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Biocatalytic hydrogen atom transfer: an invigorating approach to free-radical reactions

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Initiating and terminating free-radical reactionis via hydrogen atom transfer (HAT) is an attractive means of avoiding substrate prefunctionalization. Small molecule catalysts and reagents, however, struggle to execute this fundamental step with useful levels of diastereoselectivity and enantioselectivity. In contrast, nature often carries out HAT with exquisite levels of selectivity for even electronically unactivated carbon–hydrogen bonds. By understanding how enzymes exploit and control this fundamental step, new strategies can be developed to address several long-standing challenges in free-radical reactions. This review will cover recent discoveries in biocatalysis that exploit a HAT mechanism to either initiate or terminate novel one-electron reactions.

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Introduction

Reactions involving open-shell radical intermediates provide complementary reactivity to classic two-electron pathways [1]. Unfortunately, traditional approaches to radical formation often require preactivation strategies that complicate synthetic designs. Alternatively, abstraction of a hydrogen atom from a substrate's carbon–hydrogen (C–H) bond offers a more streamlined approach to radical formation as it mitigates the need for substrate preactivation [2]. Controlling hydrogen atom transfer (HAT) can, however, be problematic on substrates containing electronically unbiased C–H bonds [3]. Conversely, termination of carbon-centered radicals is often accomplished by HAT to provide a new C–H bond. While the selective delivery of a hydrogen atom can determine the product's stereoconfiguration, it is currently challenging to control using small molecules [4]. In order to overcome these obstacles we propose searching beyond small molecule catalysts and reagents.

Biocatalysis offers an approach for overcoming the aforementioned barriers. Many of nature's transformations, such as anaerobic metabolism [5] and DNA repair [6], are understood to proceed by radical mechanisms. Additionally, metal-cofactor dependent enzymes (such as P450s and non-heme iron/α-ketoglutarate-dependent enzymes) that catalyze highly selective C–H functionalizations (such as hydroxylation, amination or halogenation) are known to initiate by the abstraction of a hydrogen atom from substrate [7]. Enzymes are also capable of differentiating prochiral intermediates with exclusive selectivity [8], making them suitable catalysts for the stereoselective delivery of hydrogen atoms to radicals. Developing biocatalytic protocols that draw inspiration from nature promises to afford general solutions to these fundamental problems.

This review will cover biocatalytic HAT transformations reported in the last two years (2016–2018) with an emphasis on newly uncovered enzymes or reactivity. To ensure concise coverage, attention will be given to transformations generating chiral products and omitting processes involving HAT with heteroatom–hydrogen sites. Though relevant, the chemistry of cytochrome P450 monooxygenase has been recently covered elsewhere [9] and will not be discussed herein.

