WILEY ChemBioEng Reviews

Anaerobic radical enzymes for biotechnology

Journal:	*ChemBioEng Reviews*
Manuscript ID	cben.201800003.R1
Wiley - Manuscript type:	Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Jäger, Christof; University of Nottingham, Chemical and Environmental Engineering Croft, Anna; University of Nottingham, Chemical and Environmental Engineering
Keywords:	Radicals, Enzymes, Catalysis, Biotechnology, Anaerobic reactions

Christof M. Jäger and Anna K. Croft**

Anaerobic radical enzymes for biotechnology

AUTHORS: *Dr Christof Martin Jäger* and Dr Anna Kristina Croft**

ADDRESS: Department of Chemical and Environmental Engineering, University of Nottingham, Nottingham, NG7 2RD, United Kingdom. christof.jaeger@nottingham.ac.uk, anna.croft@nottingham.ac.uk

ABSTRACT:

Enzymes that proceed through radical intermediates have a rich chemistry that includes functionalisation of otherwise unreactive carbon atoms, carbon-skeleton rearrangements, aromatic reductions, and unusual eliminations. Especially under anaerobic conditions, organisms have developed a wide range of approaches for managing these transformations that can be exploited to generate new biological routes towards both bulk and specialty chemicals. These routes are often either much more direct or allow access to molecules that are inaccessible through standard (bio)chemical approaches. This review gives an overview of some of the key enzymes in this area: benzoyl-CoA reductases (that effect the enzymatic Birch reduction), ketyl radical dehydratases, coenzyme B_{12}-dependant enzymes, glycyl radical enzymes, and radical SAM (AdoMet radical) enzymes. These enzymes are discussed alongside biotechnological applications, highlighting the wide range of actual and potential uses. With the increased diversity in biotechnological approaches to obtaining these enzymes and information about them, even more of these amazing enzymes can be expected to find application in industrial processes.

(1190/1200 characters)

KEYWORDS: Radicals, Enzymes, Catalysis, Biotechnology, Anaerobic reactions.

1. Introduction

Radical chemistry is extremely versatile in functionalizing otherwise unreactive molecules. This is specifically owed to the typically high reactivity of radical species, but often comes at the cost of poor regioselectivity and stereoselectivity, and the requirement for redox-active agents, such as potentially toxic metal complexes, to initiate the reaction. As such, radical chemistry has found utility in areas such as polymer production, where either the incipient radical or its reaction can be closely controlled, although reactions ranging from carbon-carbon bond formation, carbon-heteroatom bond formation, cyclisations (including cascade cyclisations), rearrangements, and C-H bond cleavages are all well documented.[1]

Nature has transcended the challenges of radical chemistry, whilst retaining the benefits of the broad range of reactivity, through enzymatic control. The chiral, three-dimensional structure of enzymes helps to specifically locate substrates in relation to radical activating agents and can thus control both the targeted bond (regio-control), and the stereochemical outcome of the radical process. Radical enzymes are likely to have had a significant contribution in directing chemistry in the pre-oxygen era,[2, 3] and continue to play important roles in life processes, such as in the synthesis of DNA precursors,[4] detoxifying the body,[5] degradation of biological materials,[6] carrying out oxidations and epoxidations in the production of metabolites,[7, 8] decarboxylation reactions of key metabolic pathways,[9, 10] and in many other biosynthetic pathways.[11-13]

Practically, radical enzymes can be broken into two major classes; aerobic and anaerobic. Aerobic radical enzymes are typically oxidases, and utilise either oxygen or other reactive oxygen species to achieve their transformations. Examples of industrially-relevant aerobic radical enzymes include laccases,[14, 15] Cytochrome P450 enzymes (CYPs, P450s),[16-18] horseradish peroxidase,[19] and oxygenases,[20] with applications ranging from bioremediation, detoxification of wastewater streams, food preservation, fuel cells, and bleaching, to biosensors and diagnostics. Aerobic radical enzymes are thus well-established catalysts in biotechnology.

Of increasing industrial interest are the anaerobic radical enzymes, especially for fine and bulk chemicals. Importantly, these enzymes have the potential to functionally modify a substrate without oxygen incorporation, requiring less (expensive) adaptation of downstream synthetic methodologies than from oxygen-rich biomass-derived feedstocks.[21] As such, anaerobic radical enzymes could either act as a bio-based stop-gap in catalytic schemes while other new synthetic approaches are more fully developed for these sustainable resources, or as a replacement for harsher chemical conversions. Anaerobic radical enzymes possess great scope, effecting hydrocarbon and aromatic reactions,[22-25] providing routes to methane activation[25] and heteroatom insertions,[26] and in the catabolism of amino acids to generate a broad range of branched and unbranched hydrocarbon chains.[12, 13] Investments in developing such reactions with anaerobic radical enzymes have been made by such well-known companies (and their subsidiaries) as Cargill,[27, 28] Novozymes,[29, 30] BASF,[31-35] Roche,[36-39] DuPont,[40-43] INVISTA,[44-46] and Ajinomoto,[47] amongst others.

This review focuses on the current applications and prospects for anaerobic radical enzymes in biotechnology. Historically, relatively little had been known about these enzymes due to their sensitivity to oxygen, and thus the necessity of stringent handling conditions for purification, mechanistic study and further development. This capacity exists in only a few specialist groups around the world and had limited the extent of the data that was available. However, the increasing growth in genomic information, and identification of specific signatures for a number of classes of radical enzyme has led to an intensified awareness of the sheer range of enzymes now available.[48, 49] A flavour, therefore, of different enzyme types and their reactions is presented below to highlight this diversity. With the broadening of approaches to obtaining mechanistic and functional data on anaerobic radical enzymes, more of these underexploited biocatalysts can be expected to enter the biotechnology market in the future.

2. General classes of anaerobic radical enzymes

2.1 Iron-sulfur based enzymes

Before the 'Great Oxidation Event',[50] the biologically induced enrichment of terrestrial oceans and atmosphere with dioxygen, reduced forms of minerals, such as those rich in sulfide and divalent ferrous iron, were prevalent. Initial prototype reactions may have occurred on exposed iron-sulfide mineral deposits, suggesting a route by which protein scaffolds could enhance these iron-sulfur-catalysed reactions to develop them into life-processes, often referred to as the iron-sulfur world hypothesis.[51-53] Today, these anaerobic, radical-generating enzymes are often seen as a functional relic of this era, before the rapid genetic development of other forms of electron transport.[54] The fact that they still persist is indicative of the core reactions that these enzymes catalyse,[2] some of which are impossible to achieve without the extreme reactivity of radicals.

2.1.1 Different types of cluster

There are a range of iron sulfur clusters in enzymes with more than a single iron centre; Fe_2S_2, Fe_3S_4, Fe_4S_4, and $Fe_8S_{7/8}$ are typical as electron carriers.[55] Within proteins, such clusters are typically held in place by either cysteinyl or histidine residues, with aspartate, serine or backbone amide groups also acting as ligands. Of interest to radical enzymes are the ferrodoxin-like class of low-potential iron clusters, consisting of both Fe_2S_2 and Fe_4S_4 (Figure 1). In Fe_2S_2 clusters, the iron atoms are bridged by the sulfide moieties, generating a butterfly-like structure. Such clusters are found in an oxidised divalent state, with no overall spin (singlet state, S=0), or reduced singly charged state with spin of either 1/2 or

9/2. The observation has been made that these clusters can act as the basic unit for the assembly of larger clusters, although cluster biogenesis within enzymes is often complicated.[55, 56]

[FIGURE 1]

Fe_4S_4 clusters are cuboid in structure, with a broader range of oxidation states from zero to trivalent. Through complexation of three or four cysteine residues they are also able to impart structural stability, in addition to their chemical role. In general, low-potential iron sulfur clusters provide a highly reducing environment, with redox potentials normally between -200 to -650 mV, although this range is strongly dependent on the ligands surrounding the cluster.[57] It is these low potentials that facilitate the radical chemistry in the examples described below.

2.1.2 Enzymatic Birch reduction

One of the classic reactions of organic chemistry, the Birch reduction of aromatic rings was reported by the Australian chemist Arthur Birch in 1944.[58] This reaction is especially important in the production of functionalised cyclohexenyl derivatives, which are precursors for steroids and their analogues,[59] polyketide derivatives,[60] and Diels-Alder reactions.

Nature too utilises a birch-like reduction as part of the group of enzymes that are termed benzoyl-CoA reductases (EC 1.3.7.8 and 1.3.7.9).[61-63] The global importance of such enzymes comes from their role in the degradation of monocyclic aromatics in the environment under anaerobic conditions, including chemicals toxic to human health such as xylenes, ethylbenzene and benzenes.[64] More recently these enzymes have been shown to have a role in the degradation pathway of phthalates.[65] The extremely low potentials required to achieve reduction of the aromatic moieties are achieved by remarkable combination of iron sulfur chemistry with a combination of other metal ions, such as zinc, tungsten and molybdenum. These enzymes are proposed to catalyse a two-step reduction, leading to the dearomatisation of benzoyl derivatives, and formation of the diene derivatives in a reaction analogous to the synthetic birch reduction (Scheme 1a).[62, 66]

[SCHEME 1]

2.1.3 Aromatics as sources of hydrocarbon derivatives

One example of the commercially-oriented utilisation of benzoyl CoA reductase, patented by INVISTA, comes in the production of 7-carbon containing chemicals from aromatics.[44] Here, a variety of synthetically useful building blocks, including pimelic acid, 7-aminoheptanoate, 7-hydroxyheptanoate, heptamethylenediamine and 1,7-heptanediol, are generated from aromatic derivatives by the creation of new biological pathways from existing enzymes. Specifically, pimeloyl-CoA is the common intermediate for these straight-chain derivatives, and this molecule is generated *via* a five-step enzymatic process from the benzoyl CoA precursor, which in turn is generated from chorismate through one of two different three-step enzymatic processes. The key reaction to ensure that the aromatic derivatives are amenable to linearization is carried out by benzoyl CoA reductase, where reduction of benzoyl CoA generates the cyclohexa-1,5-diene-1-carboxyl-CoA derivative, which can be ring-opened after further oxidation (Scheme 1b).

2.1.4 Ketyl radical dehydratases

Dehydration of 1,3-ketoalcohols to generate alkenes through elimination is a classical reaction taught in high school and undergraduate chemistry classes, and central to fatty acid metabolism. The mechanism for dehydration of a 1,3-ketoalcohol relies on the increased acidity of the α-hydrogen, and thus stability of the intermediate carbanion *via* conjugation, to drive this reaction thermodynamically (Scheme 2a). For dehydrations of either 1,2- or

1,4-ketoalcohol though, the hydrogen atom to be removed is not as acidic, eliminating the driving force and requiring nature to come up with an alternative approach.[67] For these reactions, a radical is proposed enact an umpolung reaction *via* an intermediate ketyl radical, which allows subsequent deprotonation and dehydration. The deprotonation in 1,2- and 1,4-dehydratases is typically initiated by a single electron reduction *via* either an 'Archerase'[68] or an FAD-dependent oxidation,[69, 70] respectively, both mediated with an Fe_4S_4 cluster. The 'Archerase' activators of 2-hydroxyacyl-CoA dehydratases are so named because they evoke the image of an archer in their action, shooting an electron into the dehydratase, driven by ATP hydrolysis. This electron, required for catalysis, is returned after each turnover back to the Fe_4S_4 of the enzyme (Scheme 2a(ii)), so only one shot of the archer is required to initiate many turnovers.

[SCHEME 2]

The characterised ketylalcohol dehydratases include: (*R*)-2-hydroxyglutaryl-CoA dehydratase (utilised in glutamate metabolism in *Clostridia* and *Acidaminococcus*, amongst others, EC 4.2.1.167),[71] lactyl-CoA dehydratase (EC 4.2.1.54), involved in lactate and amino acid fermentation, (*R*)-phenyllactyl-CoA dehydratase (phenylalanine, tyrosine and tryptophan degradation, EC 4.2.1.B25), (*R*)-2-hydroxyisocaproyl-CoA dehydratase (leucine fermentation, EC 4.2.1.157),[72, 73] and 4-hydroxybutyryl-CoA dehydratase (involved in a number of pathways, including metabolite degradation and CO_2 fixation, EC 4.2.1.120).[69] Very recently, the radical SAM (see section 2.4, below) dehydratase AprD4 has also been proposed to catalyse the 1,2-diol dehydration of the antibiotic intermediate paromamine to 4'-oxolividamine *via* a ketyl radical-based mechanism.[74]

2.1.5 Unsaturated organic acid synthesis and derivatives therefrom

Applications of 2-hydroxyglutaryl-CoA dehydratase focus on the bioproduction of unsaturated dicarboxylic acids. BASF has patented glutaconate production utilizing a recombinant organism containing the enzyme, with feedstocks of either glutamate or glucose.**[31]** A similar approach to the bioproduction of adipic acid has also been described, relying on the broad substrate specificity of clostridial-derived 2-hydroxyglutaryl-CoA dehydratase expressed in *E. coli* to generate the unsaturated adipic acid precursor, 2-hexenedioic acid.[75]

Crotonyl-CoA is another target for the production of polymers and as an intermediate towards biofuels. Production of this derivative follows very similar biochemical routes to that for glutaconate, with the added step of the decarboxylation of the glutaconyl-CoA produced from 2-hydroxyglutaryl-CoA dehydratase before either deesterificaction,[76] or further biochemical transformations.[45, 77]

The generation of a variety of 7-carbon containing chemicals useful for the production of nylons and related polymers has been described utilising 2-hydroxyglutaryl-CoA dehydratase.[46] Reduction of 2-hydroxypimeloyl-CoA to the corresponding unsaturated derivative 2(*E*)-heptenedioyl-CoA is carried out with the 2-hydroxyglutaryl-CoA dehydratase en route to either 2(*E*)-heptenedioate, pimeloyl-CoA or pimelate semialdehyde, which can be further functionalised.[46] Celexion take an alternative approach to generating related difunctional hexanes by claiming not only 2-hydroxyglutaryl-CoA dehydratase, but also the ketyl-radical enzyme lactyl-CoA dehydratase,[78] as enzymes that can be used to convert 6-amino-2-hydroxyhexanoic acid (and/or the corresponding CoA ester) to (*E*)-6-aminohex-2-enoic acid, with subsequent hydrogenation to generate ε-aminocaproate.

