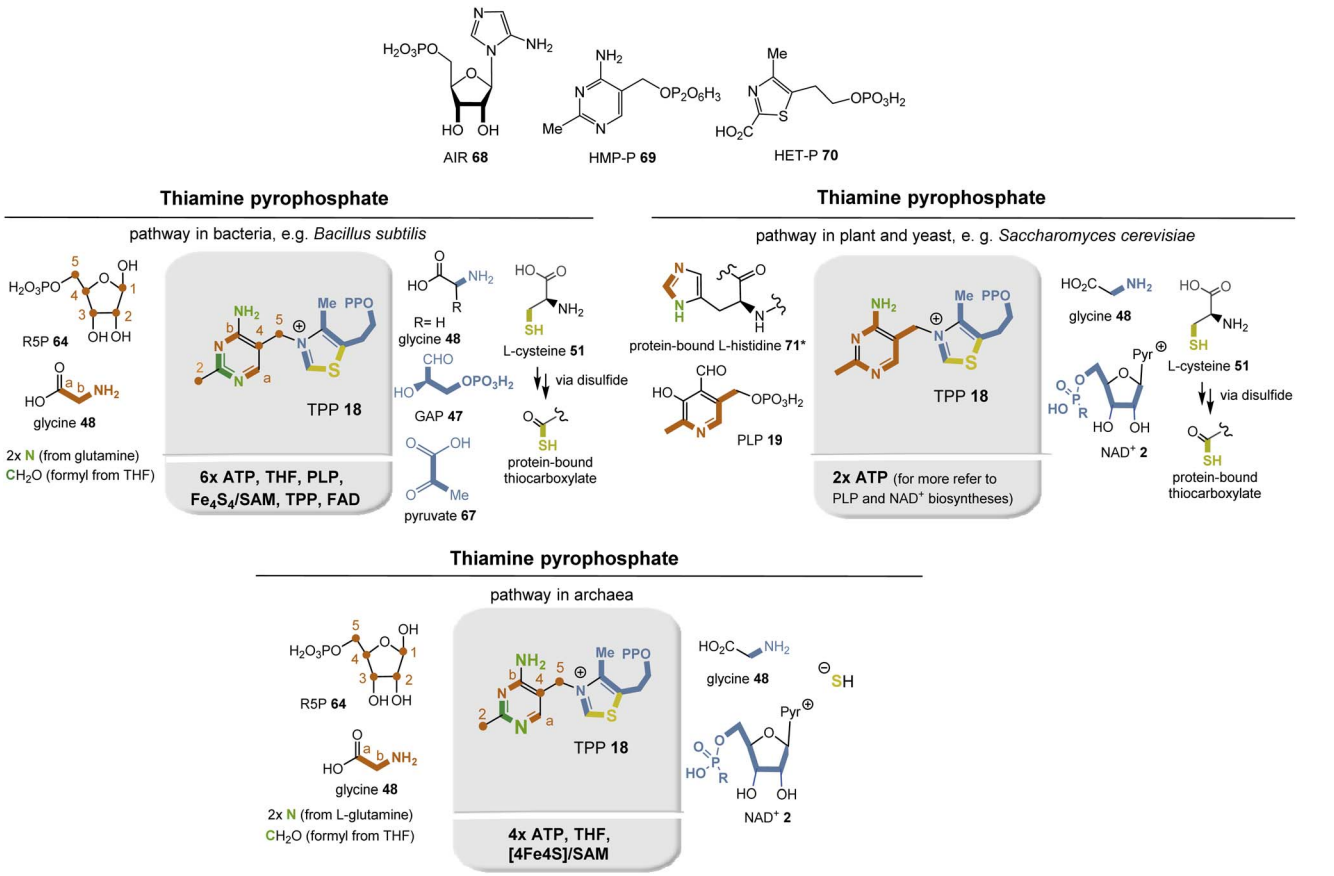
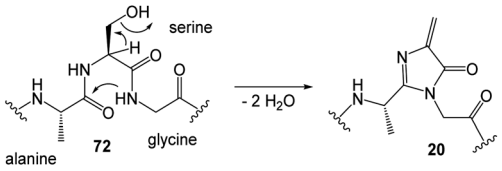


Fig. 27 Summary of three thiamine pyrophosphate biosyntheses: building blocks are marked in sienna, blue, green, orange and yellow at positions where they end up in TPP **18**. Structures of key intermediates **68**–**70** (Pyr = nicotinamide). Coenzyme-dependent enzymes (top left): ATP: ribose phosphate pyrophosphokinase (PRS-1); ATP: glycinamide ribonucleotide synthetase (GARS); THF: phosphoribosylglycinamide formyltransferase (GART formylase); ATP/glutamine: phosphoribosylformyl-glycinamidine synthase (FGMAS); ATP: AIR synthetase (FGMA cyclase); Fe_4S_4/SAM: pyrimidine synthase (ThiC); ATP: hydroxymethylpyrimidine/phosphomethylpyrimidine kinase (ThiD); ATP: thiocarboxylate synthase (ThiF); PLP: cysteine desulfurase (IscS-SH); TPP: DXP synthase (Dxs); FAD: glycine oxidase (ThiO). Coenzyme-dependent enzymes (top right): ATP: phosphomethylpyrimidine kinase (Thi21); ATP: thiamine pyrophosphokinase (Thi80). Coenzyme-dependent enzymes (bottom): ATP: ribose phosphate pyrophosphokinase (PRS-1); ATP: glycinamide ribonucleotide synthetase (GARS); THF: phosphoribosylglycinamide formyltransferase (GART formylase); ATP/glutamine: phosphoribosylformyl-glycinamidine synthase (FGMAS); ATP: AIR synthetase (FGMA cyclase); Fe_4S_4/SAM: pyrimidine synthase (ThiC). All three routes: ATP: thiamine phosphate kinase (ThiL) as final phosphorylation step.



Scheme 2 Formation of protein-bound coenzyme MIO **62**.

mechanism has also been proposed.[78] A Diels–Alder type cycloaddition is suggested, followed by radical oxidations promoted by Fe^{3+} and O_2. It is noteworthy that coenzyme **19** acts as a building block here. HET-P **70** is formed from glycine **48**, the coenzyme NAD^+ **2**, and the sulfur atom is introduced as described above. S-transfer is mediated by iron leaving a dehydroalanine residue in the protein. Remarkably, no coenzyme-dependent enzymes other than ATP-promoted phosphorylations are involved in this biosynthetic pathway. Nevertheless, the coenzymes required for the biosynthesis of the building blocks PLP **19** and NAD^+ **2** must, of course, be included in the analysis.

Archaea harbor structural homologs of both bacterial and eukaryotic proteins for biosynthesis (Fig. 27, bottom). HMP-P **69** is essentially fed by the same building blocks as in bacteria (Fig. 27, top left), while biosynthesis of HET-P **70** follows the pathway of TPP biosynthesis in fungi (Fig. 27, top right).[79] For a long time, the source of the sulfur atom remained in the dark. But thermophilic methanogens from hydrothermal vents, in which the sulfide content is high, helped to unravel this mystery. In fact, it was found that sulfide serves as a sulfur donor, with iron acting as a cofactor.

Preliminary analysis reveals that the first biosynthetic pathway found in bacteria occurred much later in evolution, requiring TPP for the synthesis of DXP **66**, which is a precursor for the non-mevalonate pathway to terpenes. The second pathway uses histidine and PLP and appears to be simple in terms of the number of coenzymes required. However, the biosynthesis of histidine starts with ATP **13** and PRPP **32** and requires the coenzyme PLP **19** and 2× NAD(P)+ **2b**. The third biosynthetic pathway, which occurs in archaea, is a hybrid of the other two biosynthetic routes because it feeds on their fragment biosyntheses. The recruitment of sulfur may also provide a hint on the evolution of these pathways. The protein Thi4 is required for thiazole biosynthesis, and in *Methanococcus jannaschii* the ortholog of Thi4 has a histidine at the site where cysteine is normally found. Consequently, sulfide had to serve as the S source. Thus, it could be argued that this mode of recruitment is the oldest, as hydrothermal vents are thought to be the habitat for early forms of life.

2.5.3 4-Methylideneimidazole-5-one (MIO) (20). 4-Methylideneimidazol-5-one (MIO) **20** is a prosthetic group that serves as a catalytic component of the ammonia lyases enzyme class. This family of enzymes is responsible for the processing of amino acids by elimination of ammonia to the unsaturated intermediate and re-addition of ammonia in the β-position.[80] The biosynthesis of MIO **62**, *e.g.* in histidine ammonia lyase, occurs by a self-processing mechanism in which water is eliminated from a serine residue (in **72**) and does not require additional coenzymes (Scheme 2).

2.6. Coenzymes of methanogenesis

Methanogens use several unique coenzymes **21–26**, which are listed in Fig. 2. The biosynthesis of coenzymes F_0 **21a** and F_{420} **21b** and of THMPT **24** has been described in earlier sections because of their similarities to the biosynthesis of flavins **3** and THF **16**, respectively.[81]

2.6.1 Coenzyme M (22). Coenzyme M is found in methanogenic archaea where it has a key role in methane formation.[82] The *S*-methyl derivative is generated from coenzyme M (**22**) in methyl transfer reactions catalyzed by proteins containing zinc. Coenzyme M is also involved in the bacterial metabolism (*e.g.* in proteobacterium *Xanthobacter autotrophicus*) of alkenes and oxiranes.[83]

In methanogens, two biosynthetic pathways for coenzyme M (**22**) are known, with the carbon skeleton derived from either phosphoenolpyruvate **46** or L-phosphoserine **73** (Fig. 28).[84] The PEP-dependent pathway begins with the Michael addition of sulfite to PEP **46**, which, including an oxidation step requiring NAD^+, leads to the key intermediate 2-oxo-3-sulfopropanoic acid. Decarboxylation and reductive introduction of H_2S eventually forms coenzyme M (**22**). Details on the active electron

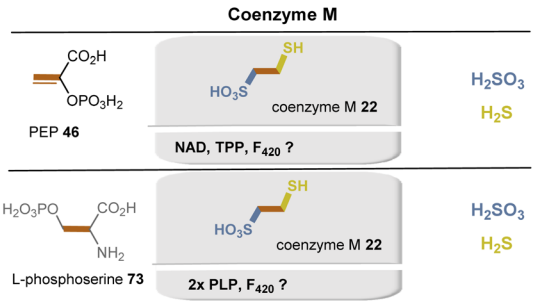


Fig. 28 Summary of coenzyme M biosyntheses: building blocks are marked in sienna, blue and yellow at positions where they end up in CoM **22**. Coenzyme-dependent enzymes: Top: NAD^+: L-sulfolactate dehydrogenase (ComC); TPP: sulfopyruvate decarboxylase (ComDE); bottom: PLP: cysteate synthase and aspartate aminotransferase; F_{420}: not unequivocally established.