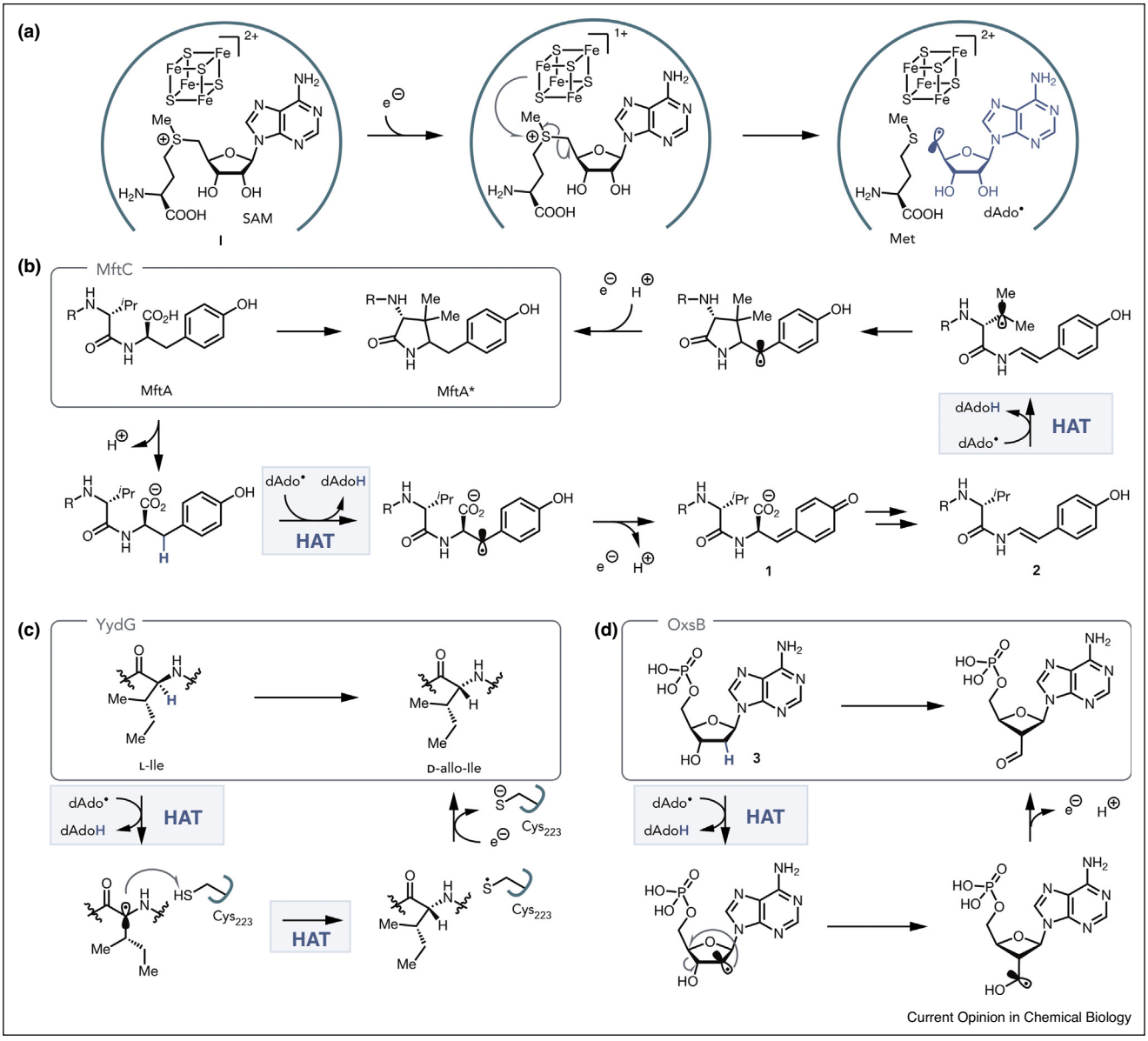
Biocatalytic transformations initiated by HAT

Radical *S*-adenosyl-L-methionine dependent enzymes

The largest enzyme superfamily, with over 100 000 annotated members, is the radical *S*-adenosyl-L-methionine (SAM) family [10]. Structurally they are defined by the presence of an iron–sulfur cluster $[4Fe–4S]^{2+}$ and SAM cofactor (as in **I**, Figure 1). Delivery of an electron results in $[4Fe–4S]^{1+}$ mediated homolytic cleavage of the S–C5′ bond, expelling methionine (Met) and unmasking the 5′-deoxyadenosyl radical (dAdo$^{\bullet}$) [11] that serves as the catalytically competent intermediate in radical SAM transformations (Figure 1a). The dAdo$^{\bullet}$ radical most frequently instigates HAT on a C–H bond on the substrate, producing dAdoH as by-product, and a substrate-centered radical that can undergo rearrangements, cyclizations, addition reactions or fragmentation reactions [12].

Peptides constitute a significant portion of natural products identified in a wide variety of microorganisms. Of these, ribosomally synthesized post-translationally

Figure 1



(a) Formation of dAdo• from SAM and [4Fe–4S] cluster. **(b)** MftC effects peptide modification of MftA to MftA•. **(c)** Epimerase YydG mechanism. **(d)** OxsB-catalyzed ring contraction of 2′-deoxyadenosylphosphate.

modified peptides (RiPPs) have garnered significant attention with whole-genome sequencing technologies allowing rapid identification of gene clusters encoding for both precursor peptides and modification enzymes. Many of these biosynthetic clusters have been found to encode for radical SAM enzymes [13]. For example, Bandarian and Bruender showed that MftC effects decarboxylation at the C-terminus of the MftA peptide in the biosynthetic pathway to mycofactotcin [14•]. Here, the dAdo• radical is implicated in a HAT with the C–H bond at the C_β-position on the terminal tyrosine residue. Following a second oxidation event, unsaturated *para*-quinone **1** is generated whereupon decarboxylation forms enamine **2**. Another equivalent of the dAdo• radical then facilitates a second HAT event at a neighbouring valine residue, with a 5-exo-trig cyclization installing the γ-butyrolactam moiety in MftA* (Figure 1b). Isotopic labelling and spectroscopic experiments by the Latham group provided support for this mechanism [15].