More commonly, lactyl-CoA dehydratase has been described in the production of for example 3-hydroxypropionic acid (3-HP) and other derivatives *via* the formation of an acryl-CoA (propenoyl-CoA) intermediate (Scheme 2b).[29, 45, 79-81] These 3-carbon units are extremely versatile for the production of a range of industrially useful building blocks, such as for example 1,3-propanediol (1,3-PD), methacrylic acid,[82] 1,3-butadiene, and this can be achieved in principle with relatively good conversions from lactate.[83] This type of conversion also gives access to these materials from a range of renewable resources including glucose,[27] and lignocellulosic biomass.[80]

An alternative route to derivatives such as 1,3-butadiene can be achieved through crotonyl-CoA formed *via* the action of 4-hydroxybutyryl-CoA dehydratase, with an additional isomerisation from vinylacetyl-CoA delta isomerase (Scheme 2c).[45] Similarly, this reaction, has been the basis for biological routes to 1,3-butanediol,[84] methacrylic acid, and methacrylate esters. The production of 1,4-butanediol[85] suggests that the additional isomerase activity of 4-hydroxybutyryl-CoA dehydratase can be utilised to generate the 1,4 diol motif (Scheme 2d).[86]

For these dehydratases, the selected examples above indicate the wide scope in the production of both bulk and specialty chemicals that is made accessible. They will certainly continue to play an important role in bioproduction for the foreseeable future.

2.2 B_{12}-dependent enzymes

Coenzyme B_{12} (Figure 2a) is one of the more widely-studied radical generating agents in nature, having the unique feature of also being an organometallic.[87] Coenzyme B_{12} is one of the most complex cofactors, requiring over 30 biosynthetic steps to produce de novo.[88] The highly complex structure contributes to the electronic control over the bound cobalt and provides numerous points for biological recognition and specificity, helping to anchor the coenzyme in a precise position for generation of the highly reactive adenosyl radical intermediate. In fact, the tight control of this radical, and others involved in the reaction pathways, is a defining feature of B_{12}-dependent enzymes, as many of the intermediates are both highly oxygen sensitive and extremely reactive.[89]

[FIGURE 2]

2.2.1 Cofactor Chemistry

The carbon-cobalt bond of coenzyme B_{12} is the defining catalytic motif from which the adenosyl radical (Ado•) is generated upon cleavage (Figure 2b). This cleavage is thought to be triggered upon binding of the substrate to the coenzyme-containing enzyme, induced by structural changes.[89] This ensures that the intermediate radicals are shielded from reactive oxygen species and other molecules that may interact with the highly reactive intermediate, and helps to ensure the recyclability of the cofactor. This approach for radical catalysis is thus practical for a range of specialist reactions of industrial interest, including carbon-skeleton rearrangements, aminomutases (Table 1) and eliminases (Table 2).

[TABLE 1] [TABLE 2]

2.2.2 1,3-Propanediol production

The related enzymes diol dehydratase (EC 4.2.1.28) and glycerol dehydratase (EC 4.2.1.30) catalyse the key step in the enzymatic generation of 1,3-propanediol (PDO or 1,3-PD), from glycerol. In a process used by DuPont Tate&Lyle,[40],[41] more than 60,000 tonnes/annum of this bio-generated material is produced and marketed as either Susterra®, with application to a number of industrial products such as polyurethanes, unsaturated polyester resins, engine coolants, and either heat-transfer, low-temperature food-safe, or deicing fluids; or as Zemea® with food, personal care and pharmaceutical applications. Because the *E. coli* used in the biotransformation does not naturally produce the cofactor coenzyme-B_{12} de novo, the pathway for its synthesis is included in the modified organism. DuPont Tate&Lyle have also carried out cradle-to-gate LCA analyses to demonstrate the effectiveness in this bio-based process with respect to reduction of greenhouse gas emissions and non-renewable energy use, relative to the petroleum-based derivatives.[90]

The radical-catalysed isomerisation of the 1,2-diol portion of glycerol to the corresponding aldehyde (3-HPA), followed by reduction, affords the 1,3-diol (Scheme 3).

[SCHEME 3]

The utility of this conversion for short-chain 1,2-diols means that diol dehydratase has also been employed in other reaction schemes for renewable bulk derivatives, including production of intermediates butanone (and thus also 2-butanol)[42] from 2,3-butanediol,[43, 45, 91] propanal from 1,2-propanediol,[45] and many applications where 3-HPA is a desired intermediate.

2.2.3 Branched to straight-chain derivatives

B_{12}-Dependant carbon-skeleton mutase enzymes take branched chain derivatives and convert them to straight chain derivatives for further metabolic processing (Table 1).[92] Characterised versions include methylmalonyl CoA mutase (EC 5.4.99.2), ethylmalonyl CoA mutase (EC 5.4.99.63), isobutyryl CoA mutase (EC 5.4.99.13), 2-hydroxyisobutyryl-CoA mutase (EC 5.4.99.64), methylene glutarate mutase (EC 5.4.99.4), and glutamate (methylaspartate) mutase (EC 5.4.99.1). Genomatica have also described the possibility of using isobutyryl-CoA mutase as a 4-hydroxybutyryl-CoA mutase *via* substrate promiscuity, en route to methacrylate,[82, 93] and isobutanol.[94]

The ability to transform a carbon backbone is invaluable in the preparation of new chemicals. This is particularly highlighted through the deployment of the four-carbon precursor succinyl-CoA as a readily accessible intermediate to access the three-carbon precursor propanoyl-CoA, through initial conversion to the branched methylmalonyl-CoA using methylmalonyl-CoA mutase, followed by epimerisation and decarboxylation.[45] Genomatica describe the same initial steps, conversion of succinyl-CoA to methylmalonyl-CoA using the mutase, and subsequent epimerisation to access methacrylic acid *via* a range of routes.[82]

Mutases are described extensively in a number of possible routes to the important bulk chemical methacrylic acid.[82] Glutamate mutase can be utilised to convert glutamate to 3-methylaspartate, which after elimination of ammonia and subsequent decarboxylation can generate methacrylic acid. In another alternative approach that also utilises a mutase reaction 2-hydroxyglutarate is used as a substrate to generate the corresponding 3-methylmalate, which is then similarly dehydrated to mesaconate and decarboxylated to methacrylic acid. Further, catalysis by 3-hydroxybutyryl-CoA mutase is described, in a route from acetyl-CoA, to generate 2-hydroxyisobutyryl-CoA, which, through subsequent radical-mediated dehydration, generates methacroyl-CoA as a methacrylic acid precursor. In another process starting from acetyl-CoA, isobutyryl-CoA mutase converts butyryl-CoA, generated from reduction of crotonyl-CoA, into isobutyryl-CoA, which is dehydrogenated to also form methacryl-CoA. These suggested transformations show how a full range of mutases can be creatively utilised to access similar building blocks from a wide range of starting carbon-chain lengths and configurations (Scheme 4).

[SCHEME 4]

The production of ethylmalonyl-CoA mutase has been described for the preparation of 3-hydroxyisobutyric acid and related derivatives through a multistep pathway going through crotonyl-CoA.[95, 96] After carboxylation of crotonyl-CoA to form the ethylmalonyl-CoA, the mutase generates the corresponding methylsuccinyl-CoA, which is converted in three further steps to the propanoyl-CoA precursor. Alternative routes to the desired 3-hydroxyisobutyric acid, starting from different precursors, can also be achieved by utilizing either methylmalonyl-CoA mutase or isobutyryl-CoA mutase.[96]

Mutases are described in the production of biofuel and small alcohols, with isobutyryl-CoA used particularly where introduction of a branched-chain is valuable.[97, 98] Isopropanol can be produced from the appropriate four-carbon straight-chain CoA derivative.[98] Similarly, the bulk chemical *n*-propanol can be produced by the action of methylmalonyl-CoA mutase on succinyl-CoA. Here, the produced methylmalonyl-CoA is subsequently decarboxylated to form the propanoyl-CoA intermediate for *n*-propanol synthesis.[30]

As can be seen, there is a strong contribution from B_{12}-dependent enzymes in targeting highly relevant bulk chemicals, with a major facilitator of their biotechnological role being the close control of the radical. This control means that they are typically more resistant to oxygen than many other anaerobic radical enzymes,[12] and as such more robust to process conditions. Refinement of the activities, for example by selection of improved enzymes from alternate organisms, may provide further advances in the contributions of these enzymes to overcoming our reliance on oil-based technologies.

2.3 Glycyl radical enzymes

As an alternative to directly cofactor-generated radicals, the active radical required for catalysis can be harboured within the enzyme. This is the situation with glycyl-radical enzymes, where the radical is stored on the enzyme backbone, which is proposed to be then transferred to an active-site cysteine for active catalysis. Generation of this stable backbone radical is achieved through a family of radical SAM (see section 2.4)-activating enzymes,[99] that selectively abstract hydrogen from a semi-conserved motif (Table 3).[100] This means that, for activity, both enzymes need to be present in any constructs that are developed, adding additional complexity to their deployment. For these enzymes, anaerobic conditions are essential as reaction of the glycyl radical with oxygen results in cleavage of the protein, and thus permanent inactivation.

[TABLE 3]

2.3.1 Scope of reactions

Although the number of glycyl radical enzymes characterised to date is small, together they cover a range of useful reactivities. This includes carbon-carbon bond cleavage activities of pyruvate formate lyase (EC 2.3.1.54) and homologue 2-oxobutyrate formate lyase (EC 2.3.1.-); carbon-nitrogen bond cleavage catalysed by choline trimethylamine-lyase (EC 4.3.99.4); decarboxylation activity of 4-hydroxyphenylacetate decarboxylase (EC 4.1.1.83); the reductase activity of anaerobic ribonucleotide reductase (EC 1.1.98.6); the dehydratase activity of the B_{12}-independent glycerol dehydratase (EC 4.2.1.30), and recently uncovered 4-hydroxyproline dehydratase;[101] and carbon-carbon bond-forming reactions of benzylsuccinate synthase (EC 4.1.99.11) and methylpentylsuccinate synthase (EC 4.1.99.-).

2.3.2 Hydrocarbon metabolism

A significant industrially-relevant role of glycyl radical enzymes is in environmental protection, through the ability of benzylsuccinate synthase and methylpentylsuccinate synthase to degrade hydrocarbons under anaerobic conditions.[24] In each case, degradation of either toluene (and derivatives, including methylnaphthyl derivatives) or long chain saturated hydrocarbons (C6-C16) is achieved by first coupling to the double bond of fumarate through generating either a tolyl-based or 2-alkyl radical, respectively (Scheme 5). The resulting succinyl derivatives can then be further degraded through a series of standard anaerobic metabolic routes. Recent work to explore the extent to which these enzymes are found suggest that there should be ample scope to select systems compatible with the desired host environment.

[SCHEME 5]

2.4 Radical SAM (Adomet radical) enzymes

S-Adenosyl methionine is a key cofactor not only for heterolytic methylation, for which it is well known,[102] but also in the generation of substrate radicals *via* an adenosyl radical intermediate.[103] This mechanism for radical generation echoes that discussed above for the B_{12}-dependent enzymes (Section 2.2), with these enzymes likely the earlier precursor of the complex B_{12} cofactor. As such, it has been designated the "poor-man's B_{12}" due to its relative simplicity,[104] although the range and scope of reactions uncovered to date for radical *S*-adenosyl methionine-dependent enzymes eclipses those of their B_{12}-dependent cousins substantially. Currently there are around one hundred different enzyme subtypes recognised, with at least twenty more uncharacterised genetic groupings.[49] Recently, hybrid B_{12} (see section 2.2) / radical SAM enzymes have been attracting interest, although the detailed mechanisms of radical catalysis for these enzymes is only just emerging.[105]

2.4.1 General chemistry

All radical SAM enzymes share common features and thus some common chemistry, even though the variety of outcomes is expansive. The structural information to date highlights a common TIM-barrel structure, although this can vary between enzymes from a full-$(\beta/\alpha)_8$ barrel architecture to more common partial barrels (often $(\beta/\alpha)_6$) (Figure 3a),[106] with size most often dependent on the substrate. The key motif is a $CX_3CX\phi C$ motif (with ϕ a conserved aromatic),[107] with these three cysteine residues being crucial for the recruitment of the Fe_4S_4 cluster, with the fourth site complexed to the sulfur of SAM. Other variations with different numbers of residues between the cysteines also exist, highlighting the diversity of primary structure utilised to generate this three-dimensional functional unit.

[FIGURE 3]

The basic and common reaction catalysed by radical SAM enzyme is the cleavage of *S*-Adenosyl methionine to generate an adenosyl radical (Ado$^\bullet$) (Figure 3b), which subsequently abstracts a hydrogen atom from the substrate. Depending on the reaction outcome, either the AdoH product is regenerated to form the radical, and subsequently reconstitutes the cofactor (catalytic action), or the formation of product leaves AdoH as a by-product (cofactor action).

Once hydrogen has been abstracted by the adenosyl radical, this is where the mechanisms of radical SAM enzymes diverge substantially. The catalysed transformations cover a huge range of radical chemistries, including sulfur insertions, decarboxylations, challenging methylations (including at phosphorous and unactivated carbon atoms), carbon-skeleton rearrangements, dehydrogenations, carbon-carbon coupling reactions, and others. As such, they show great potential for solving challenging chemical transformations through a biotechnological approach if they can be harnessed appropriately. The recognition of this potential is reflected by the significant interest in these enzymes in the patent literature, with highlights below.

2.4.2 Antibiotic synthesis

The problem of antimicrobial resistance (AMR) has been recognised as a serious global challenge by the World Health Organisation (WHO),[108] and many other research and health-related bodies.[109-112] The complex structures of many antibiotics, which often involve unusual methylations and C-C bond-forming reactions of amino-acid based and sugar-derived structures, offer a great synthetic challenge that has already been solved by the organisms that produce these antibiotics. As such, there is an increasing focus on pathways that exploit natural biosynthetic pathways for the bioproduction of both interesting antibiotics and their derivatives, access to the latter being especially important in combatting AMR. The examples below highlight the role of radical-generating enzymes and, in particular, rSAM enzymes in the transformations required to access this next generation of compounds to tackle AMR.

Argyrins (Figure 4a) are a group of cyclic peptides consisting of eight amino acids first described by Vollbrecht *et al.*,[113] which have been shown to possess interesting immunosuppressive antibiotic activity.[114] Currently, they are mostly obtained from the natural producer organism *Archangium gephyra* as a mixture of different Argyrins. A patent for the biosynthetic pathway of Argyrins includes the genes for the whole pathway and provides the basis for manipulation of the synthetic pathway to produce Argyrins in micro-organisms.[115] It also includes the gene and amino acid sequence of the radical SAM enzyme Arg1.

[FIGURE 4]

The radical SAM-domain containing enzyme participating in the biosynthetic pathway is involved in the derivatisation of pre-Argyrin, catalysing the methylation of Argyrin A (R_1=CH_3) to Argyrin B (R_1=C_2H_5), thus belonging to the class of methyl transferases.

