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Enzyme-mediated bioorthogonal technologies: Catalysts, chemoselective reactions and recent methyltransferase applications

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Abstract

Transferases have emerged as among the best catalysts for enzyme-mediated bioorthogonal functional group installation to advance innovative in vitro, cell-based and in vivo chemical biology applications. This review introduces the key considerations for selecting enzyme catalysts and chemoselective reactions most amenable to bioorthogonal platform development and highlights relevant key technology development and applications for one ubiquitous transferase subclass – methyltransferases (MTs). Within this context, recent advances in MT-enabled bioorthogonal labeling/conjugation relevant to DNA, RNA, protein, and natural products (*i.e.*, complex small molecule metabolites) are highlighted.

Keywords

transferase; *S*-adenosyl-L-methionine (AdoMet, SAM); glycosyltransferase; natural product

INTRODUCTION

A range of innovative chemoselective bioorthogonal/biocompatible conjugation strategies have been developed to enable in situ conjugation of reporters and affinity ligands for molecular tracking and mechanistic studies in cells and even in live animals [1–3] (Figure 1). Ideally, the chemoselective functional groups employed in such applications must: afford exquisite reactivity/efficiency under physiological conditions; provide notable selectivity/compatibility within the context of living cells and in vivo; lead to conjugated products that are metabolically stable and non-toxic; and not infringe on the native biology to be

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COMPETING INTERESTS STATEMENT

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studied. While, many bioorthogonal/biocompatible conjugation strategies rely on metabolic incorporation of non-native biomolecules synthetically-modified to display a chemoselective group, the use of enzyme-mediated strategies to install the requisite chemoselective functional groups is on the rise [3–5]. This review briefly introduces the catalysts and chemistries most amenable to enzyme-mediated chemoselective functional group installation and highlights recent key technology development and applications of methyltransferase-mediated bioorthogonal/biocompatible conjugation.

DESIRED CATALYST PROPERTIES: THE CASE FOR METHYLTRANSFERASES

There are three key considerations to selecting a catalyst for chemoselective functional group installation – substrate scope, catalyst efficiency, and catalyst abundance. Specifically, an ideal catalyst requires a uniquely balanced *substrate scope* that encompasses target (probe or biomolecule) selectivity with permissivity toward non-native chemoselective group installation. For intracellular applications, such non-native substrates must also be membrane permeable or transported via active/passive transport mechanisms. Catalytic *efficiency* is an essential requirement to enable subsequent rapid reporter conjugation and detection. For in situ applications catalytic turnover ultimately contributes to both probe/assay sensitivity and temporal resolution. Finally, catalyst *abundance* refers to natural/engineered distribution (cellular, tissue and organism) and abundance (protein levels), all of which ultimately influence the range of potential applications. Considering these factors, transferases (Enzyme Commission class 2; EC2) that catalyze macromolecule/metabolite 'tailoring' reactions have typically been favored for chemoselective functional group installation, catalyst development and applications.

Of EC2 transferases, methyltransferases (MTs) are currently the catalysts most favored for chemoselective functional group installation. MTs catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM or AdoMet; the 'donor') to a substrate nucleophile (carbon, nitrogen, oxygen or sulfur; the 'acceptor') [6–8] (Figure 2a). The *substrate scope* of MTs is exceptionally broad and includes macromolecular substrates (DNA, RNA, and proteins) and small molecules (primary and secondary metabolites). MTs are also able to use non-native *S*/*Se*-alkyl-substituted AdoMet donors to afford non-native alkylation, including *S*/*Se*-alkyl substituents bearing chemoselective functional groups [4,7,8]. While AdoMet analogues are unsuitable for cell-based studies due to poor uptake and chemical stability, these reagents can be generated in cells from corresponding non-native methionine analogues and ATP via methionine adenosyltransferases (MAT) [9–12]. Alternative enzyme-based approaches for non-native AdoMet analogue synthesis [13,14] and new chemically-stable AdoMet isosteric substrates [15] have also been recently reported. MT natural *abundance* and distribution is high and MTs are critical to all walks of life [4,6–8]. Thus, MT-based platforms are anticipated to offer inroads to rich biology and support a vast array of impactful applications. MT catalytic *efficiency* is the perhaps the greatest liability in the context of chemoselective applications. For example, the catalytic efficiencies for MTs discussed in this review range from ~10 to 7,200 M^{-1} s^{-1} and the corresponding range for AdoMet-producing enzymes is ~145 – 340 M^{-1} s^{-1}.