Beyond HAT at the C_β-position of peptide residues, the C_α-position may also be modified by radical SAM to provide RiPPs. Berteau *et al.* found that non-natural D-amino acids identified in a post-translationally modified

peptide from the bacterium *Bacillus subtilis* were installed by a new class of radical SAMs termed *epimerases* [16•]. They were able to elucidate YydG as the key enzyme effecting epimerization, and further that a key cysteine residue in the active site functions in terminating the reaction mechanism (Figure 1c, L-Ile→D-allo-Ile). The authors have since reported the biochemical characterization of PoyD; a radical SAM epimerase that is responsible for the 18 epimerizations found in the RiPP polytheonamide A [17].

Of approximately 7000 cobalamin (Cbl)-dependent radical SAM enzymes that have been identified, only one has been reported to perform a non-methylating transformation. Recent work by Liu and Drennan identified OxsB in the biosynthesis of oxetanocin A, a potent antitumor, antiviral, and antibacterial nucleoside analogue possessing an oxetane functionality [18•]. OxsB from *Bacillus megaterium* was structurally and biochemically characterized to effect a ring-contraction of phosphorylated 2′-deoxyadenosine **3** via a radical mechanism initiating by HAT with a dAdo• radical (Figure 1d). The exact role of Cbl in this transformation remained unclear, with the authors speculating either a support role in stabilizing the intermediate radical species or as a conduit of electrons during catalysis.

Methylations by Cbl-dependent radical SAM methyltransferases (RSMTs) are common across numerous natural product biosyntheses. HAT by dAdo• generates a C-centered radical on the substrate that combines with methyl-Cbl (as in **II**), in turn generated from reduced Cbl(I) and another equivalent of SAM in a two-electron pathway (Figure 2a, **III**→**IV**). Bacterial organisms *Streptomyces* and *Pseudomonas* produce fosfomycin, a broad-spectrum antibiotic, however their biosyntheses differ. Eguchi and Kuzuyama reported that in *Streptomyces wedmorensis* the RSMT Fom3 is responsible for installation of a methyl group to cytidylylated hydroxyphosphonate **4** en-route to fosfomycin (Figure 2b) [19]. Later, they and van der Donk's group shed further light on the stereochemical outcome of this transformation in separate communications [20,21]. The gentamicins are among the aminoglycoside family of antiobiotic natural products. Of these, gentamicin C_1 possesses a methylated sidechain at the C6′-position, which was found by Liu *et al.* to be installed by GenK (Figure 2c, **5**→**6**) [22].