Nocathiacins (thiazole Nocardia streptozotocin, Figure 4b) belong to a class of cyclic thiazolyl peptide antibiotics first characterised 1998.[116] These highly modified sulphur-rich peptides have shown growth inhibition of *Methicillin-Resistant Staphylococcus aureus* (MRSA) and other antibiotic resistant bacteria.[117] Production of these antibiotics has been patented by Bristol Myers Squibb,[118-120] in addition to an available patent on structurally related antibiotics.[121]

The cephalosporin Nocardia thiazole biosynthetic gene cluster consists of 37 genes in total and includes a gene encoding for a thiamine radical SAM synthetase. The rSAM enzyme encoded by the gene sequence Noc27 plays its role in a crucial rearrangement step during Nocathiacin biosynthesis. As shown in Scheme 6a, this rSAM enzyme catalyses the reaction of tryptophan to 3-methyl-indole-2-carboxylic acid during the biosynthesis of the crucial 5-methyl indole subunits incorporated into the Nocathiacin structures.

[SCHEME 6]

In the biosynthesis of the closely related thiopeptide antibiotic nosiheptide (Figure 4c), a very similar rSAM enzyme is involved. A patent related to the biosynthesis of fluorinated nosiheptide derivatives describes the biotechnological fermentation synthesis of 3-methyl-2-indole acid and fluorinated derivatives thereof in *E. coli.* The radical SAM enzyme NosL could be successfully transferred from *Streptomyces actuosus* to *E.coli* and was shown to catalyse the synthesis of fluorinated 3-methyl-indole-2-carboxylic acid from fluorinated tryptophan.[122] The NosL enzyme has also recently been highlighted in terms of providing access to a wider pool of nucleoside-containing compounds.[123, 124]

Radical SAM enzymes are also involved in several steps of the biosynthesis of pactamycin (Figure 4d). This antibiotic belongs to the group of aminocyclitol antibiotics that are known for their high biological activity and which have been used as antibiotics for a long time, e.g. streptomycin, neomycin and gentamycin.[125] Pactamycin is structurally unique for an aminocyclitol antibiotic, by including two aromatic rings, a dimethylurea unit, and a five-membered aminocyclitol ring structure.[126, 127]

A patent on the pactamycin biosynthetic gene cluster (the encoded proteins therein and their use)[128] from *Streptomyces pactum* contains four sequences encoding for radical SAM enzymes (PtmC, PtmH, PtmL, PtmM). While the roles of the individual radical enzymes in the pactamycin gene cluster are still not fully clear, it is believed that PtmH, -L, and –M are acting as C-methytransferases during the biosynthesis.

Further, analysis of PtmC showed significant similarity to the radical SAM enzyme MitD (50% similarity) involved the biosynthesis of mitomycin.[129] Together with PtmG and PtmJ, PtmC is anticipated to be involved in the formation of a cyclopentitol derivative (Scheme 6b), resulting from initial deacylation, followed by a PtmC-catalysed radical rearrangement.

Within a patent for a distinct peptide (phage) display[130] another radical SAM enzyme has been included. The patent for this modified peptide display makes claim on a genetic package displaying cyclic peptides that have “at least one intramolecular cyclic bond between two heteroatoms of amino acid side chains”.[130] To create this special peptide-display system, a set of post-translationally modifying (PTM) enzymes are used to modify the natural amino acids. One of them is the radical SAM enzyme TpdU. This enzyme is involved in the biosynthesis of another thiopeptide antibiotic thiomuracin (Figure 4e). Like the nocathiacins, thiomuracin also belongs to the class of macrocylic thiazolyl peptide antibiotics but with the special characteristic of a highly modified central six-membered heterocyclic ring system. The catalytic role of TpdU is as a C-methytransferase during biosynthesis, through the methylation of thiazole.

Radical enzymes are also involved in the biosynthesis of tunicamycin (Figure 4f) a fatty acyl nucleoside antibiotic containing uracil and *N*-acetylglucosamine (GlcNAc) moieties, which was first isolated in 1971.[131] The gene cluster for the biosynthesis of tunicamycins has been identified from *Streptomyces chartreusis* and patented.[132] The gene cluster contains only 14 genes of which two are thought to produce enzymes involved in radical reaction catalysis. TunB encodes for a radical SAM FeS-Oxidoreductase that is proposed to catalyse the central C-C coupling between the galactosamine and uridine moieties, together with the Methyltransferase TunM. This mechanism has been proposed based on labelled precursor feeding experiments, however, there have been no additional, clear mechanistic studies that could yet fully confirm this process.

The selected examples of patented radical SAM enzymes involved in the biosynthesis of various antibiotics gives an insight into possible broader industrially-relevant synthetic applications. While many rSAM enzymes act as C-methyl transferases, others are involved in more complex crucial steps, such as heterocyclic ring rearrangements or C-C coupling reactions. Although many of the patented applications to date focus specifically on the preparation of a specific, biologically known antibiotic through its pathway (either in the native organism or *via* a production strain), the use of fluorinated substrates indicates already that there is some scope for a wider variety of derivatives to be prepared. Further enzyme evolution and engineering approaches therefore would logically be the next step in broadening the synthetic applicability of these enzymes further for the preparation of novel antimicrobial compounds.

2.4.3 Amino acid production

The DNA sequence coding for the radical SAM enzyme lysine-2,3-amino mutase (LAM) has already been patented in the US in 1999 for the biochemical synthesis of β-amino acids.[133] LAM converts L-α-lysine to L-β-lysine *via* a radical reaction mechanism, with the need for the additional cofactor pyridoxal phosphate (PLP). It is one of the better-investigated radical enzymes, first purified 1970[134] and crystallised in 2005.[135] The active role of PLP in 1,2-amino migrations was in particular investigated by Han and Fry[136]. Fry also claimed the patent and has been involved in many aspects of understanding the function of LAM. The reaction mechanism of LAM is meanwhile well-understood and verified. It undergoes a classical catalytic cycle with *S*-adenosylmethionine (SAM) being regenerated as a cofactor.

Lysine binds in LAM as an aldimine adduct of PLP. Upon a one electron transfer from the central iron sulfur cluster to SAM the active radical species dAdo$^{\bullet}$ is formed, which subsequently abstracts the 3-pro-*R* hydrogen from the bound lysine. The subsequent radical rearrangement goes *via* an azacyclopropylcarbinyl ring formation and ring-opening, followed by re-abstraction of a hydrogen atom from dAdoH to form the β-amino acid product and close the catalytic cycle.

Even though this patent was meant to be used as an alternative synthesis of L-β-lysine, it has not been industrialised until now. The many challenging requirements for this enzyme, like the need for anaerobic handling due to disruption of the central iron sulfur cluster under oxygen, and the need for the additional cofactor PLP, does not make it commercially viable at this stage.

Due to the discovery and structural characterisation of 2,3-lysine aminomutase, the interest in other amino mutases for the production of β-amino acids has increased. Two more recent patents claim for the disclosure of gene sequences encoding for alanine 2,3 aminomutase[28] and glutamate 2,3 aminomutase activity.[137] Both enzymes have not yet been characterised structurally, but direct transformation of α- to β-amino acids could be verified in both cases. Based on sequence comparisons and evidence for radical intermediates, Ruzicka and Frey directly describe glutamate-2,3-aminomutase as a radical SAM enzyme similar to LAM but with no activity toward lysine.[138] This characterisation has not been presented for alanine-2,3-aminomutase. Further, the patent for the non-natural alanine-2,3-aminomutase is specifically linked to the biosynthesis of 3-hydroxypropionic acid (3-HP). The biosynthesis of 3-HP goes through a beta-alanine intermediate, which is normally rather inefficient for biotechnological applications using high value precursors (see Ref.[28]) but the patent describes direct amino mutase activity on L-α-alanine.

2.4.4 Fine chemicals manufacture

The biotechnological usage of a genetically modified bacterium belonging to the genera *Methylobacterium* or *Hyphomicrobium* for the production of Pyrroloquinoline Quinone (PQQ) has been patented in 2013.[47] The patent claims improved PQQ biosynthesis *via* enhanced expression of the responsible pqq gene cluster in these bacteria.

PQQ is an essential redox cofactor for various bacterial dehydrogenases such as glucose and methanol dehydrogenase. It is mainly found in gram-negative bacteria, but could also be detected in high concentration in breast milk and mouse studies indicate an essential role for proper development and growth.

The biosynthetic pathway of PQQ is still not completely understood, but it is known that this cofactor is derived from the amino acids tyrosine and glutamic acid that are present in a small peptide (23 to 39 amino acids depending on organism) thought to be both the precursor of PQQ and donor of the amino acids needed.[139] Also here, a radical SAM enzyme plays a crucial role during biosynthesis. PqqE (EC 5.-.-.-) is thought to catalyse another radical C-C bond formation between the two amino acids (Scheme 6c). However, the detailed mechanism is still far from clear. In particular it could be demonstrated that PqqE is directly interacting with PqqD but the role of this interaction remains unclear.[140] One hypothesis is that PqqD may influence the active site of PqqE in order to position the 5'-deoxyadenosyl radical for the subsequent hydrogen abstraction from the tyrosine residue in PqqA.

Lipoic acid is a sulfur-containing cofactor essential in organisms that undergo aerobic respiration, behaving as an electron acceptor in oxidative reactions, such as decarboxylation or carbon-carbon bond cleavage.[141] It has a particular role in amino acid degradation, and has additionally been highlighted as an excellent antioxidant. The mechanism biosynthesis of this molecule by the radical SAM enzyme LipA (EC 2.8.1.8) has attracted a significant degree of attention as it involved insertion of two sulfur atoms in an otherwise unactivated carbon backbone chain, a highly chemically challenging reaction (Scheme 6d).[26, 142-144] Structural and kinetic evidence has been provided that at least one, and probably both, sulfur atoms are derived from an auxiliary Fe_4S_4 cluster located near the *N*-terminus of the LipA enzyme,[141, 142, 145-147] meaning that reconstitution of this cluster limits the turnover.

Interest in both the manufacture[148, 149] and cellular up-regulation of lipoic acid has been described,[33, 150-153] the latter with a view to either improving other cellular processes by improving the metabolism of production organisms, or with the specific aim of branched-chain fatty acid synthesis.[154] Synthesis and isolation of lipoic acid is an attractive target, since current chemical approaches suffer from production of racemates, poor yields and are not economic.[148] As such biological routes offer the opportunity for enantiomerically pure production with improvements in process conditions.[149] Such a route has been disclosed reporting potentially more than twice the wild-type activity, with a relatively low incubation temperature of between 25-30 °C, achieved through the cloning of the relevant genes (including FeS cluster assembly/repair genes) in an acid tolerant host.[148] Examples of *Gluconobacter sp.* and *Saccharomyces sp.*, amongst others, are provided, with the target of extracellular production to facilitate isolation by crystallisation.

More recently, expression of relevant genes has been achieved in an *E. coli* construct and the associated patent claims the use of low-value raw materials and improved environmental outcomes.[149] Here, metK, a gene involved in SAM biosynthesis, was also upregulated to ensure that a ready supply of the cofactor was available, since each conversion to lipoic acid requires two molecules of SAM.[143] The disclosure claims a more than 200-fold improvement in lipoic acid production (1.4 - 2.1 mgL^{-1}, dependant on strain and conditions), as measured by UV-vis monitored HPLC, over the corresponding wild-type strain (0.005 mgL^{-1}).

Another essential sulfur-containing cofactor, biotin (vitamin B_7 or vitamin H), is an excellent target for biochemical manufacture, being used in vitamin food supplements, as a medical-grade pharmaceutical, and primarily for the enhancement of animal feed. Current production is mainly met through chemical synthesis, with similar challenges faced as in the production of lipoic acid, namely with enantiopurity and environmental issues being of paramount concern. In nature, only small quantities of biotin are required, meaning that production tends to be low in host organisms. The major bottleneck to improving biosynthetic production is the radical SAM enzyme BioB (EC 2.8.1.6),[36] with an *in vivo* rate reported of around 0.1 min^{-1}.[155] Given that this is comparable to the burst kinetics measured for the initial turnover (0.12 min^{-1}),[156] this implies that the rate limiting step is indeed the sulfur insertion reaction catalysed by BioB, rather than due to the slowness of the repair mechanism that is required to reconstitute the Fe_2S_2 auxiliary cluster cannibalized to provide the sulfur required for the reaction (Scheme 6e).[157]

To overcome the limitations of low biotin production, a number of approaches are disclosed in the patent and public literature, utilising different organisms. The *E. coli* system is the best studied to date,[32, 34, 35, 38, 39, 158-160] however, biotin overproduction has also been described in other organisms including *Agrobacterium sp.*,[159] *Bacillus sphaericus* (including chemically-induced mutants),[160] *Bacillus subtilis*,[36, 161] *Brevibacterium flavum*,[162] *Kurthia sp.*,[37] *Pseudomonas mutabilis*,[158] *Serratia marcescens*,[163-165] *Sphingomonas* sp.,[166, 167] the yeast *Candida utilis*,[168] and plants.[169] Many of the genetic manipulations centre upon modification of the expression systems and genes for proteins upstream from BioB, and/or inclusion of BioB from organisms other than the host organism. Using these approaches, production of biotin in levels ranging from 1.27 mg/L to 68 mg/L (up to 18-fold higher than controls),[166] and even around 15 g/L of 95% biotin,[158] have been claimed. *In vitro* use of purified BioB has also been carried out,[39, 159] and requires inclusion of repair enzymes and/or flavodoxins, to carry out this complex reaction. Interestingly, the rate constant ranges disclosed for the *in vivo* systems of 0.56-2.5 min^{-1},[166] including for mutants of BioB,[167] suggest that evolution, by generating a selection of modified BioBs either naturally or in an engineered fashion, may overcome some of the limitation on throughput, and be one of a number of useful approaches for industrial production in the future.

2.4.5 Antiviral applications

A Chinese patent claims for the preparation and application of an expression vector for the antiviral protein Viperin.[170] Applied in form of direct injection into fish, an increased antiviral potency of the fish was described.

Viperin itself is a protein containing a SAM binding domain,[171] and a recent crystal structure confirms that the active site is structurally similar to other radical-SAM enzymes and binds SAM.[172] Viperin interacts with farnesyl diphosphate synthase (FPPS) in the cell, an enzyme essential in isoprenoid biosynthesis and thus involved in steroid synthesis.[173] It also catalyses the central reductive cleavage of SAM, indicating that viperin exhibits radical SAM chemistry for its antiviral activity.

Very recently, Makins *et al.*[174] showed that over-production of viperin indeed reduces the rate of accumulation of FPPS, however, it does not influence the activity of FPPS. Further, they could demonstrate that mutating central cysteine residues of the FeS binding region does not have a negative effect on the reduction of FPPS levels in the cell which gives a clear indication that viperin does not act as a radical SAM enzyme in respect to FPPS regulation. Still unclear is if radical chemistry plays a role in other potential regulatory effects by viperin, although the recent structure suggests that the substrate may be a nucleotide triphosphate derivative,[172] very recently supported by EPR and modelling studies that identify UDP-glucose as a substrate.[175]

2.4.6 Hyperphotosynthesis

An engineered photosynthetic cell, with increased industrial fitness such as improved pH, salt and temperature tolerance, has been claimed by Joule Biotechnologies, Inc.[176] Although radical SAM enzymes are involved in various steps of the biosynthesis of coproporphyrinogen-III (an intermediate in chlorophyll production) through the enzyme HemN (EC 1.3.99.22),[3] and the radical SAM enzyme BChE is involved in bacteriochlorophyll biosynthesis,[177] this disclosure lists an alternative set of enzymes containing radical SAM domains. There is little detailed information provided on the identified radical SAM enzymes, with the exception of CfR (EC 2.1.1.224),[178] which belongs to the group of radical *S*-adenosyl-methyltransferases, and a highlight of a B_{12}/radical SAM enzyme, also likely to be involved in methyl transfer. A number of other non-radical SAM methyltransferases are also listed, involved either in nucleotide methylation or the biosynthesis of B_{12}. This suggests a primary role for radical SAM enzymes in this patent related to improvements in nucleotide methylation, including ribosomal RNA, related to enhanced protein production *in vivo*.