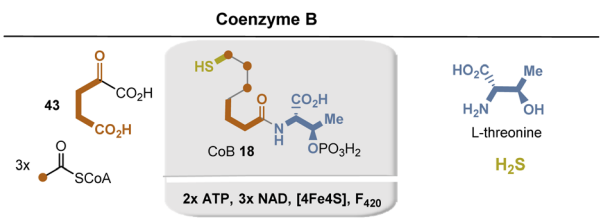


Fig. 29 Summary of coenzyme B **23** biosyntheses: building blocks marked in sienna, blue and yellow and positions where they end up in CoB **23**. Coenzyme-dependent enzymes: ATP: activation of threonine and late stage phosphorylation; enzymes not characterized yet; 3× NAD^+: *threo*-isocitrate dehydrogenases (after each formal homologization step); Fe_4S_4: enzyme not characterized yet (for details see ref. 78).

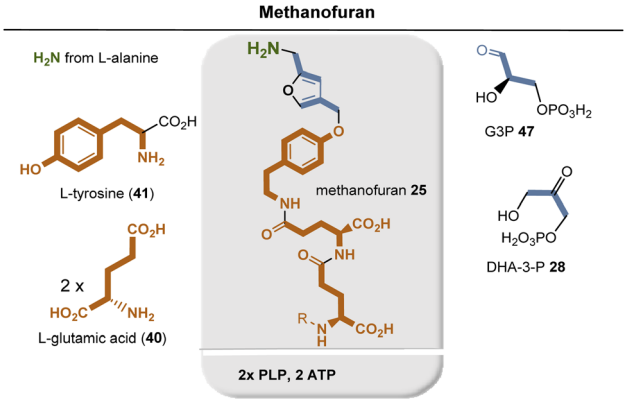


Fig. 30 Summary of methanofuran biosynthesis: building blocks are marked in red and blue including positions where they end up in methanofuran **25**. Coenzyme-dependent enzymes: PLP: 2-furaldehyde phosphate aminotransferase and L-tyrosine decarboxylase; ATP: activation of glutamic acid.

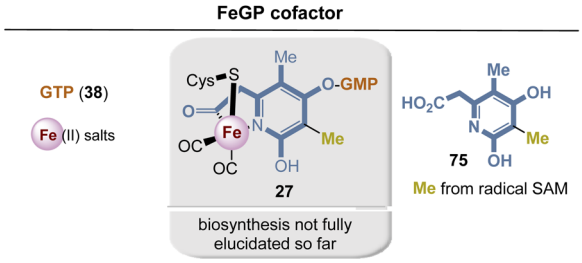
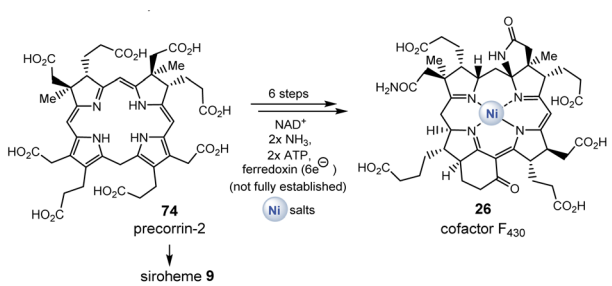


Fig. 31 Summary of FeGP cofactor biosynthesis: building blocks are marked in blue, sienna and yellow at positions where they end up in the FeGP cofactor **27**.

donor for this last step have not yet been elucidated. The L-phosphoserine-dependent pathway is based on the concerted elimination of phosphate and addition of sulfite, followed by transamination. Both steps require PLP as a coenzyme. At this point, both pathways converge *via* the intermediate 2-oxo-3-sulfopropanoic acid.

2.6.2 Coenzyme B (23). Coenzyme B (CoB) **23** is another coenzyme found in methanogenesis.[82] At the end of this process, the thiol group in coenzyme B (CoB) attacks the methyl thioether of methyl CoM **22**. In this process, methane is released with disulfide formation (CoM-S-S-CoB). For this to occur, CoB must reach the active site buried in the depth of methyl CoM reductase. Consequently, CoB consists of a linear 7-mercaptoheptanoyl chain which is linked to phosphothreonine *via* an amide bond.

The biosynthesis repeatedly follows a sequence of transformations encountered in the citric acid cycle (Krebs)[85] and in leucine biosynthesis (Fig. 29). In the present case, it is initiated by the aldol reaction of 2-oxoglutarate and acetyl-CoA. What is remarkable about the route is the iterative nature of the 2-oxoacid elongation, which leads to a formal homologization by one carbon and the release of carbon dioxide in three rounds. The isomerization sequence, based on water elimination and regioreversed hydration, is catalyzed by an iron–sulfur cluster, which acts as a Lewis acid in the present case. Biosynthesis is



Scheme 3 Biosynthesis of cofactor F_{430} **26**.

completed by decarboxylation, producing an aldehyde intermediate into which H_2S is reductively introduced and finally coenzyme B (**23**) is formed.[86] Hydrogen sulfide was found to provide the sulfur atom for the formation of 7-mercaptoheptanoic acid in a reaction that has been shown to likely obtain its reducing equivalents from hydrogen *via* an F_{420}-dependent hydrogenase.[86]

2.6.3 Biosynthesis of methanofuran (25). Methanofuran **25** is involved in the first two-electron reduction of carbon dioxide that yields the formamide derivative of methanofuran in which the primary amino group is modified.[87] Despite the involvement of methanofuran in this first step of methanogenesis, less is known about the enzymes and corresponding genes involved in the following steps of methanofuran biosynthesis. The carbon atoms of the furan moiety are recruited from G3P **47** and DHA-3-P **28** and require the coenzyme PLP for introducing the terminal amino group and to decarboxylate tyrosine (Fig. 30).

2.6.4 Cofactor F_{430} (26). Cofactor F_{430} **26** is a modified tetrapyrrole involved in the formation of methane mediated by the enzyme methyl coenzyme M reductase in methanogenesis.[88] The cofactor is found in methanogenic archaea and was first discovered in *Methanobacterium thermoautotrophicum*. Cofactor F_{430} **26** is also found in microbes that catalyse the anaerobic oxidation of alkanes. In methanogenesis, the methyl radical/Ni(II) thiolate intermediate plays a central role in catalysis. The biosynthesis of cofactor F_{430} **26** begins with precorrin-2 (**74**), and during the six-step conversion, NAD^+, ATP and presumably ferredoxins **11** are used for final electron transfer of six electrons that reduce the tetrapyrrole nucleus (Scheme 3).[89]

2.6.5 Guanylylpyridinol cofactor (FeGP) 27. The guanylylpyridinol cofactor (FeGP) **27** is the iron complex found in the [Fe] hydrogenase.[49] This enzyme has been found in many hydrogenotrophic methanogenic archaea. It catalyzes the reversible dehydrogenation of methylene-THMPT (structure of THMPT **24**, see Fig. 31) to methenyl-THMPT$^+$, which plays an important role in CO_2 reduction to methane.[5] This homodimeric enzyme contains one non-redox active iron per subunit linked to a guanylylpyridinol cofactor **27** in short the FeGP cofactor.[90]

The biosynthesis of FeGP has not yet been fully elucidated. So far, it is known that 2-(4,6-dihydroxy-3,5-dimethylpyridin-2-yl)acetic acid **75** is the biosynthetic precursor for FeGP **27**, while so far only a few preliminary feeding experiments with

^{13}C-labeled building blocks such as acetic acid and pyruvate could give some clues about the origin of the pyridine ligand.[90]