CHEMOSELECTIVE REACTION PRIORITIZATION

Chemoselective reaction *efficiency* and *selectivity* are a cornerstone of effective bioorthogonal conjugation platforms [1–3]. Specifically, to be effective, such reactions must proceed at high reaction rates (efficiency) in cellular environments (selectivity). The *molecular properties* (*e.g*, size, stability, polarity/charge and/or hydrophobicity/hydrophilicity) of the selected non-native chemoselective functional groups are also key to selecting chemistries for transferase-mediated platforms. Specifically, these features influence an enzyme's substrate recognition, turnover and lifetime must align with the selected transferase's substrate scope. Based on these key parameters, the following chemoselective reactions are considered most amenable to transferase-mediated strategies (Figure 2b).

Modified Huisgen 1,3-dipolar cycloaddition reactions (CuAAC and SPAAC) [16].

Poor reaction rates limited the synthetic utility of Huisgen 1,3-dipolar cycloaddition prior to the advent of the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction by Sharpless and co-workers (often referred as the first conceptual example of 'click' chemistry) [17]. Despite favorable reaction rates (10–100 M^{-1} s^{-1}), chemoselectivity and stability of reaction products, the metal dependence of the CuACC reaction limited use in living cells and tissues. To circumvent this liability, Bertozzi and co-workers advanced copper-free strain-promoted azide-alkyne cycloaddition (SPAAC) reactions driven by the ring strain of cyclooctyne reactants such difluorooctyne (DIFO, 0.08 M^{-1} s^{-1}) [18]. Alternative constrained reactants and electronic activation strategies led to SPAAC reaction rate improvements of nearly two orders in magnitude [19,20].

Photoinduced tetrazole-alkene/alkyne cycloaddition reactions (PTAC).

This 'photoclick' reaction, first reported by Lin and co-workers, was also inspired by the work of Huisgen [21]. Photo-induced tetrazole cycloreversion to generate short-lived reactive nitrile imine intermediates serves the basis for this reaction. These intermediates rapidly (>50 M^{-1} s^{-1}) undergo 1,3-dipolar cycloaddition reactions with alkynes or alkenes to give fluorescent cyclic pyrazolines. Conceptual advances include optimizing ring strain and electronics, leading to rate improvements of nearly two orders in magnitude [22,23]. While this method offers exceptional utility for spatial and temporal control in cell/tissue-based applications, the dependence on light may limit in vivo applications.

Inverse electron-demand Diels–Alder reactions (IEDDA).

Unlike a classical Diels–Alder reaction in which an electron-rich diene reacts with an electron-poor dienophile, IEDDA reaction exploit an electron-rich dienophile (alkenes/alkynes) and an electron-poor diene (typically tetrazines) [24]. Early IEDDA proof of concept was reported by Fox and co-workers using *trans*-cyclooctene and tetrazine (>10^3 M^{-1} s^{-1}). Continued development has focused on tuning reactivity via electronic and steric perturbation to afford rate improvements of over two orders in magnitude [24,25]. Photoinduction (photo-IEDDA) and orthogonality, both among exclusive IEDDA reactions and between IEDDA reactions and other chemoselective reactions (*e.g.*, SPAAC and CuAAC), have also been reported [26,27].

MT-ENABLED BIOORTHOGONAL APPLICATIONS

Initial work in the field was based on MT-mediated single turnover reactions using aziridine-based AdoMet analogues to give fused AdoMet analogue-acceptor adducts [28,29]. Weinhold and Klimasăuskas were first to demonstrate MT-catalyzed transfer of non-native groups from catalytically-competent AdoMet analogues [30] which set the stage for what they subsequently described as the MT-directed “Transfer of Activated Groups” (mTAG) platform for chemoselective conjugation. This section is limited to recent applications of MTs in combination with catalytically-competent AdoMet analogues bearing non-native *S*/*Se*-alkyl groups with chemoselective functionality.

Representative recent DNA applications [31].