α-Ketoglutarate-dependent non-heme iron enzymes

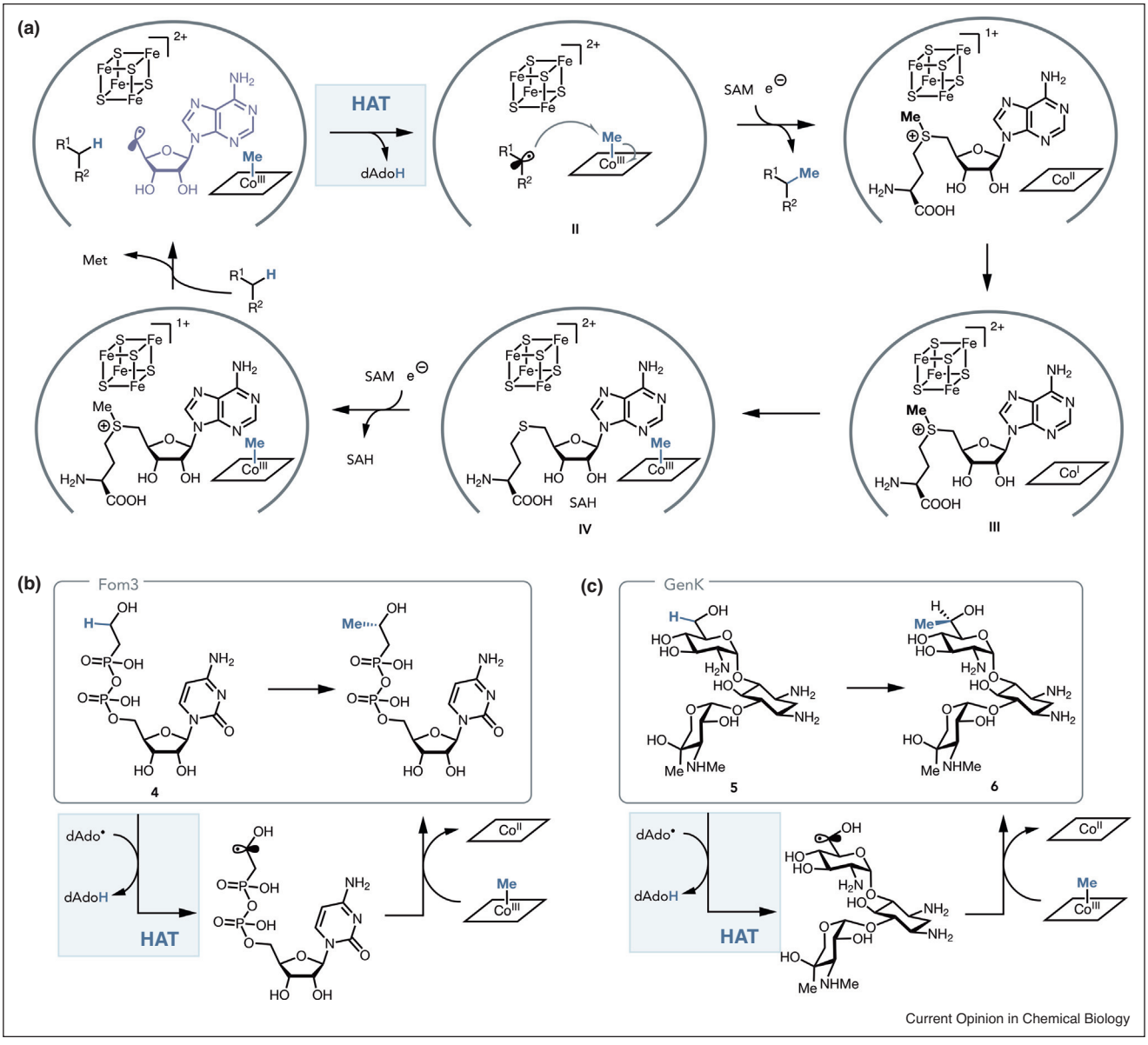
Direct functionalization of unactivated C–H bonds by oxygen-dependent non-heme iron/α-ketoglutarate (Fe/αKG) enzymes enables selective hydroxylations and halogenations [23]. Mechanistically, the enzyme's iron (II) cofactor binds αKG and O_2 to produce CO_2, succinate and an iron(IV)-oxo intermediate **V** that is poised to undergo HAT with a substrate's C–H bond (Figure 3a). Structurally, the Fe/αKG halogenases differ from their hydroxylase counterparts in the binding residues of the iron cofactor. Both classes ligate the iron through two conserved histidines, with the hydroxylase possessing a further carboxylate residue (generally in the form or aspartate or glutamate) to complete a facial triad on the iron center, whereas the halogenases allow for a chloride or bromide anion to bind in the vacant coordination site. Upon radical generation, rebound with OH or halide ligand completes C–H functionalization with concomitant reformation of the iron(II) cofactor (Figure 3a, **VI**) [24].

Regioselective functionalization of unactivated aliphatic C–H bonds on complex late-stage synthetic compounds is a key challenge in organic chemistry. The groups of Liu and Boal uncovered two cyanobacterium Fe/αKG chlorinases WelO5 and AmbO5 in the biosynthetic gene clusters of welwitindolinone and ambiguine, respectively [25,26]. With 79% sequence homology, these enzymes have closely related function and operate on structurally related complex indole natural products, albeit WelO5 operates with restricted substrate scope. Through sequence analysis and mutagenesis Liu *et al.* were able to identify that the C-terminus sequence in AmbO5 dictated the substrate tolerance, and was able to evolve WelO5 to a functional mutant with an expanded substrate scope identical to that of AmbO5 (Figure 3b) [27•]. Demonstration of the evolvable nature of these halogenases provides promise as valuable biocatalysts for selective functionalization of small molecules.