3. Can the analysis provide insight into coenzyme evolution?

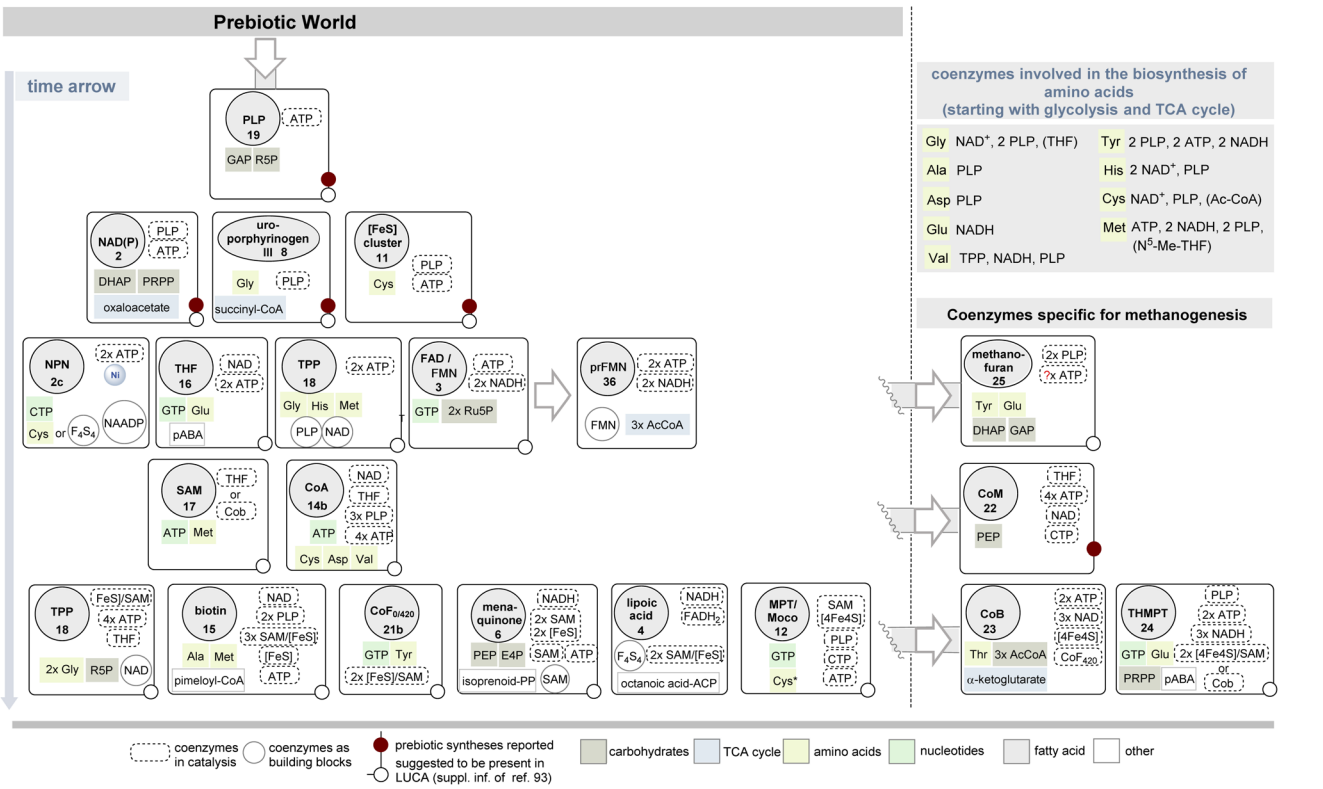
3.1 A proposal

The compilation of biosyntheses of coenzymes presented in this article searches for metabolic relationships and dependencies of these and deliberately hides questions about bioinformatic and phylogenetic relationships of the enzymes involved in the biosyntheses. Rather, the author asks which coenzymes are required for the biosynthesis of a particular coenzyme. Biosyntheses that almost do without them would thus be older than those that rely on a large number of coenzymes. It must be emphasized, however, that this analysis in no way seeks to include the possible prebiotic existence and role of coenzymes or simpler analogs that may have served as nutrients for the first organisms. Nor does it include simplifications or truncations of coenzyme biosyntheses that might conceivably occur if some of the required building blocks, such as amino acids, were of prebiotic origin.[91] Such a transitional phase on the way to life as we know it is very conceivable; it was the time before the appearance of the last unified common ancestor (LUCA).[92] A recent analysis suggests that LUCA probably already had the standard repertoire of coenzymes, like the autotrophic thermophilic anaerobes today.[93]

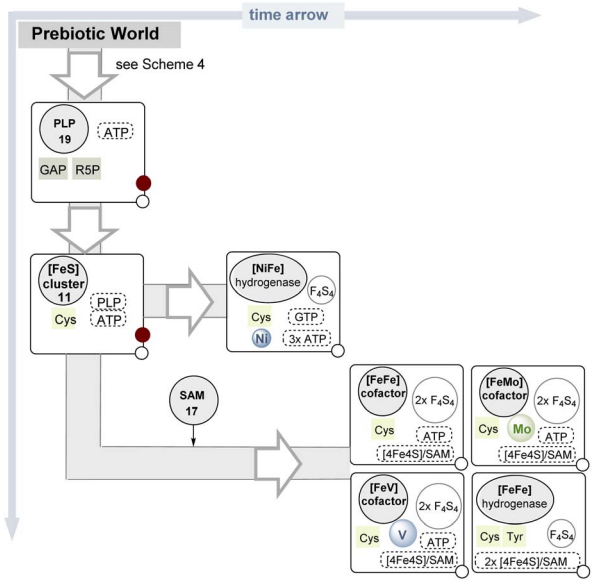
Obviously, the approach pursued in this account can represent only one of several possible perspectives; nevertheless, it is an attempt to address this question in a comprehensive manner. In Scheme 4, the most important coenzymes and their biosyntheses are arranged in such a way that they can be placed in a possible chronological evolutionary relationship. The scheme also contains four coenzymes that are dedicated to methanogenesis and acetogenesis. Some aspects of this sorting are highlighted and discussed below.

3.1.1 Coenzymes for which several biosynthetic routes are known. In some cases, nature has evolved more than one biosynthesis to coenzymes, as in the case of nicotinamide **2**, uroporphyrinogen III **8**, thiamine pyrophosphate **18** and pyridoxal phosphate **19**. In these cases, the most likely oldest biosynthetic pathway was selected based on the required coenzymes. Thus it is clear that one of the two biosynthetic pathways to PLP **19** (Fig. 26) must be very old, since it relies only on the activated nucleotide ATP **13** and two starting building blocks from carbohydrate metabolism.

Two pathways are known for the key biosynthetic precursor of uroporphyrinogen III **8** δ-ALA **50**, which differ in the required



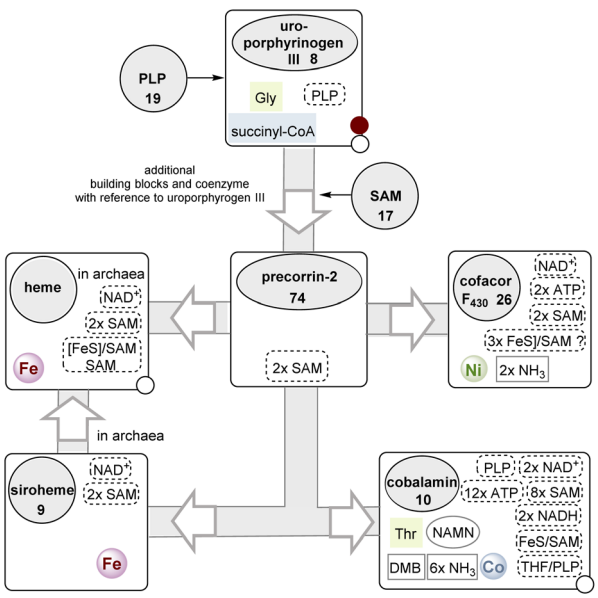
Scheme 4 Proposed appearance in time of the biosyntheses of coenzymes under consideration of the required building blocks and coenzymes.[a] Their presence in LUCA according to ref. 93 as well as plausible prebiotic synthesis are listed too. NADH and $FADH_2$ are required as coenzymes for fatty acid biosynthesis and thus for lipoic acid, as well as ATP and CoA for acyl activation; if malonyl-CoA functions as an elongation building block, biotin (**15**) would be required as an additional coenzyme. The evolutionary relationships of iron–sulfur cluster-derived cofactors and in the tetrapyrrole-based coenzyme family are summarized in Schemes 5 and 6. NAADP **54** is listed as a coenzyme building block, as it is part of NAD^+ biosynthesis. [a] The FeGP **27** cofactor is not included in the list of coenzymes specific for methanogenesis, because details on the biosynthesis of the pyridinol ligand **75** are not known yet.



Scheme 5 Proposed appearance in time of the biosyntheses of iron–sulfur cluster derived cofactors (symbols and color code are found in the legend in Scheme 4).

use of coenzymes. For route A this is PLP and for route B PLP and NADH. Similarly, eukaryotic biosynthesis of nicotinamide **2** can be evolutionarily eliminated as an early development, in part because the biosynthesis of tryptophan requires NAD^+ and PLP among other coenzymes.[2]

Metabolic evolution led to the development of three routes for TPP biosynthesis. As already indicated in section 2.5.2, the



Scheme 6 The (anaerobic) branched pathway of tetrapyrrole biosynthesis starting with uroporphyrinogen III **8** (for additional details see also Fig. 12 and 20). Fe, Ni and Co refer to metal salts actively taken from the environment. For cobalamin, the coenzymes required for the biosynthesis of DMB and the introduction of the threonine moiety are included in the list. The biosynthesis of chlorophyll is not covered here.

version in bacteria is the youngest, since it builds on the (MEP/DOXP) pathway for terpenes and starts with the coupling of pyruvate **67** and G3P **47**, which is based on the coenzyme TPP itself. The other two biosynthetic pathways, however, are embedded in Scheme 4.

3.1.2 The beginning: PLP, NAD^+, uroporphyrinogen III and iron–sulfur cluster. PLP is placed at the beginning of molecular evolution in this analysis due to its simple structure, very short and simple biosynthesis from basic building blocks of sugar metabolism and almost universal role in the biosynthesis of proteinogenic amino acids as well as other coenzymes. NAD(P)H **2** seems to be a good next candidate, since the simpler of the two biosyntheses (in prokaryotics and archaea) requires PLP as a coenzyme in addition to ATP, and the building blocks are again typical intermediates from carbohydrate metabolism and the TCA cycle. However, these considerations also suggest that one of the two pathways to uroporphyrinogen III **8**, from which various metal-binding ligands were later derived, was also formed at this early stage. These considerations also suggest that one of the two pathways to uroporphyrinogen III **8**, from which various metal-binding ligands were later derived, was also formed at this early stage.The second biosynthesis additionally requires NAD(P)H **2** as a coenzyme and probably originated a bit later.

Iron–sulfur species have been regarded to be among the oldest biological coenzymes and have strictly been conserved until today.[94] The *in vitro* chemistry of iron–sulfur clusters has been documented as several species can be generated from inorganic iron and sulfide.[95] Recently, it was demonstrated that photooxidation of ferrous ions and the photolysis of organic thiols are conditions under which polynuclear iron–sulfur clusters are generated.[96]

The development of primitive catalysts into iron–sulfur clusters could have spontaneously occurred by assembling on polypeptide templates. In all of these complexes, cysteine also plays a central role as a proteinogenic ligand, so that in the evolution of α-amino acids it must have appeared rather earlier. In prebiotic times, other ligands, probably also based on thiols (such as methanethiol), may have first taken the later role of cysteine.

Once iron–sulfur clusters were available they could have rearranged themselves to form the different clusters that are now found in (Fe–S) proteins. These also include more complex metal clusters in which iron is exchanged for other metals. As a consequence, this evolution led to enzyme classes such as nitrogenases and hydrogenases, both key enzymes in the evolution of life. Here, based on the concept of determining the coenzymes required for their biosynthetic generation, an evolutionary relationship can be proposed (Scheme 5). Noteworthy, four of the five clusters mentioned (the [FeFe], [MoFe], and [VFe] cofactors in nitrogenases and the [FeFe] 'H-cluster' found in the corresponding hydrogenase) alone had to wait for SAM **17** to appear (Scheme 4) before they could step onto the stage.