3. Future prospects

Radical enzymes have already demonstrated a broad scope in terms of applicability to industrial challenges, including the production of bulk chemicals, fine chemicals and much-needed antibiotics. Remaining is the incorporation of many of these enzymes into scaled-up and economically competitive processes, such as has been possible for the best commercially exploited example, the synthesis of 1,3-PD.[40],[41] Here, many current and burgeoning technologies are likely to play a strong role in developing these enzymes further. At the forefront, particularly in the radical field, must be the consideration of computational approaches, since these can not only be utilised cheaply as screening methodologies, but are particularly appropriate to the more oxygen-sensitive enzymes, as they by-pass the need for anaerobic handling. An additional advantage comes when coupling with the vast increase in genomic information; enzymes from difficult hosts can be examined, unknown radical enzymes can be uncovered from their genetic signatures, and mechanistic information across species can be pooled. Developments in the area of computer-led design therefore offer much promise, particularly when coupled either in experimental feedback loops, or with information from complementary experimental approaches such as directed evolution.

One of the most important aspects to ensure that radical enzymes reach their desired impact in industrial applications already shines through the above-described examples. More than for any other enzyme class, the current lack of detailed mechanistic understanding limits our potential for both improving and engineering these kinds of enzymes. Many of the examples included here often recognise radical enzymes as key catalysts, although their detailed function remains unclear. The difficulties in laboratory handling of the enzymes, resulting in limited high level mechanistic studies from just a few expert groups in the world, means that best use has been made of only a limited set of well-characterised radical enzymes. Here, the combination of advanced and steadily improving laboratory techniques, combined with the predictive computational approaches highlighted above, could mark a game-changing approach in the development of stainable (bio-)synthetic approaches incorporating radical enzymes in key catalytic reaction steps in near future.

Through mechanistic understanding, both at the single-enzyme and cross-genome level, specific targets such as directed broadening of substrate specificity, reduction in oxygen sensitivity to reduce the need for stringent process conditions, and improvements in reaction rate, are made a possibility. Other areas for focus include fuller investigation of the enzyme activation repair mechanisms, such that these coupled processes can be carried out more efficiently in either cellular or multi-enzyme *in vitro* systems. Certainly, there is much work ahead for a full exploitation of anaerobic radical enzymes in the same way that is seen for other classes of enzyme today. Yet, as more interest is shown in these unique enzymes, a significant impact on the areas of applicability will no doubt follow.

Acknowledgement

CMJ and AKC acknowledge support through the European Cooperation in Science and Technology (COST) network CM1201. AKC acknowledges support from the Wellcome Trust [091162/Z/10/Z]. Both AKC and CMJ acknowledge funding through the Dean's Prize for Engineering (UoN) and EU FP7 Marie Curie Actions – People Co-funding of Regional, National and International Programmes (COFUND) under Grant Agreement number PCOFUND-GA-2012-600181. CMJ and AKC also acknowledge Prof. Alex Conradie for helpful discussions.

CURRICULUM VITAE:

Christof Jäger is an Assistant Professor at the Faculty of Engineering of the University of Nottingham working on computational chemistry in the field of enzyme design and engineering. He studied Molecular Science at the Friedrich-Alexander Universität Erlangen-Nürnberg in Germany and received his PhD in 2010 working on specific ion effects on molecular self-assembly and radical clock reactions. He worked as a post-doctoral researcher for the Cluster of Excellence (EAM) in Erlangen on modelling, developing and improving organic electronic devices before he moved to Nottingham in 2014. He received a Marie Curie COFUND fellowship and the Nottingham Advanced Research Fellowship in 2015 and has been working on developing and applying computational strategies for predictive enzyme engineering. One key research interest is gaining detailed knowledge of the catalysis of radical enzymes in order to promote them for the usage in enzyme driven biotechnology.

Anna Croft studied organic and biochemistry at the University of Adelaide, Australia. She earned her doctorate working on amino acid radicals at the Australian National University, and subsequently moved to the University of Newcastle upon Tyne in the UK as an EU-FP5 researcher creating models for coenzyme-B12-dependent enzymes. In 1999 she moved to the University of Wales Bangor to start her own research group in biological reaction mechanisms. She is currently Associate Professor, leading a research group at the University of Nottingham, UK, with current interests in the mechanisms of radical enzymes, especially those dependent on *S*-adenosyl methionine, radical chemistry in general, and reactions in ionic liquids.

References

[1] *Radicals in Organic Synthesis,* 1st ed. (Eds: P. Renaud, M. P. Sibi), WILEY-VCH Verlag GmbH, Weinhem **2001**.

[2] E. S. Boyd, K. M. Thomas, Y. Dai, J. M. Boyd, F. W. Outten, *Biochemistry* **2014**, *53 (37)*, 5834-5847. DOI: 10.1021/bi500488r.

[3] G. Layer, J. Moser, D. W. Heinz, D. Jahn, W.-D. Schubert, *EMBO J.* **2003**, *22 (23)*, 6214-6224. DOI: 10.1093/emboj/cdg598.

[4] J. Stubbe, J. Ge, C. S. Yee, *Trends Biochem. Sci.* **2001**, *26 (2)*, 93-99. DOI: 10.1016/S0968-0004(00)01764-3.

[5] P. Anzenbacher, E. Anzenbacherová, *Cell. Mol. Life Sci.* **2001**, *58 (5)*, 737-747. DOI: 10.1007/PL00000897.

[6] G. de Gonzalo, D. I. Colpa, M. H. M. Habib, M. W. Fraaije, *J. Biotechnol.* **2016**, *236*, 110-119. DOI: 10.1016/j.jbiotec.2016.08.011.

[7] D. Leys, N. S. Scrutton, *Curr. Opin. Struct. Biol.* **2016**, *41*, 19-26. DOI: 10.1016/j.sbi.2016.05.014.

[8] M. Sono, M. P. Roach, E. D. Coulter, J. H. Dawson, *Chem. Rev.* **1996**, *96 (7)*, 2841-2888. DOI: 10.1021/cr9500500.

[9] M. I. Gibson, P. Y.-T. Chen, A. C. Johnson, E. Pierce, M. Can, S. W. Ragsdale, C. L. Drennan, *Proc. Natl. Acad. Sci. USA* **2016**, *113 (2)*, 320-325. DOI: 10.1073/pnas.1518537113.

[10] S. O. Mansoorabadi, J. Seravalli, C. Furdui, V. Krymov, G. J. Gerfen, T. P. Begley, J. Melnick, S. W. Ragsdale, G. H. Reed, *Biochemistry* **2006**, *45 (23)*, 7122-7131. DOI: 10.1021/bi0602516.

[11] J. Stubbe, W. A. van der Donk, *Chem. Rev.* **1998**, *98 (2)*, 705-762. DOI: 10.1021/cr9400875.

[12] W. Buckel, B. T. Golding, *Annu. Rev. Microbiol.* **2006**, *60*, 27-49. DOI: 10.1146/annurev.micro.60.080805.142216.

[13] W. Buckel, B. T. Golding, in *Encyclopedia of Radicals in Chemistry, Biology and Materials*, (Eds: C. Chatgilialoglu, A. Studer), John Wiley & Sons Ltd., **2012**, 1501-1546.

[14] S. Rodríguez Couto, J. L. Toca Herrera, *Biotechnol. Adv.* **2006**, *24 (5)*, 500-513. DOI: 10.1016/j.biotechadv.2006.04.003.

[15] D. M. Mate, M. Alcalde, *Microb. Biotechnol.* **2017**, *10 (6)*, 1457-1467. DOI: 10.1111/1751-7915.12422.

[16] J. Groves, *F1000Research* **2015**, *4 (178)*. DOI: 10.12688/f1000research.6314.1.

[17] V. B. Urlacher, M. Girhard, *Trends Biotechnol.* **2012**, *30 (1)*, 26-36. DOI: 10.1016/j.tibtech.2011.06.012.

[18] T. Sakaki, *Biol. Pharm. Bull.* **2012**, *35 (6)*, 844-849. DOI: 10.1248/bpb.35.844.

[19] F. W. Krainer, A. Glieder, *Appl. Microbiol. Biotechnol.* **2015**, *99 (4)*, 1611-1625. DOI: 10.1007/s00253-014-6346-7.

[20] J. B. v. Beilen, E. G. Funhoff, *Curr. Opin. Biotechnol.* **2005**, *16 (3)*, 308-314. DOI: 10.1016/j.copbio.2005.04.005.

[21] J.-P. Lange, *Catal. Sci. Technol.* **2016**, *6 (13)*, 4759-4767. DOI: 10.1039/C6CY00431H.

[22] J. Heider, A. M. Spormann, H. R. Beller, F. Widdel, *FEMS Microbiol. Rev.* **1998**, *22 (5)*, 459-473. DOI: 10.1111/j.1574-6976.1998.tb00381.x.

[23] R. Rabus, H. Wilkes, A. Behrends, A. Armstroff, T. Fischer, A. J. Pierik, F. Widdel, *J. Bacteriol.* **2001**, *183 (5)*, 1707-1715. DOI: 10.1128/JB.183.5.1707-1715.2001.

[24] R. Rabus, M. Boll, J. Heider, R. U. Meckenstock, W. Buckel, O. Einsle, U. Ermler, B. T. Golding, R. P. Gunsalus, P. M. Kroneck, M. Kruger, T. Lueders, B. M. Martins, F. Musat, H. H. Richnow, B. Schink, J. Seifert, M. Szaleniec, T. Treude, G. M. Ullmann, C. Vogt, M.

von Bergen, H. Wilkes, *J. Mol. Microbiol. Biotechnol.* **2016**, *26 (1-3)*, 5-28. DOI: 10.1159/000443997.

[25] A. V. Callaghan, *Front. Microbiol.* **2013**, *4*, 89. DOI: 10.3389/fmicb.2013.00089.

[26] M. Fontecave, S. Ollagnier-de-Choudens, E. Mulliez, *Chem. Rev.* **2003**, *103 (6)*, 2149-2166. DOI: 10.1021/cr020427j.

[27] R. R. Gokarn, O. V. Selifonova, H. Jessen, S. J. Gort, T. Selmer, W. Buckel, *World patent WO 2002042418 A3*, **2003**.

[28] H. H. Liao, R. R. Gokarn, S. J. Gort, H. J. Jessen, O. Selifonova, *World patent WO 2003062173 A8*, **2008**.

[29] A. Berry, *World patent WO 2009089457 A1*, **2009**.

[30] T. Grotkjaer, S. T. Jorgensen, T. B. Regueira, B. Christensen, A. Berry, *World patent WO 2012058603 A1*, **2012**.

[31] O. Zelder, W. Buckel, I. Djurdjevic, *World patent WO 2010081885 A3*, **2011**.

[32] C. K. Eddy, *US patent US 6277609 B1*, **2001**.

[33] H. Schön, O. Thimm, G. Ritte, O. Bläsing, K. Bruynseels, Y. Hatzfeld, V. Frankard, M. A. I. Sanz, C. Reuzeau, S. Vandenabeele, *World patent WO 2010046221 A1*, **2010**.

[34] H. Schroder, *Canadian patent CA 2321264 A1*, **1999**.

[35] H. Schroeder, B. Hauer, *World patent WO 9905285 A1*, **1999**.

[36] S. G. Bower, J. B. Perkins, R. R. Yocum, J. G. Pero, *US patent US 6057136 A*, **2000**.

[37] Y. Furuichi, T. Hoshino, H. Kimura, T. Kiyasu, Y. Nagahashi, *US patent US 2004137584 A1*, **2004**.

[38] T. Hoshino, A. Asakura, T. Kiyasu, Y. Nagahashi, *European patent EP 0806479 B1*, **2002**.

[39] T. Hoshino, A. Asakura, T. Kiyasu, Y. Nagahashi, *US patent US 2004063116 A1*, **2004**.

[40] V. Nagarajan, C. E. Nakamura, *World patent WO 9635795 A1*, **1996**.

[41] M. Emptage, S. Haynie, L. Laffend, J. Pucci, G. Whited, *World patent WO 2001012833 A2*, **2001**.

[42] M. G. Bramucci, D. Flint, J. E. S. Miller, V. Nagarajan, N. Sedkova, M. Singh, D. T. K. Van, *World patent WO 2008137403 A1*, **2008**.

[43] M. G. Bramucci, D. Flint, J. E. S. Miller, V. Nagarajan, N. Sedkova, M. Singh, D. T. K. Van, *World patent WO 2008137404 A1*, **2008**.

[44] A. L. Botes, A. van Eck Conradie, C. Chen, P. S. Pearlman, *World patent WO 2014105796 A2*, **2014**.

[45] P. S. Pearlman, C. Chen, A. Botes, A. van Eck Conradie, *World patent WO 2013082542 A3*, **2013**.

[46] A. L. Botes, A. van Eck Conradie, C. Chen, P. S. Pearlman, *World patent WO 2014105797 A3*, **2014**.

[47] E. R. Gak, N. V. Gorshkova, I. L. Tokmakova, *World patent WO 2012118225 A1*, **2013**.

[48] E. Akiva, S. Brown, D. E. Almonacid, A. E. Barber, 2nd, A. F. Custer, M. A. Hicks, C. C. Huang, F. Lauck, S. T. Mashiyama, E. C. Meng, D. Mischel, J. H. Morris, S. Ojha, A. M. Schnoes, D. Stryke, J. M. Yunes, T. E. Ferrin, G. L. Holliday, P. C. Babbitt, *Nucleic Acids Res.* **2014**, *42 (Database issue)*, D521-530. DOI: 10.1093/nar/gkt1130.

[49] Babbitt Lab SFLD Team, *Structure Function Linkage Database - Radical SAM superfamily*, **2017**. http://sfld.rbvi.ucsf.edu/django/superfamily/29/

[50] G. Luo, S. Ono, N. J. Beukes, D. T. Wang, S. Xie, R. E. Summons, *Sci. Adv.* **2016**, *2 (5)*. DOI: 10.1126/sciadv.1600134.

[51] G. Wachtershauser, *Philos. Trans. R. Soc., B* **2006**, *361 (1474)*, 1787-1806; discussion 1806-1808. DOI: 10.1098/rstb.2006.1904.