Fe/αKG hydroxylases have also shown regioselective functionalization of unactivated aliphatic C–H bonds. Recently the groups of Larsen and Abe demonstrated that Fe/αKG enzyme NvfI effects endoperoxidation of asnovolin A to fumigatonoid A en-route to novofumigatonin, a heavily oxygenated meroterpenoid produced by the fungus *Aspergillus novofumigatus* [28]. Mechanistically this transformation initiates by HAT between the substrate and iron(IV)-oxo species providing primary C-centered radical **7** that may be trapped by molecular oxygen via cyclization, whereupon rebound of the OH ligand formally install three oxygen atoms to the substrate (Figure 3c).

The utility of these Fe/αKG hydroxylases in synthetic chemistry has been explored by Renata [29•,30]. In their studies an Fe/αKG enzyme GriE, implicated in the biosynthesis of griselimycin from a *Streptomyces* strain [31], was found to possess relaxed substrate specificity and was adept at catalyzing the remote hydroxylation of 11 amino acids. The transformation could be employed on preparative scale with **8** in the truncated synthetic route to manzacidin C (five steps formal versus 13 previous [32]), demonstrating the power of enzymatic C–H functionalization in synthetic design (Figure 3d) [29•]. More recently, they completed the first total synthesis of

Figure 2



(a) Methylation of unactivated C–H bonds in RSMTs. **(b)** One pathway for fosfomycin biosynthesis involves methylation catalyzed by Fom3. **(c)** RSMT enzyme GenK installs a methyl group observed in aminoglycoside gentamicin C_1.

tambromycin employing a biocatalytic C–H functionalization strategy to access noncanonical amino acid tambroline [30]. Specifically, Fe/αKG hydroxylase KDO1 [33] was employed for the gram-scale hydroxylation of L-lysine with a further four-step sequence furnishing protected tambroline **9** in excellent yield and step economy (Figure 3e).

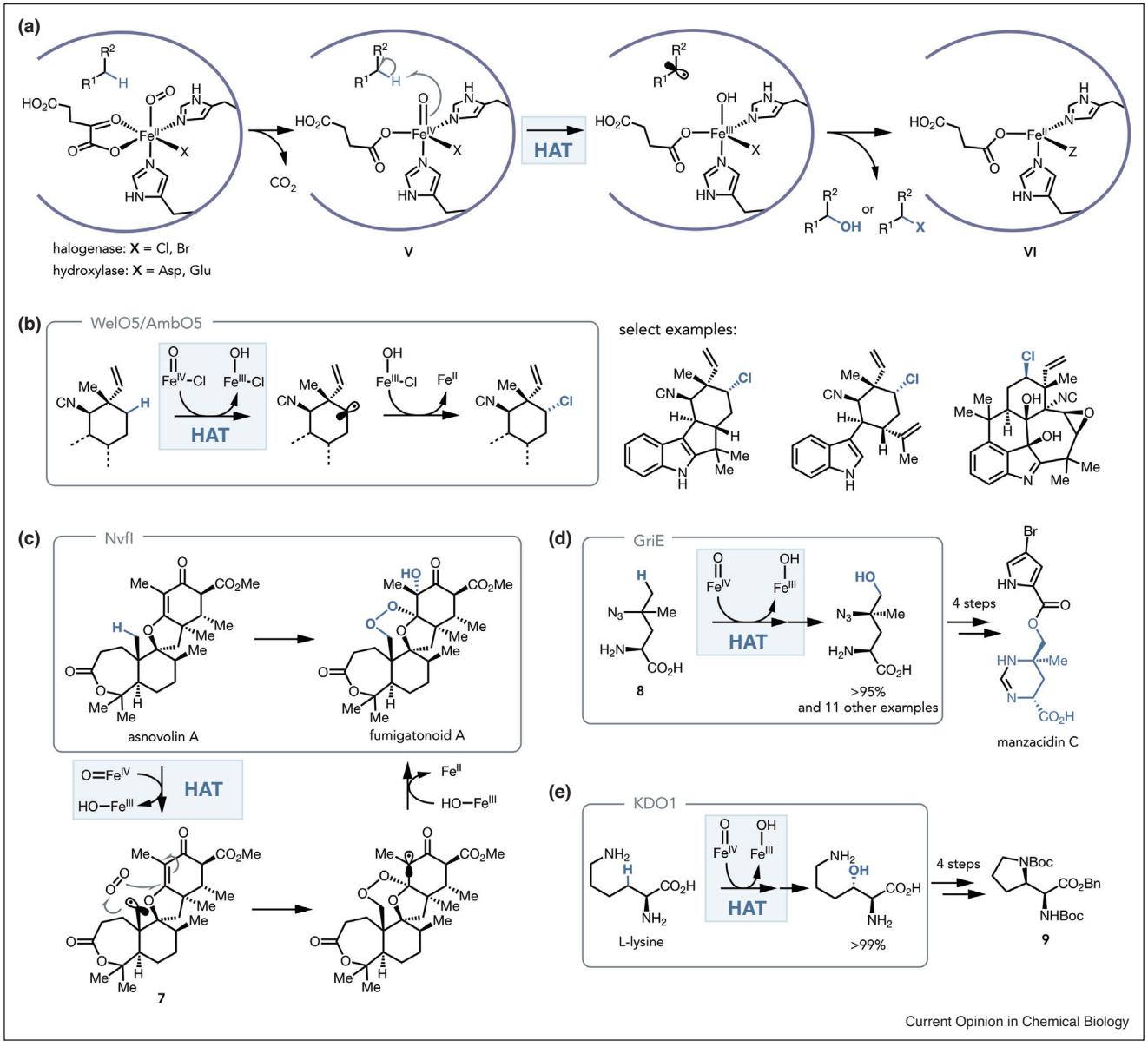
Biocatalytic protocols terminating in HAT