From the analysis compiled in Scheme 5, it is clear that the appearance of the cofactor encountered in [NiFe]-hydrogenases should be ranked earlier. And ideas about early metabolisms suggest the same as these hydrogenases have links to ancient forms of metabolism, utilizing hydrogen as the original source

of reductant on Earth.[97] It has not (yet) been chemically verified whether, from the iron–sulfur clusters very likely present in the "prebiotic" world, chemical accesses to catalytically active clusters mixed with other metals did exist.

Many (Fe–S) proteins have survived up today[98] but it is known that during evolution some of them have been replaced by other redox systems.[99] This is exemplified for the oxidation of glucose in the Entner–Doudoroff pathway (glucose to gluconate and glyceraldehyde to glycerate). Thereby, $NAD(P)^+$-dependent enzymes took over from the ferredoxins.[100,101]

Finally, an interesting finding of this analytical approach is that for these four coenzymes whose biosyntheses likely became established at an early time in evolution, chemical syntheses under plausible prebiotic conditions were also reported (Scheme 4). Or is this just a coincidence?[2,91]

3.1.3 Evolutionary straggler. Other biosyntheses of coenzymes could emerge that rely on a larger number of coenzymes and consistently require PLP **19** and/or NAD(P)H **2**. These include flavins and other pterins such as folic acid **16** that use GTP **38** as an essential starting building block. And only after the appearance of the NAD(P)H biosynthetic pathway must biotin **15** and two biosynthetic pathways to TPP **18** have been established. Folic acid **16** is required for the regeneration of *S*-adenosylmethionine (SAM, **17**), which at first glance is a simple coenzyme if the biosynthesis of methionine is excluded from the present analysis. If methionine is included in these considerations, the picture changes, because methionine is considered one of the "latecomers" among the 20 proteinogenic amino acids, partly because its biosynthesis requires a large number of coenzymes (top right; Scheme 4: ATP, 2× NAD^+, 2× PLP, THF).[2]

The analysis also shows that coenzyme A **14b**, a universal activator of carboxylic acids, must have arisen at a later stage of (proto)metabolism because its biosynthesis requires numerous coenzymes, including folic acid. Activation of carboxylic acids as CoA thioesters normally occurs *via* the mixed phosphate anhydrides with the participation of ATP **13**. These, in turn, represent particularly strongly activated carboxylate derivatives, so that the development of CoA thioesters can be regarded as an evolutionary advance. This is simply because thioesters are chemically more stable than the corresponding phosphate anhydrides, *i.e.* more "manageable".

Furthermore, ATP **13** is part of the nucleotide metabolism. So, if we assume that life without nucleotide biochemistry is unthinkable, also with respect to the first steps in the emergence of life from a prebiotic world, as manifested in the RNA world theory,[102] then ATP **13** will be much older than CoA **14b**. However, it cannot be ruled out that the reverse is true, as has been proposed for the prebiotic thioester world, but then based on chemically simpler thiols than CoA **14b**.[103] The (bio)molecular and physiological basis of LUCA was analyzed by genetic analyses of protein clusters from sequenced prokaryotic genomes of different phylogenetic trees.[93] Its metabolism was likely dominated by FeS clusters and radical chemistry.[104] Analysis suggests the presence of biosynthetic pathways for almost all coenzymes, including flavins (molybdopterin), 5-deazaflavins (coenzyme F_{420}, **21b**), *S*-adenosylmethionine (SAM, **17**), coenzyme A (CoA, **14b**), coenzyme M (CoM, **22**), thiamine pyrophosphate (TPP, **18**), ferredoxin (Fe–S proteins), siroheme, and corrin (Scheme 4).

3.1.4 Implementation of TCA cycle, sugar metabolism and amino acid biosynthesis. The origin of the starting building blocks for the biosyntheses to the coenzymes has hardly been covered up to this point of the present report. The basic metabolic systems from which these building blocks are recruited are glycolysis, the citric acid cycle, nucleotide and amino acid metabolism.[2,105]

Recent systems chemistry approaches have argued that the basic metabolic networks of life,[106,107] such as the TCA cycle and especially its reverse counterpart, the rTCA cycle, but also others, existed long before the appearance of LUCA.[108–110] These networks arose in parallel with RNA, which receives special attention in the RNA-world theory because of its catalytic and self-replicating capabilities.[101]

While there is hypothetical and experimental evidence for the presence of sugar-like building blocks and small carboxylic acids generated under prebiotic conditions (*e.g.* formose reaction,[111] Sutherland's cyanosulfidic protometabolism[112]),[91] the picture for amino acids is more complex. Thus, no plausible prebiotic approaches to methionine, tyrosine[113] and histidine[114] are known. In addition, the biosynthesis of methionine requires diverse coenzymes, and other considerations (*e.g.*, evolution of the genetic code[115]) also suggest it to be one of the last established amino acid biosyntheses. This gives additional support for the argument that the coenzymes TPP **18**, and biotin **15** are evolutionarily among the late arrivers.

Glycine is not only the structurally simplest amino acid, but is produced under almost all known plausible prebiotic conditions.[91] Interestingly, it appears preferentially as a building block of the coenzyme uroporphyrinogen III (**8**) and cobalamin (**10**), which are classified as evolutionary ancient in Scheme 4.

3.1.5 Coenzymes specific for methanogenesis. Following a similar pattern of reasoning, four coenzymes found only in methanogenesis, methanofuran (**25**), THMPT (**24**), as well as coenzymes B (**23**) and M (**22**) are also embedded in Scheme 4. The analysis suggests that methanogenesis is a process that should have occurred later in evolution although recently it was claimed that it could have existed already 3.5 billion years ago.[5c] Instead, the biosynthetic pathways of coenzymes described here provide additional support for the now favored hypothesis that the evolutionarily oldest C1 fixation pathway is the Wood–Ljungdahl pathway and specifically acetogenesis here. In this metabolic pathway, two equivalents of carbon dioxide are reductively combined to form acetyl-CoA and tetrahydrofolate as well as iron–sulfur and iron–nickel–sulfur clusters play important key roles here.[116]

3.2 The evolution of tetrapyrrole biosynthesis

Finally, the branched metabolic pathway responsible for the synthesis of tetrapyrrole-containing natural products will be discussed in more detail. Some aspects have already been dealt with in sections 2.2.1, 2.4.1 and Scheme 3.[117] Scheme 6 summarizes the evolutionary relationships between tetrapyrrole scaffold-

containing coenzymes, which include cobalamin.[118] Precorrin-2 (**74**) is the central branching site for the biosynthesis of heme,[119] cofactor F_{430} **26**, siroheme (**9**), and cobalamin (**10**).

In the further course of the biosyntheses, starting with uroporphyrinogen III (**8**), SAM **17** plays a central role both as a methyl-transferring coenzyme and in radical SAM-mediated reactions[120] that also allow oxidations in the absence of molecular oxygen. The analysis shows that cobalamin can by no means be an ancient coenzyme, which is also reflected in the fact that its biosynthesis is based on about 30 enzymes.[121,122]

This is also reflected in the fact that cobalamin was not necessarily required to be involved in the development of DNA, specifically as part of ribonucleotide reductase, since this radical deoxygenation process can instead be promoted by radical SAM (ribonucleotide reductase type III).[123]

4. Conclusions

The evolution of the biosynthesis of coenzymes is proposed by analyzing the individual biosynthetic pathways in terms of their demand for (other) coenzymes. Although this approach contains various imponderables, since it only chooses one particular point of view, *i.e.*, it leaves out the topic of phylogenetic analyses of proteins and bioinformatic approaches. Indeed, it must be emphasized that the chronological-evolutionary classification of coenzymes developed here and *e.g.* summarised in Scheme 4 has a certain theoretical character, since it analyzes the need for coenzymes and building blocks of individual biosyntheses. This does not necessarily mean that the appearance on the scene happened at the theoretically possible moment. This shall be explained for the Ni-pincer nucleotide **2c**, lipoic acid (**4**) and for the prFMN **36**. These could have existed at a very early time based on the analysis done here. But with respect to their chemical properties, was their existence necessary in early organisms or could they have appeared much later? Martin's analysis of LUCA, compiled for coenzymes and cofactors in Scheme 4, may provide clues here. According to this, the metabolism of LUCA was not based on these coenzymes. The same is true for the FeM cofactors of the hydrogenases.

Still, this report represents one of the first comprehensive attempts – another important one by Warren and coworkers[7] must be mentioned here – to place coenzymes in an evolutionary context. Future "thought experiments" would need to take a more holistic approach. For example, the present report has left open the essential question of enzymatic electrophilic and radical methylations, for which nature has found various solutions, such as in the Wood–Ljungdahl C1-fixation pathway,[116] methionine metabolism,[124] and the methylation of uridine.[125] Associated with this are the coenzymes SAM **17**, THF **16**, THMPT **24** and cobalamin **10**. However, a deeper contemplation on this may imply the starting point for the narration of another story.[126]

5. Conflicts of interest

There are no conflicts to declare.

6. Acknowledgements

I would like to thank Carsten Zeilinger (Center for Biomolecular Drug Research (BMWZ), Leibniz University Hannover) for the fruitful exchange of ideas. I am indebted to the reviewers for their comprehensive comments and suggestions for improvement, which were very helpful and contributed to a considerable improvement in the quality of the manuscript.