[52] G. Wächtershäuser, *Proc. Natl. Acad. Sci. USA* **1990**, *87 (1)*, 200-204.

[53] G. Wächtershäuser, *Prog. Biophys. Mol. Biol.* **1992**, *58 (2)*, 85-201. DOI: 10.1016/0079-6107(92)90022-X.

[54] L. A. David, E. J. Alm, *Nature (London)* **2011**, *469 (7328)*, 93-96. DOI: 10.1038/nature09649.

[55] D. C. Johnson, D. R. Dean, A. D. Smith, M. K. Johnson, *Annu. Rev. Biochem.* **2005**, *74*, 247-281. DOI: 10.1146/annurev.biochem.74.082803.133518.

[56] R. Lill, *Nature (London)* **2009**, *460 (7257)*, 831-838. DOI: 10.1038/nature08301.

[57] J. Liu, S. Chakraborty, P. Hosseinzadeh, Y. Yu, S. Tian, I. Petrik, A. Bhagi, Y. Lu, *Chem. Rev.* **2014**, *114 (8)*, 4366-4469. DOI: 10.1021/cr400479b.

[58] A. J. Birch, *J. Chem. Soc.* **1944**, 430-436. DOI: 10.1039/JR9440000430.

[59] A. J. Birch, *J. Proc. R. Soc. N.S.W.* **1974**, *107 (3-4)*, 100-113.

[60] G. S. R. Subba Rao, *Pure Appl. Chem.* **2003**, *75 (10)*, 1443-1451. DOI: 10.1351/pac200375101443.

[61] M. Boll, *Biochim. Biophys. Acta, Bioenerg.* **2005**, *1707 (1)*, 34-50. DOI: 10.1016/j.bbabio.2004.01.009.

[62] T. Weinert, S. G. Huwiler, J. W. Kung, S. Weidenweber, P. Hellwig, H.-J. Staerk, T. Biskup, S. Weber, J. J. H. Cotelesage, G. N. George, U. Ermler, M. Boll, *Nat. Chem. Biol.* **2015**, *11 (8)*, 586-591. DOI: 10.1038/nchembio.1849.

[63] B. Thiele, O. Rieder, B. T. Golding, M. Muller, M. Boll, *J. Am. Chem. Soc.* **2008**, *130 (43)*, 14050-14051. DOI: 10.1021/ja805091w.

[64] M. Boll, C. Loeffler, B. E. L. Morris, J. W. Kung, *Environ. Microbiol.* **2014**, *16 (3)*, 612-627. DOI: 10.1111/1462-2920.12328.

[65] C. Ebenau-Jehle, M. Mergelsberg, S. Fischer, T. Bruels, N. Jehmlich, M. von Bergen, M. Boll, *ISME J.* **2017**, *11 (1)*, 224-236. DOI: 10.1038/ismej.2016.91.

[66] M. Boll, *J. Mol. Microbiol. Biotechnol.* **2005**, *10 (2-4)*, 132-142. DOI: 10.1159/000091560.

[67] D. M. Smith, W. Buckel, H. Zipse, *Angew. Chem., Int. Ed.* **2003**, *42 (16)*, 1867-1870. DOI: 10.1002/anie.200250502.

[68] J. Kim, M. Hetzel, C. D. Boiangiu, W. Buckel, *FEMS Microbiol. Rev.* **2004**, *28 (4)*, 455-468. DOI: 10.1016/j.femsre.2004.03.001.

[69] W. Buckel, J. Zhang, P. Friedrich, A. Parthasarathy, H. Li, I. Djurdjevic, H. Dobbek, B. M. Martins, *Biochim. Biophys. Acta* **2012**, *1824 (11)*, 1278-1290. DOI: 10.1016/j.bbapap.2011.11.009.

[70] J. Zhang, P. Friedrich, A. J. Pierik, B. M. Martins, W. Buckel, *Appl. Environ. Microbiol.* **2015**, *81 (3)*, 1071-84. DOI: 10.1128/AEM.03099-14.

[71] W. Buckel, M. Hetzel, J. Kim, *Curr. Opin. Chem. Biol.* **2004**, *8 (5)*, 462-467. DOI: 10.1016/j.cbpa.2004.07.001.

[72] J. Kim, D. Darley, T. Selmer, W. Buckel, *Appl. Environ. Microbiol.* **2006**, *72 (9)*, 6062-6069. DOI: 10.1128/aem.00772-06.

[73] J. Kim, D. J. Darley, W. Buckel, A. J. Pierik, *Nature (London)* **2008**, *452 (7184)*, 239-U85. DOI: 10.1038/nature06637.

[74] W. Q. Liu, P. Amara, J. M. Mouesca, X. Ji, O. Renoux, L. Martin, C. Zhang, Q. Zhang, Y. Nicolet, *J. Am. Chem. Soc.* **2018**, *140 (4)*, 1365-1371. DOI: 10.1021/jacs.7b10501.

[75] A. Parthasarathy, A. J. Pierik, J. Kahnt, O. Zelder, W. Buckel, *Biochemistry* **2011**, *50 (17)*, 3540-3550. DOI: 10.1021/bi1020056.

[76] J. W. Schmid, K. Mauch, *World patent WO 2009046828 A1*, **2009**.

[77] B. Guentner, *World patent WO 2016034691 A1*, **2016**.

[78] B. M. Baynes, J. M. Geremia, S. Lippow, *World patent WO 2014028026 A1*, **2014**.

[79] J. Hughes, K. C. Symes, *World patent WO 2005090586 A1*, **2005**.

[80] A. J. Shaw, V. Rajgarhia, *World patent WO 2010105194 A2*, **2010**.

[81] F. Valle, N. J. Agard, C. Noriega, *World patent WO 2013044076 A1*, **2013**.

[82] M. J. Burk, A. P. Burgard, R. E. Osterhout, P. Pharkya, *World patent WO 2009135074 A3*, **2009**.

[83] W. Fenghuan, M. Qingqing, Y. Huimin, W. Yuhai, *Chinese patent CN 103409447 B*, **2015**.

[84] M. J. Burk, A. P. Burgard, R. E. Osterhout, J. Sun, *World patent WO 2010030711 A3*, **2010**.

[85] D. S. J. Van, A. P. Burgard, R. Haselbeck, C. J. Pujol-Baxley, W. Niu, J. D. Trawick, H. Yim, M. J. Burk, R. E. Osterhout, J. Sun, *World patent WO 2010141920 A2*, **2011**.

[86] B. M. Martins, H. Dobbek, I. Çinkaya, W. Buckel, A. Messerschmidt, *Proc. Natl. Acad. Sci. USA* **2004**, *101 (44)*, 15645-15649. DOI: 10.1073/pnas.0403952101.

[87] K. L. Brown, *Chem. Rev.* **2005**, *105 (6)*, 2075-2150. DOI: 10.1021/cr030720z.

[88] J. H. Martens, H. Barg, M. J. Warren, D. Jahn, *Appl. Microbiol. Biotechnol.* **2002**, *58 (3)*, 275-285. DOI: 10.1007/s00253-001-0902-7.

[89] D. P. Dowling, A. K. Croft, C. L. Drennan, *Annu. Rev. Biophys.* **2012**, *41*, 403-427. DOI: 10.1146/annurev-biophys-050511-102225.

[90] DuPont Tate & Lyle Bio Products, *Life Cycle Analysis Overview - Susterra® Propanediol*, **2006**. http://www.duponttateandlyle.com/sites/default/files/Susterra LCA.pdf (Accessed on 31/07/17)

[91] G. R. Eastham, D. W. Johnson, A. J. J. Straathof, M. W. Fraaije, R. T. Winter, *World patent WO 2013179005 A1*, **2013**.

[92] R. Banerjee, *Chem. Rev.* **2003**, *103 (6)*, 2083-2094. DOI: 10.1021/cr0204395.

[93] M. J. Burk, A. P. Burgard, R. E. Osterhout, J. Sun, P. Pharkya, *World patent WO 2012135789 A3*, **2014**.

[94] A. P. Burgard, *World patent WO 2010127303 A1*, **2010**.

[95] M. Pötter, A. Marx, G. Fuchs, B. Alber, T. J. Erb, *World patent WO 2008119738 A1*, **2008**.

[96] B. Alber, S. Buchholz, L. Eggeling, G. Fuchs, A. Marx, A. May, M. Poetter, H. Siegert, *World patent WO 2007141208 A2*, **2008**.

[97] B. Green, N. Reppas, D. Robertson, *World patent WO 2010044960 A1*, **2010**.

[98] B. Bhadra, R. Bhalla, A. L. Kruckeberg, V. Nagarajan, R. Patnaik, W. Suh, *World patent WO 2013102084 A2*, **2013**.

[99] K. A. Shisler, J. B. Broderick, *Arch. Biochem. Biophys.* **2014**, *546*, 64-71. DOI: 10.1016/j.abb.2014.01.020.

[100] J. L. Vey, J. Yang, M. Li, W. E. Broderick, J. B. Broderick, C. L. Drennan, *Proc. Natl. Acad. Sci. USA* **2008**, *105 (42)*, 16137-16141. DOI: 10.1073/pnas.0806640105.

[101] B. J. Levin, Y. Y. Huang, S. C. Peck, Y. Wei, A. M.-d. Campo, J. A. Marks, E. A. Franzosa, C. Huttenhower, E. P. Balskus, *Science* **2017**, *355 (6325)*, eaai8386. DOI: 10.1126/science.aai8386.

[102] G. D. Markham, *eLS* **2010**. DOI: 10.1002/9780470015902.a0000662.pub2.

[103] J. B. Broderick, B. R. Duffus, K. S. Duschene, E. M. Shepard, *Chem. Rev.* **2014**, *114*, 4229-4317. DOI: 10.1021/cr4004709.

[104] P. A. Frey, M. D. Ballinger, G. H. Reed, *Biochem. Soc. Trans.* **1998**, *26 (3)*, 304. DOI: 10.1042/bst0260304.

[105] J. Bridwell-Rabb, C. L. Drennan, *Curr. Opin. Chem. Biol.* **2017**, *37*, 63-70. DOI: 10.1016/j.cbpa.2017.01.013.

[106] D. P. Dowling, J. L. Vey, A. K. Croft, C. L. Drennan, *Biochim. Biophys. Acta* **2012**, *1824 (11)*, 1178-1195. DOI: 10.1016/j.bbapap.2012.04.006.

[107] H. J. Sofia, G. Chen, B. G. Hetzler, J. F. Reyes-Spindola, N. E. Miller, *Nucleic Acids Res.* **2001**, *29 (5)*, 1097-1106. DOI: 10.1093/nar/29.5.1097.

[108] World Health Organisation, *Antimicrobial resistance*, **2017**. http://www.who.int/antimicrobial-resistance/en/ (Accessed on 27/07/2017)

[109] National Institute of Allergy and Infectious Diseases, *Antimicrobial (Drug) Resistance*, **2017**. https://www.niaid.nih.gov/research/antimicrobial-resistance (Accessed on 27/07/17)

[110] Medical Research Council, *Antimicrobial Resistance*, **2017**. https://www.mrc.ac.uk/research/initiatives/antimicrobial-resistance/ (Accessed on 27/07/2017)

[111] European Commission, *Antimicrobial Resistance*, **2017**. https://ec.europa.eu/health/amr/antimicrobial-resistance_en (Accessed on 27/07/2017)

[112] K. McLaughlin, *China tackles antimicrobial resistance*, **2016**. http://www.sciencemag.org/news/2016/08/china-tackles-antimicrobial-resistance (Accessed on 27/07/2017)

[113] L. Vollbrecht, H. Steinmetz, G. Hofle, *J. Antibiot.* **2002**, *55 (8)*, 715-721. DOI: 10.7164/antibiotics.55.715.

[114] F. Sasse, H. Steinmetz, T. Schupp, F. Petersen, K. Memmert, H. Hofmann, C. Heusser, V. Brinkmann, P. Von Matt, G. Hofle, H. Reichenbach, *J. Antibiot.* **2002**, *55 (6)*, 543-551. DOI: 10.7164/antibiotics.55.543.

[115] R. Müller, S. Wenzel, R. Garcia, *World patent WO 2010000601 A1*, **2010**.

[116] T. Sasaki, T. Otani, H. Matsumoto, N. Unemi, M. Hamada, T. Takeuchi, M. Hori, *J. Antibiot.* **1998**, *51 (8)*, 715-721. DOI: 10.7164/antibiotics.51.715.

[117] W. Liu, Y. Ding, Y. Yu, H. Pan, *World patent WO 2011009416 A1*, 27/01/2011, **2011**.

[118] W. Li, J. Leet, X. Huang, K. S. Lam, A. Regueiro-Ren, *World patent WO 2002013834 A1*, **2002**.

[119] W. Li, J. E. Leet, K. S. Lam, *World patent WO 2000014100 A1*, **2000**.

[120] J. E. Leet, H. A. Ax, D. R. Gustavson, D. M. Brown, L. Turner, K. Brown, W. Li, K. S. Lam, *World patent WO 2000003722 A1*, **2000**.

[121] W. Li, J. E. Leet, K. S. Lam, *World patent WO 2000068413 A1*, **2000**.

[122] W. Liu, Q. Zhang, S. Chen, *Chinese patent CN 102453077 A*, 16/05/2012, **2013**.

[123] Q. Zhang, X. Ji, *Synlett* **2016**, *28 (02)*, 143-147. DOI: 10.1055/s-0036-1588677.

[124] X. Ji, Y. Li, L. Xie, H. Lu, W. Ding, Q. Zhang, *Angew. Chem. Int. Ed. Engl.* **2016**, *55 (39)*, 11845-11848. DOI: 10.1002/anie.201605917.

[125] E. J. Begg, M. L. Barclay, *Br. J. Clin. Pharmacol.* **1995**, *39 (6)*, 597-603. DOI: 10.1111/j.1365-2125.1995.tb05719.x.

[126] K. L. Rinehart, M. Potgieter, D. L. Delaware, *J. Am. Chem. Soc.* **1981**, *103 (8)*, 2099-2101. DOI: 10.1021/ja00398a042.

[127] P. F. Wiley, H. K. Jahnke, F. A. MacKellar, R. B. Kelly, A. D. Argoudelis, *J. Org. Chem.* **1970**, *35 (5)*, 1420-1425. DOI: 10.1021/jo00830a035.

[128] T. Mahmud, *World patent WO 2012018854 A8*, **2013**.

[129] Y. Mao, M. Varoglu, D. H. Sherman, *Chem. Biol. (Oxford, U. K.)* **1999**, *6 (4)*, 251-263. DOI: 10.1016/S1074-5521(99)80040-4.

[130] G. Christiansen, *World patent WO 2012019928 A1*, **2013**.

[131] A. Takatsuki, K. Arima, G. Tamura, *J. Antibiot.* **1971**, *24 (4)*, 215-223. DOI: 10.7164/antibiotics.24.215.