NAD(P)H as cofactor

Though the regioselective and chemoselective HAT from substrate to cofactor is well established in biocatalysis, few examples exist where HAT terminates a substrate radical in an enzyme's active site. Owing to the prochiral nature of C-centered radicals, an opportunity exists for controlling the stereochemical outcome of this operation — a feat that has eluded a general solution in small molecule catalysis [4].

We accomplished a highly stereoselective dehalogenation of halolactones **10** by exploiting the interesting photophysical properties of nicotinamide [34••]. Irradiation of an electron donor–acceptor complex formed between NAD(P)H and the substrate within the enzyme active

Figure 3

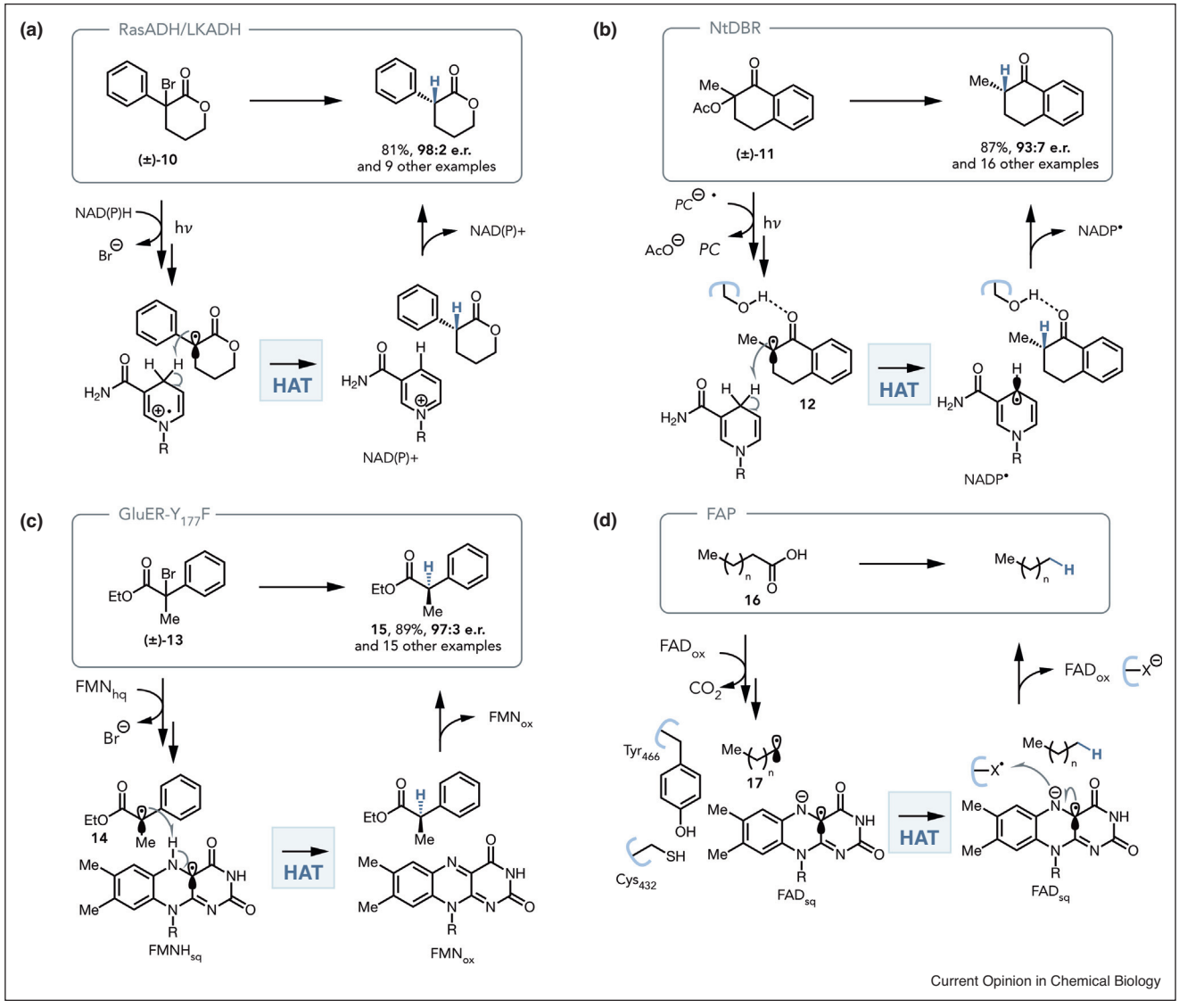


(a) Mechanism for C–H functionalization with Fe/αKG enzymes. **(b)** AmbO5/WelO5 chlorination of ambiguine indole alkaloids. **(c)** Endoperoxidation catalyzed by NvfI. **(d)** Formal synthesis of manzacidin C using δ-hydroxylation by GriE. **(e)** Total synthesis of tambromycin enabled by rapid access to noncanonical amino acid tambroline, enabled by KDO1 catalysis.

site results in electron transfer to the substrate [35], whereupon the radical cation on the cofactor possesses a weakened C4–H bond enabling this species to act as a hydrogen atom donor [36]. Performing HAT inside the enzyme active site ensures facially selective delivery of the hydrogen atom to the prochiral radical. Nicotinamide-dependent ketoreductases from *Ralstonia* sp. (RasADH) [37] and *Lactobacillus kefiri* (LKADH) [38] were able to effect this dehalogenation with divergent stereoselectivites. Together this study demonstrates the catalytic adaptability of these enzymes to carry out non-native transformations (Figure 4a).