7. Notes and references

1 J. Nielsen, *Annu. Rev. Biochem.*, 2017, **86**, 245–275.
2 (*a*) A. Kirschning, *Chem.–Eur. J.*, 2022, DOI: **10.1002/chem.202201419**; (*b*) A. Kirschning, *Nat. Prod. Rep.*, 2021, **38**, 993–1010.
3 (*a*) T. P. Begley, *Nat. Prod. Rep.*, 2006, **23**, 15–25; (*b*) M. E. Webb, A. Marquet, R. R. Mendel, F. Rébeillé and A. G. Smith, *Nat. Prod. Rep.*, 2007, **24**, 988–1008; (*c*) T. D. Begley, A. Chatterjee, J. W. Hanes, A. Hazra and S. E. Ealick, *Curr. Opin. Chem. Biol.*, 2008, **12**, 118–125.
4 T. F. Schwede, J. Rétey and G. E. Schulz, *Biochemistry*, 1999, **38**, 5355–5361.
5 (*a*) T. Sato and H. Atomi, *Curr. Opin. Microbiol.*, 2011, **14**, 307–314; (*b*) A. A. DiMarco, T. A. Bobik and R. S. Wolfe, *Annu. Rev. Biochem.*, 1990, **59**, 355–394; (*c*) J. M. Wolfe and G. P. Fournier, *Nat. Ecol. Evol.*, 2018, **2**, 897–903.
6 (*a*) P. N. Evans, J. A. Boyd, A. O. Leu, B. J. Woodcroft, D. H. Parks, P. Hugenholtz and G. W. Tyson, *Nat. Rev. Microbiol.*, 2019, **17**, 219–232; (*b*) L. G. Gorris and C. van der Drift, *BioFactors*, 1994, **4**, 139–145.
7 G. L. Holliday, J. M. Thornton, A. Marquet, A. G. Smith, F. Rebeille, R. R. Mendel, H. L. Schubert, A. D. Lawrence and M. J. Warren, *Nat. Prod. Rep.*, 2007, **24**, 972–987.
8 (*a*) A. Mattevi, *Nat. Struct. Mol. Biol.*, 2006, **13**, 563–564; (*b*) G. Noctor, G. Queval and B. Gakière, *J. Exp. Bot.*, 2006, **57**, 1603–1620; (*c*) B. Gakière, J. Hao, L. de Bont, P. Pétriacq, A. Nunes-Nesi and A. R. Fernie, *Crit. Rev. Plant Sci.*, 2018, **37**, 259–307; (*d*) P. Belenky, K. L. Bogan and C. Brenner, *Trends Biochem. Sci.*, 2007, **32**, 12–19; (*e*) J. C. Morales, L. Li, F. J. Fattah, Y. Dong, E. A. Bey, M. Patel, J. Gao and D. A. Boothman, *Crit. Rev. Eukaryotic Gene Expression*, 2014, **24**, 15–28; (*f*) B. Lüscher, M. Bütepage, L. Eckei, S. Krieg, P. Verheugd and B. H. Shilto, *Chem. Rev.*, 2018, **118**, 1092–1136.
9 S. Y. Gerdes, M. D. Scholle, M. D'Souza, A. Bernal, M. V. Baev, M. Farrell, O. V. Kurnasov, M. D. Daugherty, F. Mseeh, B. M. Polanuyer, J. W. Campbell, S. Anantha, K. Y. Shatalin, S. A. K. Chowdhury, M. Y. Fonstein and A. L. Osterman, *J. Bacteriol.*, 2002, **184**, 4555–4572.
10 G. Magni, A. Amici, M. Emanuelli, N. Raffaelli and S. Ruggieri, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1999, **73**, 135–182.
11 (*a*) A. Volbeda, C. Darnault and J.-C. Fontecilla-Camps, *J. Am. Chem. Soc.*, 2016, **138**, 11802–11809; (*b*) S. O.-de Choudens, L. Loiseau, Y. Sanakis, F. Barras and M. Fontecave, *FEBS Lett.*, 2005, **79**, 3737–3743; (*c*) T. Begley, C. Kinsland, R. Mehl, A. Osterman and

P. Dorrestein, *Vitam. Horm.*, 2001, **61**, 103–119; (*d*) H. Sakuraba, T. Satomura, R. Kawakami, S. Yamamoto, Y. Kawarabayasi, H. Kikuchi and T. Ohshima, *Extremophiles*, 2002, **6**, 275–281; (*e*) F. Gazzaniga, R. Stebbins, S. Z. Chang, M. A. McPeek and C. Brenner, *Microbiol. Mol. Biol. Rev.*, 2009, **73**, 529–541.

12 (*a*) Y. Zhang, K. L. Colabroy, T. P. Begley and S. E. Ealick, *Biochemistry*, 2005, **44**(21), 7632–7643; (*b*) W. C. Lima, A. M. Varani and C. F. Menck, *Mol. Biol. Evol.*, 2009, **26**, 399–406; (*c*) For an evolutionary view on both pathways see W. C. Lima, A. M. Varani and C. F. Menck, *Mol. Biol. Evol.*, 2009, **26**, 399–406; (*d*) H. B. White III, *The pyridine nucleotide coenzyme*, Academic Press Inc., 1982, ch. 17, pp. 225–248.

13 (*a*) H. J. Cleaves and S. L. Miller, *J. Mol. Evol.*, 2001, **52**, 73–77; (*b*) H. Kim and S. A. Benner, *Chem.–Eur. J.*, 2018, **24**, 581–584.

14 (*a*) F. Ahmad and A. G. Moat, *J. Biol. Chem.*, 1966, **241**, 775–780; (*b*) H. D. Heilmann and F. Lingens, *Hoppe-Seyler's Z. Physiol. Chem.*, 1968, **349**, 231–236.

15 (*a*) M. Fischer and A. Bacher, *Nat. Prod. Rep.*, 2005, **22**, 324–350; (*b*) A. Bacher, S. Eberhardt, M. Fischer, K. Kis and G. Richter, *Annu. Rev. Nutr.*, 2000, **20**, 153–167; (*c*) M. Mack and S. Grill, *Appl. Microbiol. Biotechnol.*, 2006, **71**, 265–275; (*d*) M. Fischer, A.-K. Schott, W. Römisch, A. Ramsperger, M. Augustin, A. Fidler, A. Bacher, G. Richter, R. Huber and W. Eisenreich, *J. Mol. Biol.*, 2004, **343**, 1267–1278; (*e*) M. Fischer, W. Romisch, S. Schiffmann, M. Kelly, H. Oschkinat, S. Steinbacher, R. Huber, W. Eisenreich, G. Richter and A. Bacher, *J. Biol. Chem.*, 2002, **277**, 41410–41416; (*f*) S. Steinbacher, S. Schiffmann, G. Richter, R. Huber, A. Bacher and M. Fischer, *J. Biol. Chem.*, 2003, **278**, 42256–42265.

16 (*a*) M. Zhang, L. Wang and D. Zhong, *Arch. Biochem. Biophys.*, 2017, **632**, 158–174; (*b*) A. Sancar, *Biochemistry*, 1994, **33**, 2–9; (*c*) C. A. Brautigam, B. S. Smith, Z. Ma, M. Palnitkar, D. R. Tomchick, M. Machius and J. Deisenhofer, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 12142–12147.

17 (*a*) D. Leys, *Curr. Opin. Chem. Biol.*, 2018, **47**, 117–125; (*b*) A. Saaret, A. Balaikaite and D. Leys, *Enzymes*, 2020, **47**, 517–549.

18 P.-H. Wang, A. N. Khusnutdinova, F. Luo, J. Xiao, K. Nemr, R. Flick, G. Brown, R. Mahadevan, E. A. Edwards and F. Yakunin, *Cell Chem. Biol.*, 2018, **25**, 560–570.

19 (*a*) B. Reuke, S. Korn, W. Eisenreich and A. Bacher, *J. Bacteriol.*, 1992, **174**, 4042–4049; (*b*) C. Greening, F. H. Ahmed, A. E. Mohamed, B. M. Lee, G. Pandey, A. C. Warden, C. Scott, J. G. Oakeshott, M. C. Taylor and C. J. Jackson, *Microbiol. Mol. Biol. Rev.*, 2016, **80**, 451–493.

20 (*a*) C. Walsh, *Acc. Chem. Res.*, 1986, **19**, 216–221; (*b*) W. Friedrich, *Vitamins*, Walter de Gruyter&Co., Berlin, Germany, 1988.

21 (*a*) R. Jaenchen, P. Schoenheit and R. K. Thauer, *Arch. Microbiol.*, 1984, **137**, 362–365; (*b*) B. Reuke, S. Korn, W. Eisenreich and A. Bacher, *J. Bacteriol.*, 1992, **174**, 4042–4049.

22 (*a*) L. Decamps, B. Philmus, A. Benjdia, R. White, T. P. Begley and O. Berteau, *J. Am. Chem. Soc.*, 2012, **134**(44), 18173–18176; (*b*) F. Forouhar, M. Abashidze, H. Xu, L. L. Grochowski, J. Seetharaman, M. Hussain, A. Kuzin, Y. Chen, W. Zhou, R. Xiao, T. B. Acton, G. T. Montelione, A. Galinier, R. H. White and L. Tong, *J. Biol. Chem.*, 2008, **283**, 11832–11840.

23 M. R. Challand, F. T. Martins and P. L. Roach, *J. Biol. Chem.*, 2010, **285**, 5240–5248.

24 A. Pagnier, L. Martin, L. Zeppieri, Y. Nicolet and J. C. Fontecilla-Camps, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 104–109.

25 (*a*) S. J. Booker, *Chem. Biol.*, 2004, **11**, 10–12; (*b*) A. Marquet, B. T. S. Bui and D. Florentin, *Vitam. Horm.*, 2001, **61**, 51–101.

26 J. E. Cronan, *Microbiol. Mol. Biol. Rev.*, 2016, **80**, 429–450.

27 R. M. Cicchillo and S. J. Booker, *J. Am. Chem. Soc.*, 2005, **127**, 2860–2861.

28 (*a*) S. Puehringer, Mh. Metlitzky and R. Schwarzenbacher, *BMC Biochem.*, 2008, **9**, 8; (*b*) A. M. Martins, J. A. Latham, P. J. Martel, I. Barr, A. T. Iavarone and J. P. Klinman, *J. Biol. Chem.*, 2019, **294**, 15025–15036; (*c*) I. Barr, J. A. Latham, A. T. Iavarone, T. Chantarojsiri, J. D. Hwang and J. P. Klinman, *J. Biol. Chem.*, 2016, **291**, 8877–8884; (*d*) W. Zhu, A. M. Martins and J. P. Klinman, *Methods Enzymol.*, 2018, **606**, 389–420.

29 O. T. Magnusson, H. Toyama, M. Saeki, R. Schwarzenbacher and J. P. Klinman, *J. Am. Chem. Soc.*, 2004, **126**, 5342–5343.

30 M. D. Collins and D. Jones, *Microbiol. Rev.*, 1981, **45**, 316–354.

31 R. Meganathan and O. Kwon, *EcoSal Plus*, 2009, **3**(2), DOI: **10.1128/ecosalplus.3.6.3.3**.

32 (*a*) T. Hiratsuka, K. Furihata, J. Ishikawa, H. Yamashita, N. Itoh, H. Seto and T. Dairi, *Science*, 2008, **321**, 1670–1673; (*b*) A. M. Gobe, R. Toro, X. Li, A. Ornelas, H. Fan, S. Eswaramoorthy, Y. Patskovsky, B. Hillerich, R. Seidel, A. Sali, B. K. Shoichet, S. C. Almo, S. Swaminathan, M. E. Tanner and F. M. Raushel, *Biochemistry*, 2013, **52**, 6525–6536; (*c*) X. Li, D. Apel, E. C. Gaynot and M. E. Tanner, *J. Biol. Chem.*, 2011, **286**, 19392–19398.