[132] F. Wyszynski, B. G. Davis, M. Bibb, A. Hesketh, *World patent WO 2012013960 A2*, **2012**.

[133] P. A. Frey, F. J. Ruzicka, *US patent US 6248874 B1*, **2001**.

[134] V. Zappia, H. A. Barker, *Biochim. Biophys. Acta, Protein Struct.* **1970**, *207 (3)*, 505-513. DOI: 10.1016/S0005-2795(70)80013-7.

[135] B. W. Lepore, F. J. Ruzicka, P. A. Frey, D. Ringe, *Proc. Natl. Acad. Sci. USA* **2005**, *102 (39)*, 13819-13824. DOI: 10.1073/pnas.0505726102.

[136] O. Han, P. A. Frey, *J. Am. Chem. Soc.* **1990**, *112 (24)*, 8982-8983. DOI: 10.1021/ja00180a054.

[137] P. A. Frey, F. J. Ruzicka, *World patent WO 2007044839 A3*, **2008**.

[138] F. J. Ruzicka, P. A. Frey, *Biochim. Biophys. Acta, Proteins Proteomics* **2007**, *1774 (2)*, 286-296. DOI: 10.1016/j.bbapap.2006.11.008.

[139] S. Puehringer, M. Metlitzky, R. Schwarzenbacher, *BMC Biochem.* **2008**, *9 (1)*, 8. DOI: 10.1186/1471-2091-9-8.

[140] S. R. Wecksler, S. Stoll, A. T. Iavarone, E. M. Imsand, H. Tran, R. D. Britt, J. P. Klinman, *Chem. Commun.* **2010**, *46 (37)*, 7031-7033. DOI: 10.1039/C0CC00968G.

[141] N. D. Lanz, M. E. Pandelia, E. S. Kakar, K. H. Lee, C. Krebs, S. J. Booker, *Biochemistry* **2014**, *53 (28)*, 4557-4572. DOI: 10.1021/bi500432r.

[142] R. M. Cicchillo, S. J. Booker, *J. Am. Chem. Soc.* **2005**, *127 (9)*, 2860-2861. DOI: 10.1021/ja042428u.

[143] R. M. Cicchillo, D. F. Iwig, A. D. Jones, N. M. Nesbitt, C. Baleanu-Gogonea, M. G. Souder, L. Tu, S. J. Booker, *Biochemistry* **2004**, *43 (21)*, 6378-6386. DOI: 10.1021/bi049528x.

[144] N. D. Lanz, K. H. Lee, A. K. Horstmann, M. E. Pandelia, R. M. Cicchillo, C. Krebs, S. J. Booker, *Biochemistry* **2016**, *55 (9)*, 1372-1383. DOI: 10.1021/acs.biochem.5b01216.

[145] N. D. Lanz, J. M. Rectenwald, B. Wang, E. S. Kakar, T. N. Laremore, S. J. Booker, A. Silakov, *J. Am. Chem. Soc.* **2015**, *137 (41)*, 13216-13219. DOI: 10.1021/jacs.5b04387.

[146] M. I. McLaughlin, N. D. Lanz, P. J. Goldman, K. H. Lee, S. J. Booker, C. L. Drennan, *Proc. Natl. Acad. Sci. USA* **2016**, *113 (34)*, 9446-9450. DOI: 10.1073/pnas.1602486113.

[147] Jenny E. Harmer, Martyn J. Hiscox, Pedro C. Dinis, Stephen J. Fox, A. Iliopoulos, James E. Hussey, J. Sandy, Florian T. Van Beek, Jonathan W. Essex, Peter L. Roach, *Biochem. J.* **2014**, *464 (1)*, 123. DOI: 10.1042/BJ20140895.

[148] M. Kandula, M. E. V. Rao, *World patent WO 2009091582 A1*, **2008**.

[149] Y. Sun, H. Wang, H. Pang, W. He, *Chinese patent CN 104450762 A*, **2015**.

[150] F. Schneider, S. Molck, B. Bathe, *World patent WO 2012098042 A1*, **2012**.

[151] I. Ochrombel, B. Bathe, M. Hasselmeyer, J. Kalinowski, C. Rückert, M. Persicke, *World patent WO 2015165746 A1*, **2015**.

[152] F. Schneider, C. Gerth, *World patent WO 2013120685 A1*, **2013**.

[153] B. Bathe, S. Molck, H. Priefert, *World patent WO 2014029592 A1*, **2014**.

[154] F. Zhang, W. Jiang, G. Bentley, *World patent WO 2017106747 A1*, **2017**.

[155] E. Choi-Rhee, J. E. Cronan, *Chem. Biol.* **2005**, *12 (4)*, 461-468. DOI: 10.1016/j.chembiol.2005.02.006.

[156] C. E. Farrar, K. K. W. Siu, P. L. Howell, J. T. Jarrett, *Biochemistry* **2010**, *49 (46)*, 9985-9996. DOI: 10.1021/bi101023c.

[157] J. T. Jarrett, in *Iron-Sulfur Clusters in Chemistry and Biology*, (Ed: T. Rouault), Walter de Gruyter GmbH, **2014**, 107-131.

[158] B. A. Clack, A. B. Youngblood, *World patent WO 2007046943 A2*, **2005**.

[159] O. Birch, J. Brass, M. Fuhrmann, N. Shaw, *World patent WO 9408023 A3*, **2000**.

[160] M. Ikuro, F. Nobuko, K. Koichi, *Japanese patent JPH 0761267 B2*, **1995**.

[161] I. Ohsawa, D. Speck, T. Kisou, K. Hayakawa, M. Zinsius, R. Gloeckler, Y. Lemoine, K. Kamogawa, *Gene* **1989**, *80 (1)*, 39-48. DOI: 10.1016/0378-1119(89)90248-5.

[162] K. Hatakeyama, S. Nara, M. Terasawa, H. Yugawa, *Japanese patent JPH 07177895 A*, **1995**.

[163] N. Sakurai, Y. Imai, M. Masuda, S. Komatsubara, T. Tosa, *Appl. Environ. Microbiol.* **1993**, *59 (9)*, 2857-2863.

[164] D. I. Jang, C. H. Kim, B. J. Ha, M. G. Yoo, D. J. Bae, C. S. Koo, H. H. Lee, K. M. Kim, *Korean patent KR 101023788 B1*, **2011**.

[165] S. Komatsubara, Y. Imai, M. Masuda, N. Sakurai, *US patent US 5374554 A*, **1994**.

[166] T. Kobayashi, H. Honda, *Japanese patent JP 2000236895 A*, **2000**.

[167] F. Mukumoto, S. Nishio, J. Akimaru, S. Mitsuda, *World patent WO 9839452 A1*, **1998**.

[168] D. Shiuan, *US patent US 20030104584 A1*, **2003**.

[169] D. A. Patton, *World patent WO 9617944 A3*, **1996**.

[170] J.-Y. Seo, R. Yaneva, P. Cresswell, *Cell Host Microbe* **2011**, *10 (6)*, 534-539. DOI: 10.1016/j.chom.2011.11.004.

[171] K. S. Duschene, J. B. Broderick, *FEBS Lett.* **2010**, *584 (6)*, 1263-1267. DOI: 10.1016/j.febslet.2010.02.041.

[172] M. K. Fenwick, Y. Li, P. Cresswell, Y. Modis, S. E. Ealick, *Proc. Natl. Acad. Sci. USA* **2017**, *114 (26)*, 6806-6811. DOI: 10.1073/pnas.1705402114.

[173] X. Wang, E. R. Hinson, P. Cresswell, *Cell Host Microbe* **2007**, *2 (2)*, 96-105. DOI: 10.1016/j.chom.2007.06.009.

[174] C. Makins, S. Ghosh, G. D. Román-Meléndez, P. A. Malec, R. T. Kennedy, E. N. G. Marsh, *J. Biol. Chem.* **2016**. DOI: 10.1074/jbc.M116.751040.

[175] K. H. Ebrahimi, S. B. Carr, J. McCullagh, J. Wickens, N. H. Rees, J. Cantley, F. A. Armstrong, *FEBS Lett.* **2017**, *591 (16)*, 2394-2405. DOI: 10.1002/1873-3468.12769.

[176] E. J. Devroe, S. Kosuri, D. A. Berry, N. B. Afeyan, F. A. Skraly, D. E. Robertson, B. Green, C. P. Ridley, *World patent WO 2009062190 A3*, **2009**.

[177] S. P. Gough, B. O. Petersen, J. Ø. Duus, *Proc. Natl. Acad. Sci. USA* **2000**, *97 (12)*, 6908-6913. DOI: doi: 10.1073/pnas.1701687114.

[178] T. L. Grove, J. Livada, E. L. Schwalm, M. T. Green, S. J. Booker, A. Silakov, *Nat. Chem. Biol.* **2013**, *9 (7)*, 422-427. DOI: 10.1038/nchembio.1251.

FIGURES:

Figure 1. The Fe_2S_2 (butterfly-like structure, left) and Fe_4S_4 (cubane-like structure, right) iron sulfur clusters are important to many radical-based enzymatic reactions.

Figure 2. (a) The organometallic coenzyme B_{12} features a corrin ring and an adenosyl moiety (R) bound to cobalt, poised to form **(b)** the reactive 5'-adenosyl radical (Ado•), which is an important radical catalyst generated in both B_{12}-dependant enzymes and radical SAM enzymes.

Figure 3. The structures of radical SAM enzymes show a distinctive TIM-barrel architecture **(a)**, with either a full-$(\beta/\alpha)_8$ barrel (left, biotin synthase, pdb ID 1R30, with second monomer of the dimer structure transparent) or, more commonly, a partial-$(\beta/\alpha)_6$ barrel (right, pyruvate formate lyase activating enzyme, pdb ID 3C8F). The common Fe_4S_4 motif is highlighted as spheres. **(b)** Cleavage of Fe_4S_4-bound *S*-adenosylmethionine to generate the 5'-adenosyl radical ($Ado^{\bullet}$).

Figure 4. Antibiotic scaffolds naturally fabricated with the help of radical enzymes in key steps of biosynthesis. **(a)** The cyclic peptides Argyrins are antimicrobials obtained from *Archangium gephyra*. Argyrin B (R_1=CH_2CH_3) is synthesised *via* a radical-SAM mediated methylation of Argyrin A (R_1=CH_3). **(b)** The antimicrobial Nocathiacin contains a modified indole ring, derived from the radical-SAM mediated rearrangement of tryptophan. **(c)** The thiazole antibiotic nosiheptide has a key biosynthetic step catalysed by the radical enzyme NosL to generate the substituted indole ring. **(d)** Pactamycin is biosynthesised with the

involvement of a number of different radical enzymes. **(e)** The antimicrobial secondary metabolite Thiomuracin A involves a radical methyltransferase in its synthesis. **(f)** Formation of the glycosidic antibiotic tunicamycin is thought to involve radical-SAM enzymes in the central C-C coupling between the sugar units.

SCHEMES:

Scheme 1. Conversion of benzoyl-CoA to the diene derivative is proposed via the enzymatic Birch reduction, catalysed by benzoyl-CoA reductase. **(a)** The initial single electron reduction occurs at -1.9 V, compared with the -3 V required for the chemical birch reduction of benzene.[66] **(b)** Diene formation opens up the possibility for further functionalisation leading to ring cleavage, to form pimeloyl-CoA.[44]

Scheme 2. Dehydration of ketoalcohols is a generally important reaction. **(a)** The general mechanism differs depending on the location of the alcohol **i.**) an example of simple base-catalysed dehydration of a 1,3-ketoalcohol. **ii.**) an example of the proposed mechanism for dehydration of a 1,2-ketoalcohol, as might be expected for 2-hydroxyglutaryl-CoA dehydratase, proceeding through an umpolung reaction *via* a ketyl-radical intermediate. **(b)** Lactoyl-CoA dehydratase forms the precursor molecule propenoyl-CoA, which can undergo further conversion into a range of highly useful synthetic building blocks including 1,3-propanediol (1,3-PD), butadiene, 3-hydroxypropanoic acid (3-HP) and methacrylic acid. **(c)** Formation of crotonyl-CoA through dehydration of 4-hydroxybutyryl-CoA using 4-hydroxybutyryl-CoA dehydratase and subsequent isomerisation. **(d)** Isomerisation of crotonyl-CoA is also catalysed by 4-hydroxybutyryl-CoA dehydratase, and can be used to generate the 1,4-oxygenation motif required for the production of 1,4-butanediol.

Scheme 3. Formation of the bulk chemical 1,3-propanediol (1,3-PD) from glycerol through radical-catalysed dehydration by B_{12}-dependant dehydratase.

Scheme 4. Routes to methacrylic acid have been proposed using a number of carbon-skeleton mutases.

Scheme 5. **(a)** Aromatic and **(b)** long-chain hydrocarbons can be coupled to succinate as part of initial steps towards degradation.[22, 24]

Scheme 6. Example radical-SAM catalysed reactions. **(a)** The radical rearrangement of tryptophan, catalysed by the enzyme Noc27, to generate the key 5-methyl indole intermediate in the biosynthesis of nocathiacin. **(b)** Suggested synthesis of the cyclopentitol intermediate that forms the core of the pactamycin structure, first through PtmG-catalysed deacylation followed by a radical rearrangement mediated by PtmC. **(c)** The radical-SAM enzyme PqqE is involved in the C-C bond coupling required to generate the ring structure of the cofactor PQQ. **(d)** Sulfur is inserted into the otherwise unactivated carbon backbone of octanoic acid through the action of LipA. The sulfur atoms derive from a Fe_4S_4 cluster within LipA, which must be regenerated after reaction. **(e)** The reaction of dethiobiotin (DTB) with the radical-SAM enzyme BioB. The substrate radical reacts with the auxiliary enzyme-bound Fe_2S_2 cluster, with subsequent hydrogen abstraction by a second adenosyl radical resulting in sulfur-ring formation to form biotin.

TABLES:

Table 1. Key B_{12}-dependant mutases and their reactions.