More recently, we reported an alternate approach for forming radicals within enzyme active sites accompanied by subsequent HAT from a nicotinamide cofactor [39•]. A difficult single electron transfer from a photocatalyst (PC) to substrate was achieved through redox activation provided to the substrate by binding to the enzyme. Calculations revealed hydrogen-bonding interactions attenuated

Figure 4



(a) Dehalogenation of bromolactones catalyzed by RasADH or LKADH. **(b)** Deacetoxylation of tetralones by enzymatic redox activation. **(c)** Dehalogenation of bromoesters with GluER-$Y_{177}F$. **(d)** Fatty acid photodecarboxylase invoked in lipid metabolism of microalga.

the reduction potential of a model α-acetoxy ketone by approximately +157 mV. Thus, through cooperative action of photocatalyst Rose Bengal and double-bond reductase (NtDBR), a single-electron reduction of α-acetoxytetralone **11** by the Rose Bengal radical anion (generated *in situ* through absorption of light and a single electron oxidant) was followed by a rapid spin-center shift [40] to eliminate acetate and afford a prochiral α-acyl radical **12**. HAT with ground state NADPH completes the stereoselective deacetoxylation (Figure 4b). This strategy proved general, enabling the enantioselective dehalogenation of previously challenging amides and esters using Eosin Y as a photocatalyst and a KRED variant from *L. kefiri*.