33 (*a*) S. Joshi, D. Fedoseyenko, N. Mahanta, H. Manion, S. Naseem, T. Dairi and T. P. Begley, *Curr. Opin. Chem. Biol.*, 2018, **47**, 134–141; (*b*) X.-Y. Zhi, J.-C. Yao, S.-K. Tang, Y. Huang, H.-W. Li and W.-J. Li, *Genome Biol. Evol.*, 2014, **6**, 149–160.

34 S. S. Abby, K. Kazemzadeh, C. Vragniau, L. Pelosi and F. Pierrel, *Biochim. Biophys. Acta, Bioenerg.*, 2020, **1861**, 148259.

35 H. Maeda and N. Dudareva, *Annu. Rev. Plant Biol.*, 2012, **63**, 73–105.

36 (*a*) R. H. White, *Biochemistry*, 2004, **43**, 7618–7627; (*b*) R. H. White and H. Xu, *Biochemistry*, 2006, **45**, 12366–12379; (*c*) R. H. White, *Biochemistry*, 2008, **47**, 5037–5046; (*d*) D. V. Miller, M. Ruhlin, W. K. Ray, H. Xu and R. H. White, *FEBS Lett.*, 2017, **591**, 2269–2278; (*e*) T. Soderberg, *Archaea*, 2005, **1**, 347–352.

37 (*a*) G. Layer, J. Reichelt, D. Jahn and D. W. Heinz, *Protein Sci.*, 2010, **19**, 1137–1161; (*b*) F. J. Leeper, *Nat. Prod. Rep.*, 1989, **6**, 171–203; (*c*) D. R. Nelson, T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda and D. W. Nebert, *DNA Cell Biol.*, 1993, **12**, 1–51.

38 (*a*) H. A. Dailey, T. A. Dailey, S. Gerdes, D. Jahn, M. Jahn, M. R. O'Brian and M. J. Warren, *Microbiol. Mol. Biol. Rev.*, 2017, **81**, e00048-16; (*b*) T. Fan, B. Grimm and G. Layer, *Adv. Bot. Res.*, 2019, **91**, 89–131.

39 (*a*) M. Richter, *Nat. Prod. Rep.*, 2013, **30**, 1324–1345; (*b*) J. D. Fischer, G. L. Holliday, S. A. Rahman and J. M. Thornton, *J. Mol. Biol.*, 2010, **403**, 803–824; (*c*) T. P. Begley, *Nat. Prod. Rep.*, 2006, **23**, 15–25; (*d*) M. E. Webb, A. Marquet, R. R. Mendel, F. Rébeillé and A. G. Smith, *Nat. Prod. Rep.*, 2007, **24**, 988–1008; (*e*) M. Richter, *Nat. Prod. Rep.*, 2013, **30**, 1324–1345; (*f*) D. E. Scott, A. Ciulli and C. Abell, *Nat. Prod. Rep.*, 2007, **24**, 1009–1026.

40 Y. Li, N. Kitadai and R. Nakamura, *Life*, 2018, **8**, 46.

41 (*a*) L. Belmonte and S. S. Mansy, *Elements*, 2016, **12**, 413–418; (*b*) N. Kitadai, R. Nakamura, M. Yamamoto, K. Takai, N. Yoshida and Y. Oono, *Sci. Adv.*, 2019, **5**, eaav7848.

42 S. Storbeck, S. Rolfes, E. Raux-Deery, M. J. Warren, D. Jahn and G. Layer, *Archaea*, 2010, 175050.

43 H. Beinert, R. H. Holm and E. Münck, *Science*, 1997, **277**, 653–659.

44 (*a*) S. Bandyopadhyay, K. Chandramouli and M. K. Johnson, *Biochem. Soc. Trans.*, 2008, **36**, 1112–1119; (*b*) C. Ayala-Castro, A. Saini and F. W. Outten, *Microbiol. Mol. Biol. Rev.*, 2008, **72**, 110–125.

45 (*a*) C. J. Schwartz, O. Djaman, J. A. Imlay and P. J. Kiley, *Proc. Natl. Acad. Sci.*, 2000, **97**, 9009–9014; (*b*) D. C. Johnson, D. R. Dean, A. D. Smith and M. K. Johnson, *Annu. Rev. Biochem.*, 2005, **74**, 247–281.

46 (*a*) R. R. Eady, *Chem. Rev.*, 1996, **96**, 3013–3030; (*b*) B. K. Burgess and D. J. Lowe, *Chem. Rev.*, 1996, **96**, 2983–3012; (*c*) Y. Hu, C. C. Lee and M. W. Ribbe, *Dalton Trans.*, 2012, **41**, 1118–1127.

47 D. Harris, D. Lukoyanov, S. Shaw, P. D. Compton, M. Tokmina-Lukaszewska, B. Bothner, N. L. Kelleher, D. R. Dean, B. M. Hoffman and L. C. Seefeldt, *Biochemistry*, 2018, **57**(5), 701–710.

48 (*a*) J. Raymond, J. L. Siefert, C. R. Staples and R. E. Blankenship, *Mol. Biol. Evol.*, 2004, **21**, 541–554; (*b*) N. Gruber and J. N. Galloway, *Nature*, 2008, **451**, 293–296; (*c*) Y. Hu and M. W. Ribbe, *Coord. Chem. Rev.*, 2011, **255**, 1218–1224.

49 (*a*) W. Lubitz, H. Ogata, O. Rüdiger and E. Reijerse, *Chem. Rev.*, 2014, **114**, 4081–4148; (*b*) P. M. Vignais and B. Billoud, *Chem. Rev.*, 2007, **107**, 4206–4272.

50 (*a*) L. Tao, S. A. Pattenaude, S. Joshi, T. P. Begley, T. B. Rauchfuss and R. D. Britt, *J. Am. Chem. Soc.*, 2020, **142**, 10841–10848; (*b*) G. Rao, L. Tao, D. L. M. Suess and R. D. Britt, *Nat. Chem.*, 2018, **10**, 555–560; (*c*) J. B. Broderick, A. S. Byer, K. S. Duschene, B. R. Duffus, J. N. Betz, E. M. Shepard and J. W. Peters, *J. Biol. Inorg Chem.*, 2014, **19**, 747–757; (*d*) D. W. Mulder, E. S. Boyd, R. Sarma, R. K. Lange, J. A. Endrizzi, J. B. Broderick and J. W. Peters, *Structure*, 2011, **19**, 1038–1052; (*e*) R. C. Driesener, M. R. Challand, S. E. McGlynn, E. M. Shepard, E. S. Boyd, J. B. Broderick, J. W. Peters and P. L. Roach, *Angew. Chem., Int. Ed.*, 2010, **49**, 1687–1690; (*f*) J. M. Kuchenreuther, W. K. Myers, D. L. M. Suess, T. A. Stich, V. Pelmenschikov, S. A. Shiigi, S. P. Cramer, J. R. Swartz, R. D. Britt and S. J. George, *Science*, 2014, **343**, 424–427.

51 R. R. Mendel, A. G. Smith, A. Marquet and M. J. Warren, *Nat. Prod. Rep.*, 2007, **24**, 963–971.

52 (*a*) R. R. Mendel, *J. Biol. Chem.*, 2013, **288**, 13165–13172; (*b*) C. Nivol and S. Leimkühler, *Biochim. Biophys. Acta, Bioenerg.*, 2013, **1827**, 1086–1101; (*c*) B. M. Hover, N. K. Tonthat, M. A. Schumacher and K. Yokoyama, *Proc. Natl. Acad. Sci.*, 2015, **112**, 6347–6352; (*d*) G. Schwarz, *Cell. Mol. Life Sci.*, 2005, **62**, 2792–2810.

53 (*a*) G. Gutzke, B. Fischer, R. R. Mendel and G. Schwarz, *J. Chem. Biol.*, 2001, **276**, 36268–36274; (*b*) M. M. Wuebbens and K. V. Rajagopalan, *J. Biol. Chem.*, 2003, **278**, 14523–14532; (*c*) J. Sloan, J. R. Kinghorn and S. E. Unkles, *Nucleic Acids Res.*, 1999, **27**, 854–858; (*d*) M. J. Rudolph, M. M. Wuebbens, K. V. Rajagopalan and H. Schindelin, *Nat. Struct. Biol.*, 2001, **8**, 42–46.

54 R. P. Hausinger, B. Desguin, M. Fellner, J. A. Rankin and J. Hu, *Curr. Opin. Chem. Biol.*, 2018, **47**, 18–23.

55 Review:S. Chatterjee, S. Gatreddi, S. Gupta, J. L. Nevarez, J. A. Rankin, A. Turmo, J. Hu and R. P. Hausinger, *Biochem. Soc. Trans.*, 2022, **50**, 1187–1196, DOI: **10.1042/BST20220490.55**.

56 (*a*) C. Doering, B. Ermentrout and G. Oster, *Biophys. J.*, 1995, **69**, 2256–2267; (*b*) K. W. Beyenbach and H. Wieczorek, *J. Exp. Biol.*, 2006, **209**, 577–589; (*c*) R. L. Cross and V. Müller, *FEBS Lett.*, 2004, **576**, 1–4.

57 U. Genschel, *Mol. Biol. Evol.*, 2004, **21**, 1242–1251.

58 (*a*) R. Leonardi and S. Jackowski, *EcoSal Plus*, 2007, **2**(2), DOI: **10.1128/ecosalplus.3.6.3.4**; (*b*) T. P. Begley, C. Kinsland and E. Strauss, *Vitam. Horm.*, 2001, **61**, 157–171.

59 (*a*) S. Lin and J. E. Cronan, *Mol. Biosyst.*, 2011, **7**, 1811–1821; (*b*) S. Lin, R. E. Hanson and J. E. Cronan, *Nat. Chem. Biol.*, 2010, **6**, 682–688.