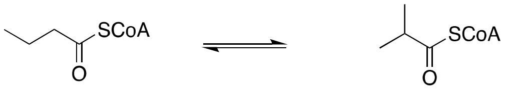
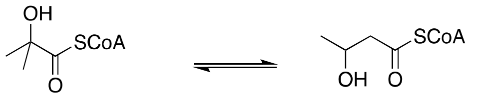
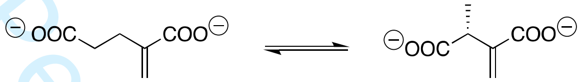
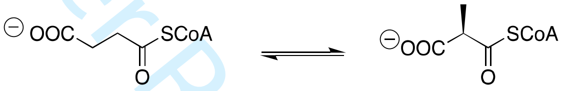
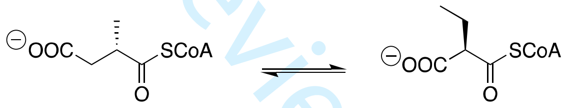
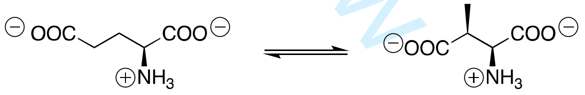
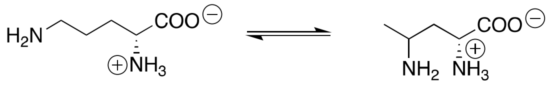
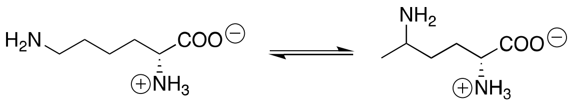
Enzyme name	**Reaction catalysed**	**EC reference**
Carbon skeleton mutases		
Isobutyryl CoA mutase	 Butyryl-CoA ⇌ Isobutyryl-CoA	5.4.99.13
2-Hydroxyisobutyryl CoA mutase	 2-Hydroxy-2-methylpropanoyl-CoA ⇌ 3-Hydroxybutyryl-CoA	5.4.99.64
2-Methylene glutarate mutase	 2-Methyleneglutarate ⇌ (*R*)-3-Methylitaconate	5.4.99.4
Methylmalonyl CoA mutase	 Succinyl-CoA ⇌ (*R*)-Methylmalonyl-CoA	5.4.99.2
Ethylmalonyl CoA mutase	 (2S)-Methylsuccinyl-CoA ⇌ (*R*)-Ethylmalonyl-CoA	5.4.99.63
Glutamate mutase	 (*S*)-Glutamate ⇌ (2*S*,3*S*)-3-Methylaspartate	5.4.99.1
Aminomutases		
4,5-Ornithine aminomutase	 (*R*)-Ornithine ⇌ 2,4-Diaminopentanoate (2,4-DAP)	5.4.3.5
5,6-Lysine aminomutase	 (*R*)-Lysine ⇌ 2,5-Diaminohexanoate (2,5-DAH)	5.4.3.4

Table 2. Key B_{12}-dependant eliminases and their reactions.

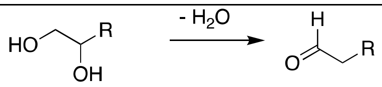
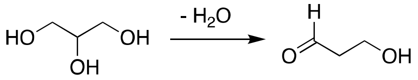
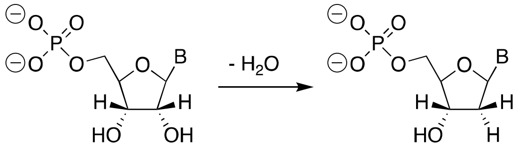
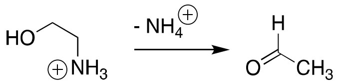
Enzyme name	**Reaction catalysed**	**EC reference**
Diol dehydratase	 R = H, CH_3	4.2.1.28
Glycerol dehydratase		4.2.1.30
Ribonucleotide reductase		1.17.4.1
Ethanolamine ammonia lyase		4.3.1.7

Table 3. Consensus sequence for glycyl radical enzymes.[100]

Target Consensus		**Enzyme**
-RVS**G**YAV-		Pyruvate formate lyase (PFL)
-RVA**G**YSA-†		Choline trimethylamine lyase (CTL)
-RVA**G**YSD-‡		*trans*-4-Hydroxy-L-proline dehydratase (*t*4LHypD)
-RXC**G**YLG-	X = V or T	Class III ribonucleotide reductase (RNR)
-RVA**G**XSZ-	X = Y or F; Z = A, D or V	B_{12}-independent glycerol dehydratase (GDH)
-RVA**G**XZB-	X = Y or F; Z = S or T; B = A, D or G	4-Hydroxyphenylacetate decarboxylase (HPAD)
-RXZ**G**BSJ-	X = V or T; Z = A or S; B = Y or F; J = A or D	Benzyl Succinate Synthase (BSS)

†Obtained through BLAST search of the *Desulfovibrio alaskensis* choline trimethylamine lyase, followed by multiple sequence alignment using Clustal Omega on sequences defined as choline trimethylamine lyase (>80% sequence identity). ‡Obtained using the same methodology as for CTL, using the base sequence Uniprot ID A0A031WDE4,[101] with Clustal Omega on sequences identified with >77% sequence identity.

SHORT TEXT:

Radicals offer the possibility of unique and efficient chemical transformations, if properly controlled. Nature can provide this control in the form of enzymes, and this review provides an overview of the types of enzymes characterised, their current applications in biotechnology, and future directions to better exploit these fascinating catalysts.

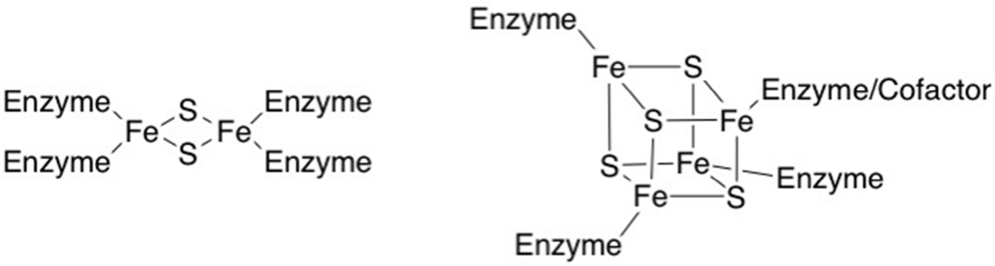


Figure 1. The Fe2S2 (butterfly-like structure, left) and Fe4S4 (cubane-like structure, right) iron sulfur clusters are important to many radical-based enzymatic reactions.

117x31mm (144 x 144 DPI)

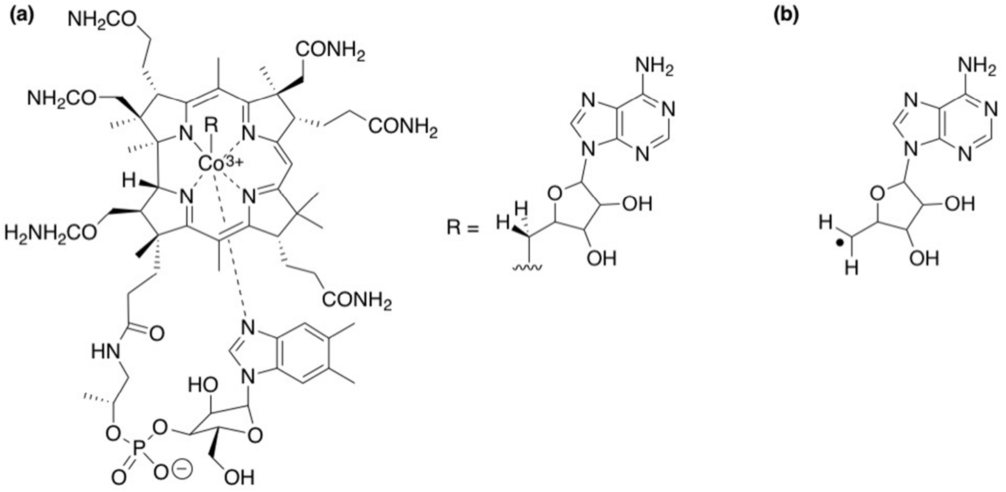


Figure 2. (a) The organometallic coenzyme B12 features a corrin ring and an adenosyl moiety (R) bound to cobalt, poised to form (b) the reactive 5'-adenosyl radical (Ado•), which is an important radical catalyst generated in both B12-dependant enzymes and radical SAM enzymes.

169x82mm (144 x 144 DPI)

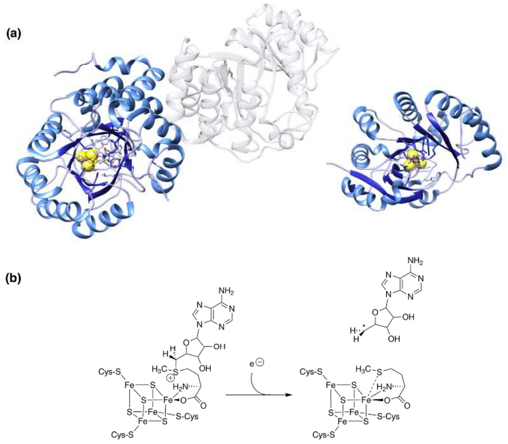


Figure 3. The structures of radical SAM enzymes show a distinctive TIM-barrel architecture (a), with either a full-(β/α)8 barrel (left, biotin synthase, pdb ID 1R30, with second monomer of the dimer structure transparent) or, more commonly, a partial-(β/α)6 barrel (right, pyruvate formate lyase activating enzyme, pdb ID 3C8F). The common Fe4S4 motif is highlighted as spheres. (b) Cleavage of Fe4S4-bound S-adenosylmethionine to generate the 5'-adenosyl radical (Ado•).

1410x793mm (72 x 72 DPI)

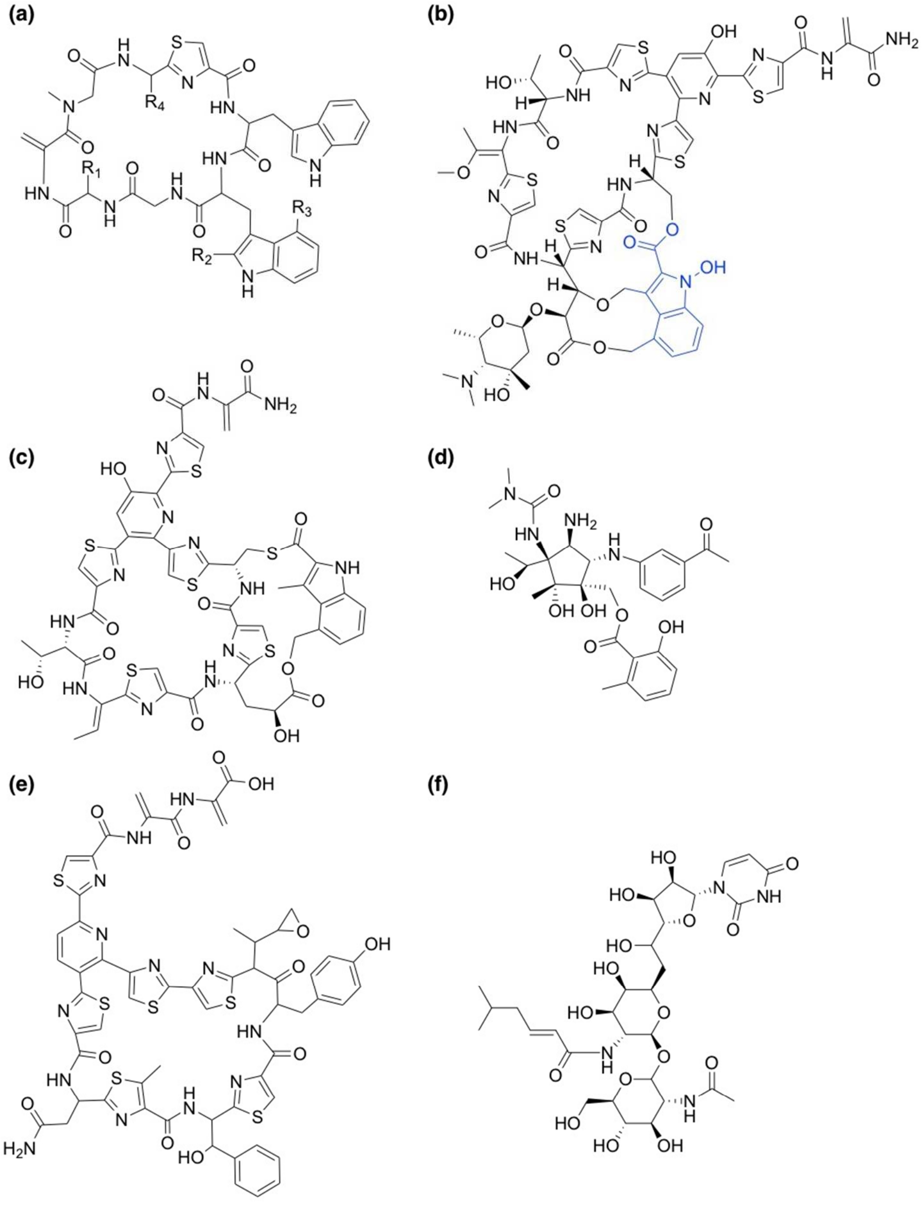
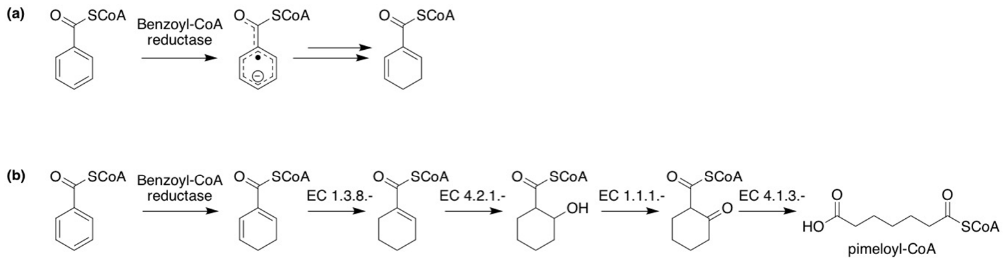