Building on the mTAG precedent, Neely and collaborators reported DNA adenine-N6 MT M.TaqI-mediated DNA CuAAC using **1** (Figure 2a) for DNA mapping [32] and a subsequent comparative study of M. TaqI-mediated *N*-hydroxysuccinimidyl (NHS) ester amide coupling or CuAAC/SPAAC using amine **2** or azide **3**, respectively [33]. These studies revealed SPAAC to outperform CuAAC and amide coupling and also noted DNA decomposition/damage in the CuAAC reactions. Weinhold and co-workers also recently reported the use of a similar M. TaqI-driven strategy with **3** and SPAAC to introduce fluorophores and affinity labels as probes to study DNA origami nanostructure folding [34]. Innovative new MT-enabled methods for DNA photocaging open the door to ‘reversible’ selective modification of DNA as exemplified by the M.TaqI-catalyzed introduction of photocleavable protecting groups (Figure 2a; **4**) [35,36]. This work demonstrated a feasible platform for photoregulation using simple in vitro transcription/translation systems where subtle changes in the photoprotecting group substitution pattern led to modulation of both MT turnover and/or photoreaction efficiency. Rentmeister and co-workers recently extended this platform via the development of new MATs (*Cryptosporidium hominus* MAT and an engineered *Methanocaldococcus jannaschii* thermostable MAT variant) to produce photocaged AdoMet analogues in situ [12]. Neely, Fernandez-Trillo and team also recently put forth tools for an innovative in vitro DNA ‘write, remove, rewrite’ approach [37]. Specifically, they demonstrated M.TaqI-mediated and the cytosine-C5 MT M.MpeI-catalyzed installation of a bifunctional DNA tag comprised of a hydrazone-linked **5** or oxime-linked terminal azide **6**. This modification allowed for SPAAC-mediated conjugation and, in the case of the hydrazone linker, selective hydrolysis in the presence of NH_2OH and chemoselective reinstallation of modifiers via the exposed hydrazide.

Representative recent RNA applications [31,38].

Rentmeister and colleagues have reported a range of mTAG RNA-based applications in recent years using three fundamental MT model systems – variants of the 5’-cap mRNA adenine-N2 MT trimethylguanosine synthase2 (GlaTgs2) [39–41,44], 5’-cap mRNA gaunine-N7 MT Ecm1 [42–44] and mRNA adenine-N6 MTs and METTL3–14 and METTL16 [45,46]. Chemoselective reactions employed in these studies include CuAAC [41,45,46], SPAAC [40–44,46], PTAC and IEDDA [39,43,44] using non-native AdoMet analogues (Figure 2a; **1**, **7**-**13**). Many of these studies evaluated the impact of mRNA non-native alkylation on RNA processing (primarily reverse transcription) and corresponding

MT-catalyzed reactions were conducted in increasingly complex reaction environments (in vitro [41,44,46], cell lysates [39,40,43] and live cells [45]). Four specific recent advances relating to MT-mediated RNA applications are particularly noteworthy. First, reminiscent to previously reported MT-enabled DNA photocaging (Figure 2a; **4**), conceptually similar RNA studies were recently reported [46]. Rentmeister and co-workers also demonstrated proof of concept of novel norbornene-based AdoMet co-substrates (*e.g.*, **12,13**) for RNA MTs to facilitate downstream IEDDA conjugation reactions [43]. Third, this same team published an innovative study that revealed the ability to use adenine-N2 MT (GlaTgs2 variant) and gaunine-N7 MT (Ecm1) in tandem, two distinct non-native AdoMet co-substrates **10** and **11**, and subsequent SPAAC and IEDDA to afford selective differential mRNA labeling and FRET [44]. Finally, these researchers also reported a cell-based platform to identify METTL3-METTL14 target sites where the resulting termination of reverse transcription and bioconjugation method faciltiated RNA fragment capture and next generation sequencing [45]. Klimašauskas, Vilkaitis and colleagues recently extended these tools to the small-RNA 2'-*O*-MT HEN1 from *Arabidopsis thaliana*, a duplex-driven MT [47]. Specifically, this work employed amines **2** and **14** (NHS ester amide coupling), azide **3** (CuAAC) and alkyne **15** (CuAAC) as tools to map the sequence/context specificity of HEN1 in vitro.

Representative recent protein applications.

A range of diverse strategies have been developed for bioorthgonal chemoselective protein modification [48]. Within this context, the Weinhold group were the first to demonstrate MT-mediated chemoselective protein modification via CuAAC using the *Neurospora crassa* histone 3 lysine 9 *N*-MT (H3K9) Dim-5 and **1** (Figure 2a) [49]. Luo and co-workers were among the first to advance similar tools for proteomics using oncogenic H3K9 *N*-MTs EuHMT1 (GLP1) and EuHMT2 (G9a) as models. Coined 'bioorthogonal profiling of protein methylation' (BPPM), their proof of concept utilized EuHMT1/2 variants engineered for improved turnover with **10** followed by SPAAC to identify non-histone EuHMT1/2 substrates in cell lysates [50]. The same group used a similar strategy to identify substrates for protein arginine *N*-MT PRMT3 using a PRMT3 variant (M233G) and **16** [51]. This team extended the concept to living cells via the inclusion of a MAT (I117A) and H3K9 *N*-MT (EuHMT1-Y1211A or EuHMT2-Y1154A) engineered to favor **1** followed by CuAAC-based labeling. Referred to as 'clickable chromatin enrichment with parallel DNA sequencing' (CliEn-seq) [52], this proof of concept study highlighted MAT-catalyzed intracellular production of **1** from cell-permeable methionine analogues, in situ chromatin modification by engineered EuHMT1/2 and subsequent enrichment of uniquely modified chromatins via CuAAC-enabled capture for sequencing. In vitro functional annotation of two putative lysine *N*-MTs METTL21A and METTL10 employed similar tactics. Specifically, the use of **8** in cell lysates followed by CuAAC confirmed METTL21A to function as a histone *N*-MT and revealed METTL10 to EF1A1 lysine 318 [53]. Zumbusch and colleagues recently reported an alternative method for conceptually similar cell-based proteomics [54]. In this study, HEK293T and HeLa S3 cells engineered to produce enhanced green fluorescent protein (eGFP)-fusions with protein targets of interest (*e.g.*, p53, Akt1, GAPDH, histones H2B, H3, H4; microtubule-associated protein RP/EB family member EB1; Foxo1; and heat shock proteins HSPA1 and HSPA8; valosin-containing protein VCP; and nucleolin) were electroporated with **8**. Subsequent CuAAC-mediated fluorophore conjugation enabled