60 (*a*) R. G. Matthews, *Acc. Chem. Res.*, 2001, **34**, 681–689; (*b*) Q. Zhang, W. A. van der Donk and W. Liu, *Acc. Chem. Res.*, 2012, **45**, 555–564; (*c*) A. B. Hazra, A. W. Han, A. P. Mehta, K. C. Mok, V. Osadchiy, T. P. Begley and M. E. Taga, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 10792–10797; (*d*) Y. Mathur, S. Sreyas, P. M. Datar, M. B. Sathian and A. B. Hazra, *J. Biol. Chem.*, 2020, **295**, 10522–10534.

61 (*a*) E. Raux, H. L. Schubert and M. J. Warren, *Cell. Mol. Life Sci.*, 2000, **57**, 1880–1893; (*b*) S. J. Moore, A. D. Lawrence, R. Biedendieck, E. Deery, S. Frank, M. J. Howard, S. E. J. Rigby and M. J. Warren, *Proc. Natl. Acad. Sci.*, 2013, **110**, 14906–14911; (*c*) H. Fang, J. Kang and D. Zhang, *Microb. Cell Fact.*, 2017, **16**, 15; (*d*) A. B. Hazra, A. W. Han,

A. P. Mehta, K. C. Mok, V. Osadchiy, T. P. Begley and M. E. Taga, *Proc. Natl. Acad. Sci.*, 2015, **112**, 10792–10797.
62 V. Gorelova, O. Bastien, O. De Clerk, S. Lespinats, F. Rébeillé and D. van Der Straeten, *Sci. Rep.*, 2019, **9**, 5731.
63 A. D. Welch, *Perspect. Biol. Med.*, 1983, **27**, 64–75.
64 A. Bermingham and J. P. Derrick, *BioEssays*, 2002, **24**, 637–648.
65 (*a*) G. M. Brown, *J. Biol. Chem.*, 1962, **237**, 536–540; (*b*) K. M. Herrmann, *Plant Cell*, 1995, **7**, 907–919; (*c*) A. R. Knaggs, *Nat. Prod. Res.*, 2003, **20**, 119–136.
66 (*a*) J. McKinney and T. Tunckanat, *FASEB J.*, 2021, **35**, S1; (*b*) K. D. Allen, H. Xu and R. H. White, *J. Bacteriol.*, 2014, **96**, 3315–3323.
67 D. M. Howell and R. H. White, *J. Bacteriol.*, 1997, **179**, 5165–5170.
68 T. Sato and H. Atomi, *Curr. Opin. Microbiol.*, 2011, **14**, 307–314.
69 (*a*) S. Roje, *Phytochemistry*, 2006, **67**, 1686–1698; (*b*) A.-W. Struck, M. L. Thompson, L. S. Wong and J. Micklefield, *ChemBioChem*, 2012, **13**, 2642–2655; (*c*) K. Yokoyama and E. A. Lilla, *Nat. Prod. Rep.*, 2018, **35**, 660–694; (*d*) J. B. Broderick, B. R. Duffus, K. S. Duschene and E. M. Shepard, *Chem. Rev.*, 2014, **114**, 4229–4317; (*e*) K. A. Shisler and J. B. Broderick, *Curr. Opin. Struct. Biol.*, 2012, **22**, 701–710; (*f*) G. L. Holliday, E. Akiva, E. C.Meng, S. D. Brown, S. Calhoun, U. Pieper, A. Sali, S. J. Booker and P. C. Babbitt, *Methods Enzymol.*, 2018, **606**, 1–71; (*g*) A. P. Mehta, S. H. Abdelwahed, N. Mahanta, D. Fedoseyenko, B. Philmus, L. E. Cooper, Y. Liu, I. Jhulki, S. E. Ealick and T. P. Begley, *J. Biol. Chem.*, 2015, **290**, 3980–3986.
70 A. C. Eliot and J. F. Kirsch, *Annu. Rev. Biochem.*, 2004, **73**, 383–415.
71 P. A. Frey, *Annu. Rev. Biochem.*, 2001, **70**, 121–148.
72 Reviews: (*a*) T. B. Fitzpatrick, N. Amrhein, B. Kappes, P. Macheroux, I. Tews and T. Raschle, *Biochem. J.*, 2007, **407**, 1–13; (*b*) T. Mukherjee, J. Hanes, I. Tews, S. E. Ealick and T. P. Begley, *Biochim. Biophys. Acta*, 2011, **1814**, 1585–1596; (*c*) M. Tambasco-Studart, O. Titiz, T. Raschle, G. Forster, N. Amrhein and T. B. Fitzpatrick, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 13687–13692.
73 B. Richts, J. Rosenberg and F. M. Commichau, *Front. Mol. Biosci.*, 2019, **6**, 32.
74 (*a*) C. T. Jurgenson, S. E. Ealick and T. P. Begley, *EcoSal Plus*, 2009, **3**(2), DOI: **10.1128/ecosalplus.3.6.3.7**; (*b*) V. I. Bunik, A. Tylicki and N. V. Lukashev, *FEBS J.*, 2013, **280**, 6412–6442; (*c*) J. A. Maupin-Furlow, *B Group Vitamins: Current Uses and Perspectives*, ed. J. G. LeBlanc and G. S. de Giori, 2018, ch. 2, DOI: **10.5772/intechopen.77170**.
75 K. Zhang, J. Bian, Y. Deng, A. Smith, R. E. Nunez, M. B. Li, U. Pal, A. M. Yu, W. Qiu, S. E. Ealick and C. Li, *Nat. Microbiol.*, 2016, **2**, 16213.
76 (*a*) L. D. Palmer and D. M. Downs, *J. Biol. Chem.*, 2013, **288**, 30693–30699; (*b*) A. Chatterjee, A. B. Hazra, S. Abdelwahed, D. G. Hilmey and T. P. Begley, *Angew. Chem., Int. Ed.*, 2010, **49**, 8653–8656.
77 T. P. Begley, D. M. Downs, S. E. Ealick, F. W. McLafferty, A. P. Van Loon, S. Taylor, N. Campobasso, H. J. Chiu, C. Kinsland, J. J. Reddick and J. Xi, *Arch. Microbiol.*, 1999, **171**, 293–300.
78 (*a*) P. C. Dorrestein, H. Zhai, F. W. McLafferty and T. P. Begley, *Chem. Biol.*, 2004, **11**, 1373–1381; (*b*) R. Y. Lai, S. Huang, M. K. Fenwick, A. Hazra, Y. Zhang, K. Rajashankar, B. Philmus, C. Kinsland, J. M. Sanders, S. E. Ealick and T. P. Begley, *J. Am. Chem. Soc.*, 2012, **134**, 9157–9159.
79 (*a*) S. Hwang, B. Cordova, N. Chavarria, D. Elbanna, S. McHugh, J. Rojas, F. Pfeiffer and J. A. Maupin-Furlow, *BMC Microbiol.*, 2014, **14**, 260; (*b*) J. Joshi, Q. Li, J. D. García-García, B. J. Leong, Y. Hu, S. D. Bruner and A. D. Hanson, *Biochem. J.*, 2021, **478**, 3265–3279; (*c*) X. Zhang, B. E. Eser, P. K. Chanani, T. P. Begley and S. E. Ealick, *Biochemistry*, 2016, **55**, 1826–1838; (*d*) B. E. Eser, X. Zhang, P. K. Chanani, T. P. Begley and S. E. Ealick, *J. Am. Chem. Soc.*, 2016, **138**, 3639–3642.
80 H. A Cooke, C. V. Christianson and S. D. Bruner, *Curr. Opin. Chem. Biol.*, 2009, **13**, 460–468.
81 (*a*) C. Welte and U. Deppenmeier, *Biochim. Biophys. Acta*, 2014, **1837**, 1130–1147; (*b*) F. Enzmann, F. Mayer, M. Rother and D. Holtmann, *AMB Express*, 2018, **8**(1); (*c*) D. E. Graham and R. H. White, *Nat. Prod. Rep.*, 2002, **19**, 133–147; (*d*) J. G. Ferry, *FEMS Microbiol. Rev.*, 1999, **23**, 13–38.
82 (*a*) R. K. Thauer, A.-K. Kaster, M. Goenrich, M. Schick, T. i. Hiromoto and S. Shima, *Annu. Rev. Biochem.*, 2010, **79**, 507–536; (*b*) P. N. Evans, J. A. Boyd, A. O. Leu, B. J. Woodcroft, D. H. Parks, P. Hugenholtz and G. W. Tyson, *Nat. Rev. Microbiol.*, 2019, **17**, 219–232.
83 (*a*) J. R. Allen, D. D. Clark, J. G. Krum and S. A. Ensign, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 8432–8437; (*b*) X. Liu and T. E. Mattes, *Appl. Environ. Microbiol.*, 2016, **82**, 3269–3279.
84 S. E. Partovi, F. Mus, A. E. Gutknech, H. A. Martinez, B. P. Tripet, B. M. Lange, J. L. DuBois and J. W. Peters, *J. Biol. Chem.*, 2018, **293**, 5236–5246.
85 D. M. Howell, M. Graupner, H. Xu and R. H. White, *J. Bacteriol.*, 2000, **182**, 5013–5016.
86 R. H. White, *Biochemistry*, 1989, **28**, 9417–9423.
87 (*a*) W. Eisenreich and A. Bacher, *Biol. Chem.*, 1992, **267**, 17574–17580; (*b*) D. Miller, Y. Wang, H. Xu, K. Harich and R. H. White, *Biochemistry*, 2014, **53**, 4635–4647.
88 (*a*) S. W. Ragsdale, *Met. Ions Life Sci.*, 2014, **14**, 125–145; (*b*) R. K. Thauer, *Microbiol.*, 1998, **144**, 2377–2406.
89 S. J. Moore, S. T. Sowa, C. Schuchardt, E. Deery, A. Lawrence, J. Vazquez Ramos, S. Billig, C. Birkemeyer, P. T. Chivers, M. J. Howard, S. E. J. Rigby, G. Layer and M. J. Warren, *Nature*, 2017, **543**, 78–82.
90 S. Schaupp, F. J. Arriaza-Gallardo, H.-j. Pan, J. Kahnt, G. Angelidou, N. Paczia, K. Costa, X. Hu and S. Shima, *Angew. Chem., Int. Ed.*, 2022, **61**, e202200994.
91 A. Kirschning, *Angew. Chem., Int. Ed.*, 2021, **60**, 6242–6269.
92 (*a*) W. Gilbert, *Nature*, 1986, **319**, 618; (*b*) G. F. Joyce, *Nature*, 2002, **418**, 214–221; (*c*) M. P. Robertson and G. F. Joyce, *Cold*

Spring Harbor Perspect. Biol., 2012, **4**, a003608; (*d*) D. Penny, *Biol. Philos.*, 2005, **20**, 633–671.