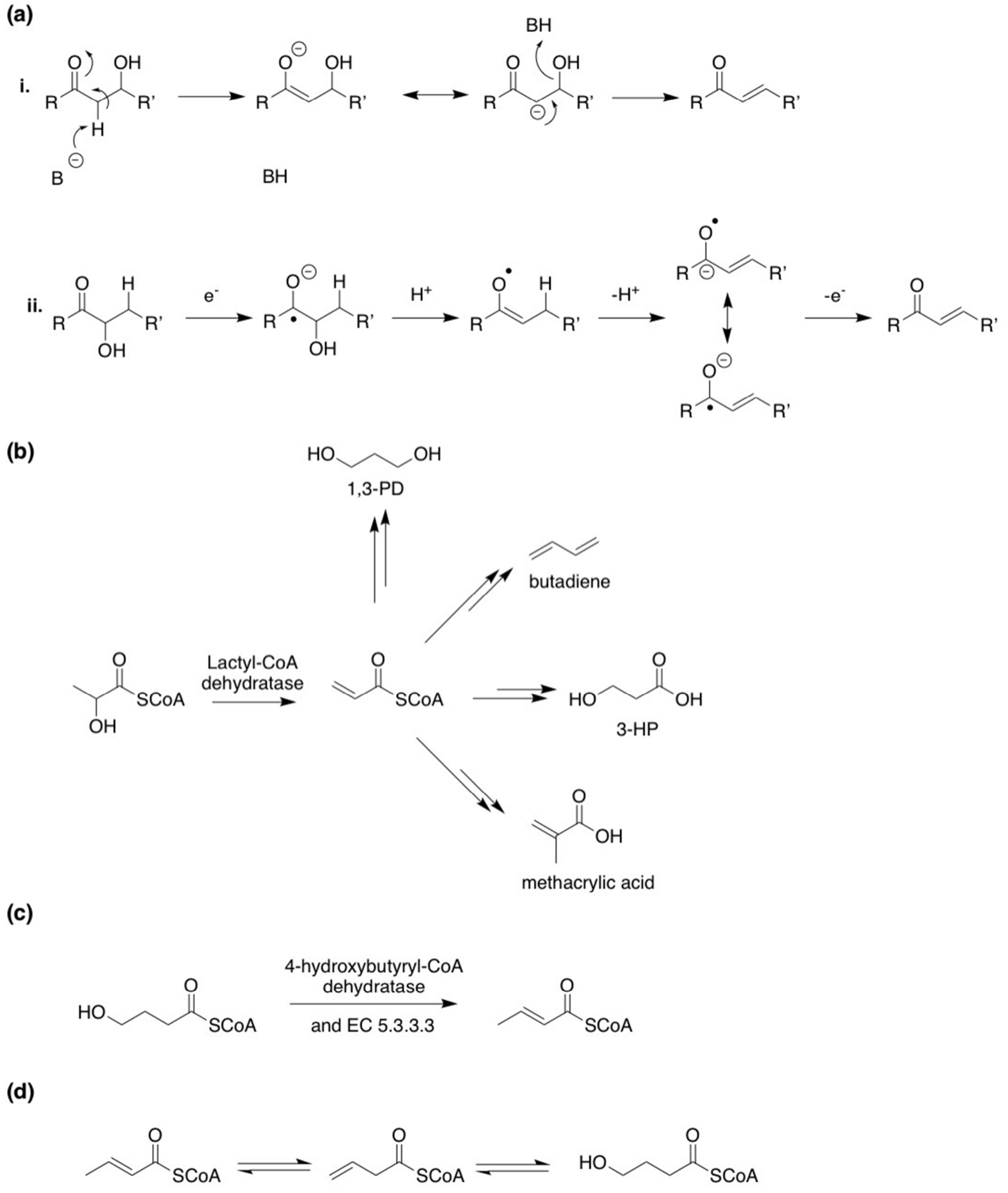
Figure 4. Antibiotic scaffolds naturally fabricated with the help of radical enzymes in key steps of biosynthesis. (a) The cyclic peptides Argyrins are antimicrobials obtained from Archangium gephyra. Argyrin B (R1=CH2CH3) is synthesised via a radical-SAM mediated methylation of Argyrin A (R1=CH3). (b) The antimicrobial Nocathiacin contains a modified indole ring, derived from the radical-SAM mediated rearrangement of tryptophan. (c) The thiazole antibiotic nosiheptide has a key biosynthetic step catalysed by the radical enzyme NosL to generate the substituted indole ring. (d) Pactamycin is biosynthesised with the involvement of a number of different radical enzymes. (e) The antimicrobial secondary metabolite Thiomuracin A involves a radical methyltransferase in its synthesis. (f) Formation of the glycosidic antibiotic tunicamycin is thought to involve radical-SAM enzymes in the central C-C coupling between the sugar units.

182x237mm (144 x 144 DPI)



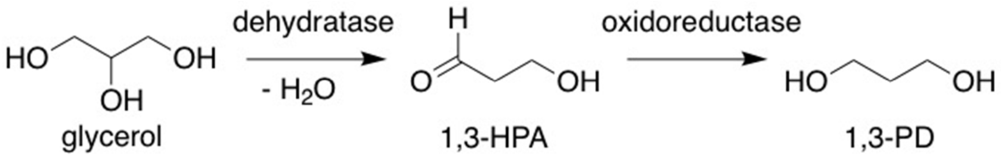
Scheme 1. Conversion of benzoyl-CoA to the diene derivative is proposed via the enzymatic Birch reduction, catalysed by benzoyl-CoA reductase. (a) The initial single electron reduction occurs at -1.9 V, compared with the -3 V required for the chemical birch reduction of benzene.[66] (b) Diene formation opens up the possibility for further functionalisation leading to ring cleavage, to form pimeloyl-CoA.[44]

238x61mm (144 x 144 DPI)



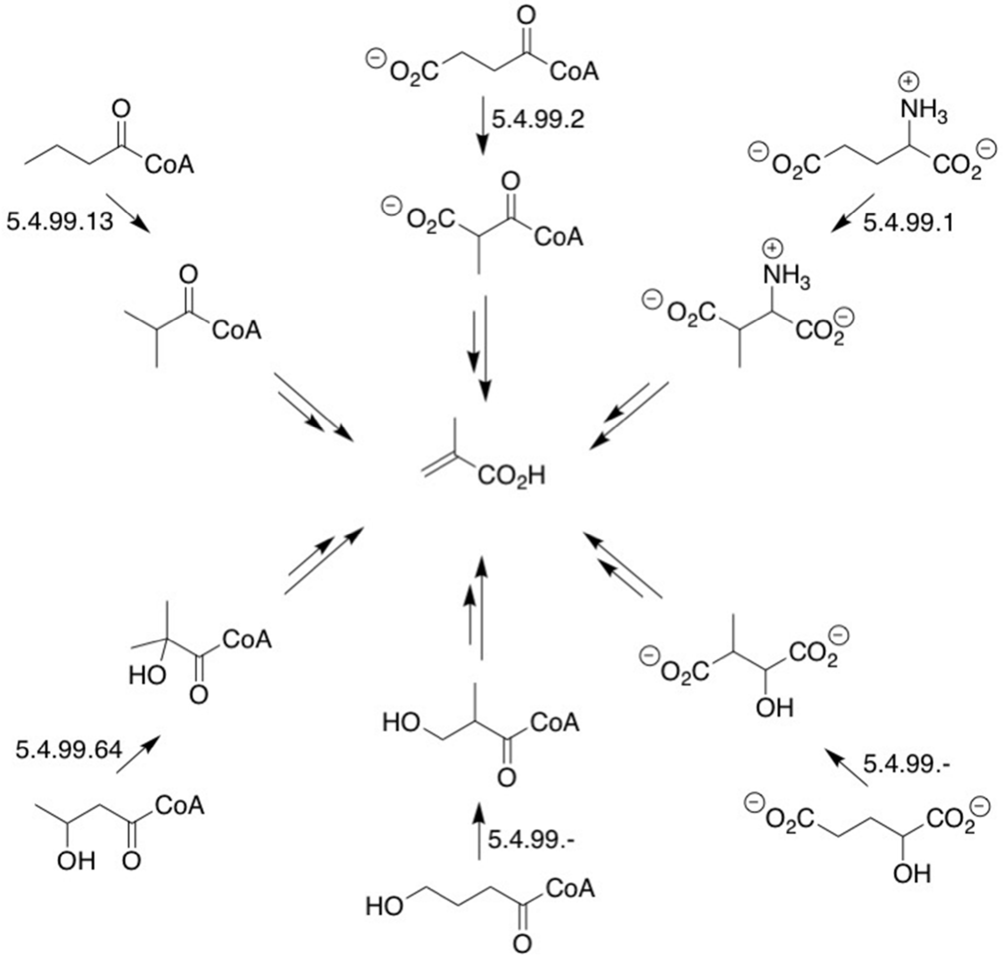
Scheme 2. Dehydration of ketoalcohols is a generally important reaction. (a) The general mechanism differs depending on the location of the alcohol i.) an example of simple base-catalysed dehydration of a 1,3-ketoalcohol. ii.) an example of the proposed mechanism for dehydration of a 1,2-ketoalcohol, as might be expected for 2-hydroxyglutaryl-CoA dehydratase, proceeding through an umpolung reaction via a ketyl-radical intermediate. (b) Lactoyl-CoA dehydratase forms the precursor molecule propenoyl-CoA, which can undergo further conversion into a range of highly useful synthetic building blocks including 1,3-propanediol (1,3-PD), butadiene, 3-hydroxypropanoic acid (3-HP) and methacrylic acid. (c) Formation of crotonyl-CoA through dehydration of 4-hydroxybutyryl-CoA using 4-hydroxybutyryl-CoA dehydratase and subsequent isomerisation. (d) Isomerisation of crotonyl-CoA is also catalysed by 4-hydroxybutyryl-CoA dehydratase, and can be used to generate the 1,4-oxygenation motif required for the production of 1,4-butanediol.

189x224mm (144 x 144 DPI)



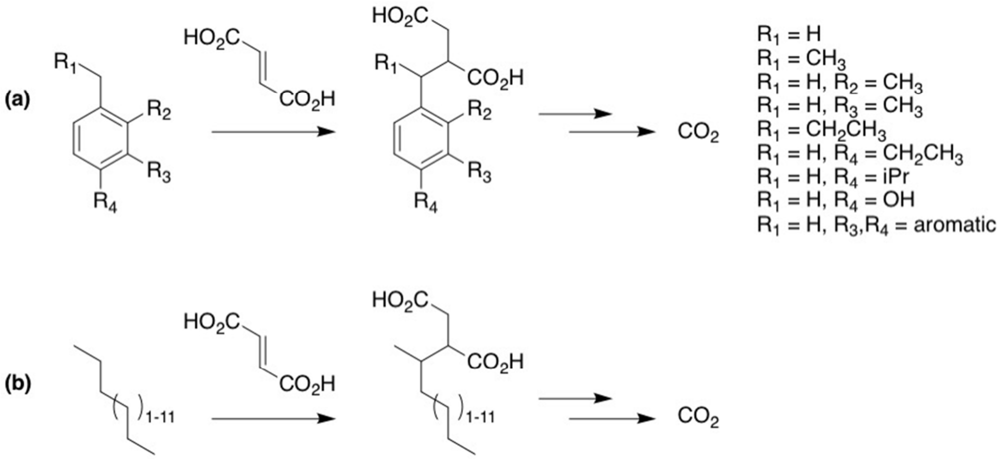
Scheme 3. Formation of the bulk chemical 1,3-propanediol (1,3-PD) from glycerol through radical-catalysed dehydration by B12-dependant dehydratase.

119x19mm (144 x 144 DPI)



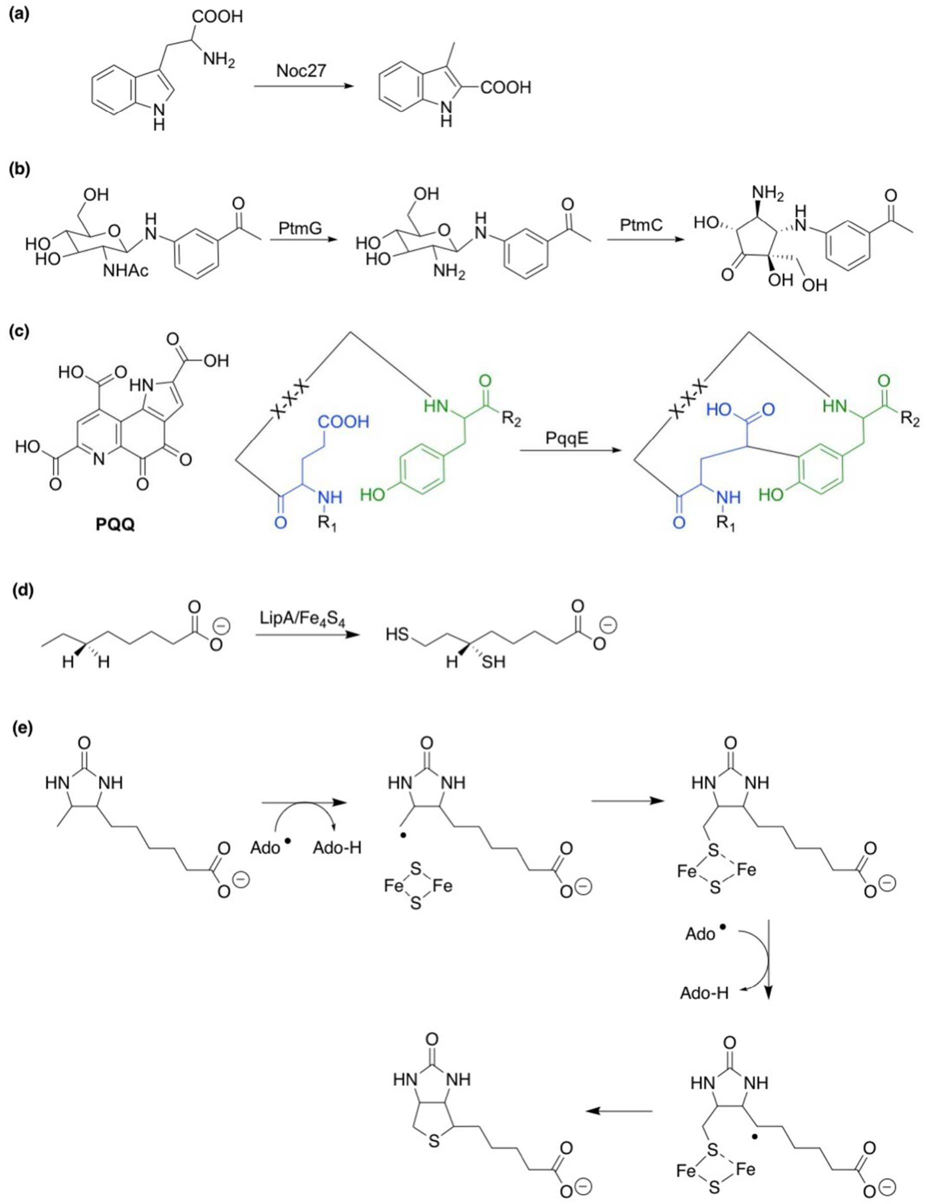
Scheme 4. Routes to methacrylic acid have been proposed using a number of carbon-skeleton mutases.

136x130mm (144 x 144 DPI)



Scheme 5. (a) Aromatic and (b) long-chain hydrocarbons can be coupled to succinate as part of initial steps towards degradation.[22, 24]

162x74mm (144 x 144 DPI)



Scheme 6. Example radical-SAM catalysed reactions. (a) The radical rearrangement of tryptophan, catalysed by the enzyme Noc27, to generate the key 5-methyl indole intermediate in the biosynthesis of nocathiacin. (b) Suggested synthesis of the cyclopentitol intermediate that forms the core of the pactamycin structure, first through PtmG-catalysed deacylation followed by a radical rearrangement mediated by PtmC. (c) The radical-SAM enzyme PqqE is involved in the C-C bond coupling required to generate the ring structure of the cofactor PQQ. (d) Sulfur is inserted into the otherwise unactivated carbon backbone of octanoic acid through the action of LipA. The sulfur atoms derive from a Fe4S4 cluster within LipA, which must be regenerated after reaction. (e) The reaction of dethiobiotin (DTB) with the radical-SAM enzyme BioB. The substrate radical reacts with the auxiliary enzyme-bound Fe2S2 cluster, with subsequent hydrogen abstraction by a second adenosyl radical resulting in sulfur-ring formation to form biotin.

185x242mm (144 x 144 DPI)