Flavin as cofactor

Flavin is a ubiquitous cofactor employed in a variety of enzyme classes. One fascinating feature of this cofactor is its ability to perform reactions via both one-electron and two-electron mechanisms. Despite this dichotomy, flavoenzymes typically engage in two-electron reactions in biocatalytic processes [41]. Stimulated by the mechanistic adaptability of flavin, we questioned whether substrate promiscuous flavoenzymes could generate and utilize free-radicals intermediates.

We found 'ene'-reductases (EREDs) to be catalytically adaptive in the stereoselective debromination of acyclic esters [42]. Mechanistically, this transformation proceeds

by single electron reduction of the bromoester **13** by reduced flavin mononucleotide hydroquinone (FMN_{hq}), generating the α-acyl radical **14** and FMN semiquinone ($FMNH_{sq}$). Notably, this one-electron dehalogenation is analogous to that implicated in the FMN-dependent human iodotyrosine deiodinase mechanism [43]. Stereodetermining HAT from the semiquinone onto the C-centered radical furnishes oxidized FMN and dehalogenated product **15** (Figure 4c). A screen of a series of EREDs as well as a mutant Baeyer-Villiger monooxygenase indicated that this reaction was general across the flavoenzyme family, with an ERED from *Gluconobacter oxydans* (GluER) [44] providing highest yield and enantioselectivity. The mutation of a conserved tyrosine residue in the active site to phenylalanine improved the stereoselectivity to synthetically useful levels. In the native mechanism of GluER the reduced flavin hydroquinone delivers a hydride to the β-position of an activated olefin, and the removed tyrosine is invoked in a proton transfer. Observation of reaction enhancement with this Tyr→Phe mutation coupled with deuterium labelling studies, as well as the observed divergent stereochemical outcome compared with the native 'ene'-reduction, conclusively showed the stereodetermining HAT arising from the protonated flavin semiquinone. While this mechanism appears to be abiotic, uncharacterized flavoenzymes found in the digestive tract are known to carry out similar dehalogenative transformations, suggesting these types of open-shell reaction mechanisms may be represented in natural pathways [45].

Flavin further gathers attention due to its unique photophysical properties. Photoexcitation of the oxidized form results in a potent single-electron oxidant in solution-phase, with decarboxylative degradation of EDTA in buffer being well-documented [46]. Oxidative decarboxylation of fatty acids with flavoenzymes were discovered by Beisson *et al.* in the light-driven lipid metabolism in microalga *Chlorella variabilis* [47••]. The fatty acid photodecarboxylase (FAP) was found to have flavin adenine dinucleotide (FAD) as a cofactor, and spectroscopic experiments determined that the photoexcited FAD underwent single electron transfer with the fatty acid **16** generating FAD semiquinone and a carboxyl radical. Rapid decarboxylation results in an alkyl radical **17** that then undergoes HAT with either a neighbouring cysteine or tyrosine residue in the active site (Figure 4d). Alternatively, back-electron transfer from the FAD semiquinone to radical **17** followed by protonation could complete the catalytic cycle, however the authors were unable to perform the experiments necessary to distinguish between these mechanisms.

Conclusion and outlook

Recent focus on single-electron pathways has transformed the organic chemists' approach to synthetic design. As we gain knowledge on how nature accomplishes radical chemistry on structurally complex compounds in a highly selective manner, opportunities arise in applying these processes toward strategic implementation. Greater understanding of the ceilings in the HAT enzymes covered here should enable novel transformations to be envisaged that are beyond the capabilities of archetypal small molecule catalysts. An immediate challenge facing the synthetic chemist is departing from current trends of initiation by sacrificial radical precursors. Shifting this paradigm to a circular, catalytic radical protocol that initiate *and* terminate via HAT will prove invaluable. Modern technologies such as directed evolution [48], high-throughput experimentation [49] and machine learning [50] will aid in engineering these powerful catalysts to perform such tailored functions. Moreover, exploration in catalytic adaptability of enzymes to repurpose two-electron cofactors for single-electron chemistry is becoming a burgeoning field in biocatalytic research, with further non-native functionalities expected to be uncovered in future.

Conflict of interest statement

Nothing declared.

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