93 (*a*) C. Weiss, F. Sousa, N. Mrnjavac, S. Neukirchen, M. Roettger, S. Nelson-Sathi and W. F. Martin, *Nat. Microbiol.*, 2016, 161; (*b*) J. L. E. Wimmer and W. F. Martin, *Bunsenmagazin*, 2022, **24**, 135–138.

94 M. J. Russell and A. J. Hall, *J. Geol. Soc.*, 1997, **154**, 377–402.

95 (*a*) R. H. Holm, *Adv. Inorg. Chem.*, 1992, **38**, 1–71; (*b*) J. M. Berg and R. H. Holm, in *Iron-sulfur proteins*, ed. T. G. Spiro, Wiley, New York, 1982, pp. 1–66.

96 (*a*) A. D. Tsaousis, *Front. Microbiol.*, 2019, **10**, 2478; (*b*) C. Bonfio, L. Valer, S. Scintilla, S. Shah, D. J. Evans, L. Jin, J. W. Szostak, D. D. Sasselov, J. D. Sutherland and S. S. Mansy, *Nat. Chem.*, 2017, **9**, 1229–1234; (*c*) Related studies: M. Dörr, J. Käßbohrer, R. Grunert, G. Kreisel, W. A. Brand, R. A. Werner, H. Geilmann, C. Apfel, C. Robl and W. Weigand, *Angew. Chem., Int. Ed.*, 2003, **42**, 1540–1543.

97 A. J. Finney and F. Sargent, *Adv. Microb. Physiol.*, 2019, **74**, 465–486.

98 J. J. Braymera, S. A. Freiberta, M. Rakwalska-Bangea and R. Lilla, *Biochim. Biophys. Acta, Mol. Cell Res.*, 2021, **1868**, 118863.

99 (*a*) C. H. Verhees, S. W. M. Kengen, J. E. Tuininga, G. J. Schut, M. W. W. Adams, W. M. de Vos and J. van der Oost, *Biochem. J.*, 2004, **377**, 819–822; (*b*) B. Siebers, B. Tjaden, K. Michalke, C. Dörr, H. Ahmed, M. Zaparty, P. Gordon, C. W. Sensen, A. Zibat, H. Klenk, S. C. Schuster and R. Hensel, *J. Bacteriol.*, 2004, **186**, 2179–2194; (*c*) H. Ahmed, T. J. G. Ettema, B. Tjaden, A. C. M. Geerling, J. van der Oost and B. Siebers, *Biochem. J.*, 2005, **390**, 529–540.

100 (*a*) A.-M. Sevcenco, M. W. H. Pinkse, E. Bol, G. C. Krijger, H. T. Wolterbeek, P. D. E. M. Verhaert and P.-L. Hagedoorn, *Metallomics*, 2009, **1**, 395–402; (*b*) H. Beinert, *JBIC, J. Biol. Inorg. Chem.*, 2000, **5**, 2–15; (*c*) T. A. Major, H. Burd and W. B. Whitman, *FEMS Microbiol. Lett.*, 2004, **239**, 117–123.

101 R. M. Daniel and M. J. Danson, *J. Mol. Evol.*, 1995, **40**, 559–563.

102 (*a*) G. Joyce and L. E. Orgel, in *The RNA World*, ed. R. Gesteland, T. Cech and J. Atkins, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 2nd edn, 1999, pp. 49–77; (*b*) G. D. McDonald and M. C. Storrie-Lombardi, *Astrobiology*, 2010, **10**, 989–1000.

103 (*a*) R. Krishnamurthy, *Acc. Chem. Res.*, 2017, **50**, 455–459; (*b*) J. E. Goldford, H. Hartman, T. F. Smith and D. Segre, *Cell*, 2017, **168**, 1126–1134.

104 A. Marquet, B. Tse Sum Bui, A. G. Smith and M. J. Warren, *Nat. Prod. Rep.*, 2007, **24**, 1027–1040.

105 In this context, it may be of interest to note that the pair of amino acids and coenzymes is a metabolic chicken-and-egg problem (see ref. 2b).

106 (*a*) H. J. Morowitz, *Complexity*, 1999, **4**, 39–53; (*b*) G. Caetano-Anolles, L. S. Yafremava, H. Gee, D. Caetano-Anolles, H. S. Kim and J. E. Mittenthal, *Int. J. Biochem. Cell Biol.*, 2009, **41**, 285–297.

107 (*a*) H. S. Bernhardt and W. M. Patrick, *J. Mol. Evol.*, 2014, **78**, 307–309; (*b*) H. S. Bernhardt and W. P. Tate, *Biol. Direct*, 2008, **3**, 53; (*c*) K. Tamura, *J. Mol. Evol.*, 2015, **81**, 69–71.

108 (*a*) A. Eschenmoser, *Chem. Biodiversity*, 2007, **4**, 554–573; (*b*) S. A. Benner, H.-J. Kim and E. Biondi, *Life*, 2019, **9**, 84; (*c*) S. Harrison and N. Lane, *Nat. Commun.*, 2018, **9**, 5176; (*d*) A. Wołos, R. Roszak, A. Żądło-Dobrowolska, W. Beker, B. Mikulak-Klucznik, G. Spólnik, M. Dygas, S. Szymkuć and B. A. Grzybowsk, *Science*, 2020, **369**, eaaw1955.

109 (*a*) E. Smith and H. J. Morowitz, *The Origin and Nature of Life on Earth: the Emergence of the Fourth Geosphere*, Cambridge University Press, New York, NY, 1st edn, 2016; (*b*) H. J. Morowitz, J. D. Kostelnik, J. Yang and G. D. Cody, *Proc. Natl. Acad. Sci.*, 2000, **97**, 7704–7708; (*c*) E. Smith and H. J. Morowitz, *Proc. Natl. Acad. Sci.*, 2004, **101**, 13168–13173; (*d*) R. T. Stubbs, M. Yadav, R. Krishnamurthy and G. Springsteen, *Nat. Chem.*, 2020, **12**, 1016–1022.

110 (*a*) M. Ralser, *Biochem. J.*, 2018, **475**, 2577–2592; (*b*) K. B. Muchowska, E. Chevallot-Beroux and J. Moran, *Bioorg. Med. Chem.*, 2019, **27**, 2292–2297.

111 (*a*) R. Breslow, *Tetrahedron Lett.*, 1959, **1**, 22–26; (*b*) C. Appayee and R. Breslow, *J. Am. Chem. Soc.*, 2014, **136**, 3720–3723.

112 (*a*) J. D. Sutherland, *Angew. Chem., Int. Ed.*, 2016, **55**, 104–121; (*b*) J. D. Sutherland, *Angew. Chem., Int. Ed.*, 2016, **55**, 104–12194.

113 N. Friedmann and S. L. Miller, *Science*, 1969, **166**, 766–767.

114 (*a*) C. Shen, L. Yang, S. Miller and J. Oró, *Origins Life Evol. Biospheres*, 1987, **17**, 295–305; (*b*) C. Shen, L. Yang, S. L. Miller and J. Oró, *J. Mol. Evol.*, 1990, **31**, 167–174.

115 (*a*) M. Barbieri, *Biosystems*, 2019, **181**, 11–19; (*b*) M. Barbieri, *Biosystems*, 2019, **185**, 104024.

116 (*a*) S. W. Ragsdale and E. Pierce, *Biochim. Biophys. Acta*, 2008, **1784**, 1873–1898; (*b*) S. W. Ragsdale, *Ann. N. Y. Acad. Sci.*, 2008, **1125**, 129–136.

117 M. J. Warren and A. I. Scott, *Trends Biochem. Sci.*, 1990, **15**, 486–491.

118 G. L. Holliday, J. M. Thornton, A. Marquet, A. G. Smith, F. Rebeille, R. R. Mendel, H. L. Schubert, A. D. Lawrence and M. J. Warren, *Nat. Prod. Rep.*, 2007, **24**, 972–987.

119 G. Layer, J. Reichelt, D. Jahn and D. W. Heinz, *Protein Sci.*, 2010, **19**, 1137–1161.

120 G. Layer, M. Jahn, J. Moser and D. Jahn, *ACS Bio Med Chem Au*, 2022, **2**, 196–204, DOI: **10.1021/acsbiomedchemau.1c00061**.

121 (*a*) F. Blanche, B. Cameron, J. Crouzet, L. Debussche, D. Thibaut, M. Vuilhorgne, F. J. Leeper and A. R. Battersby, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 383; (*b*) J. R. Roth, J. G. Lawrence and T. A. Bobik, *Annu. Rev. Microbiol.*, 1996, **50**, 137.

122 M. J. Warren, E. Raux, H. L. Schubert and J. C. Escalante-Semerena, *Nat. Prod. Rep.*, 2002, **19**, 390.

123 S. Gambarelli, F. Luttringer, D. Padovani, E. Mulliez and M. Fontecave, *ChemBioChem*, 2005, **6**, 1960–1962.

124 (*a*) M. N. Price, A. M. Deutschbauer and A. P. Arkin, *PLoS Genet.*, 2021, **17**, e100934; (*b*) D. Sean Froese, B. Fowler

and M. R. Baumgartner, *J. Inherited Metab. Dis.*, 2019, **42**, 673–685.

125 (*a*) M. J. Yebra and A. S. Bhagwat, *Biochemistry*, 1995, **34**, 14752–14757; (*b*) S. Kumar, X. Cheng, S. Klimasauskas, S. Mi, J. Posfai, R. J. Roberts and G. G. Wilson, *Nucleic Acids Res.*, 1994, **22**, 1–10; (*c*) T. V. Mishanina, E. M. Koehn and A. Kohen, *Bioorg. Chem.*, 2012, **43**, 37–43.

126 E. Smith and H. J. Morowitz, *The Origin and Nature of Life on Earth: the Emergence of the Fourth Geosphere*, Cambridge University Press, New York, NY, 1st edn, 2016.