intracellular fluorescence lifetime imaging (FLIM) FRET of each target protein's alkylation state and localization.

Representative recent natural product and small molecule applications (Figure 3).

Transferase-mediated bioactive natural product (NP) chemoselective modification was first demonstrated using the vancomycin glycosyltransferase GtfE and non-native sugars bearing azides **17** and alkoxyamines **18** to afford CuAAC [55] or alkoxyamine-based 'neoglycosylation' [56], respectively. Proof-of concept MT-catalyzed non-native NP alkylation was first established by Gruber-Khadjawi and colleagues using the novobiocin and coumermycin 8-*C*-MTs NovO and CouO [57]. Using synthetic non-native AdoMet analogues **19–23** and simple coumarin models, this study demonstrated NovO/CouO-catalyzed five non-native 8-*C*-alkyl groups and also highlighted some permissivity toward alternative acceptors. To circumvent limitations associated with synthetic AdoMets within this context, Singh, Thorson and colleagues were the first to develop MAT-MT coupled systems for natural product 'alkylrandomization' using the indolocarbazole rebeccamycin sugar 4'-*O*-MT RebM as a model [9]. This work highlighted the survey of five diverse MATs for non-native AdoMet production, use of hMAT2 to produce AdoMet analogues bearing 18 non-native *S*-substitutions and the application of coupled hMAT2-RebM reactions to generate 4 rebeccamycin analogues bearing 4'-*O* non-native alkyl groups (Figure 3; **Reb-19**, **21**, **24** and **25**). Similar NP biosynthetic MT alkyl permissivity and corresponding coupled multi-enzyme platforms have since been reported for a range of NPs including: rapamycin (16-*O*-MT RapM: coupled hMAT-RapM system to produce 16-*O*-alkyl rapalogs **Rap-19** and **24** [58]); coumarins (novobiocin 8-*C*-MT NovO: coupled SalL-NovO system to produce model coumarins including 8-*C*-isotopically-labeled coumarin-**26–28** derivatives [59,60]) and alkaloids/phenolics (coclaurine *N*-MT CNMT: coupled hMAT-CNMT system to produce 2-*N*-alkyl isoquinolone-**19** and isoquinolone-**24** [61]; carboxy-*S*-adenosyl-L-methionine synthase in conjunction with CNMT or catechol-*O*-methyltransferase COMT to produce *N*- and *O*-carboxymethylated isoquinoline-**29** molecules [62]). Micklefield and colleagues also introduced an innovative coupled fungal tyrosinase-mammalian catechol-*O*-MT (COMT) system for selective peptide *O*-alkylation with non-native alkyl groups (Figure 3; peptide-**30)** [63]. Brieke and collaborators reported the first proof of concept for MT-mediated chemoselective conjugation. This study employed two glycopeptide α-*N*-MTs (A40926 $MtfA_{dbv}$ and pekiskomycin $MtfA_{pek}$) and led to the production of teicoplanin aglycon-**19** and teicoplanin aglycon-**31** (Figure 3), the latter of which was successfully used in IEDDA conjugation reactions to tether an affinity ligand or fluorophore [64]. Enediyne MT-mediated chemoselective conjugation was also recently demonstrated using the permissive tiancimycin 7-*O*-MT TnmH [65]. This study highlighted TnmH to turnover a range of anthraquinone acceptors and, in the presence of non-native AdoMet donors (**19,21,** and **32)**, catalyze production of tiancimycin 7-*O*-alkyl analogues. The corresponding tiancimycin-**21** (Figure 3) was subsequently used in CuAAC reactions to introduce tethers for putative antibody-conjugation. This work set the stage for future production of tiancimycin-antibody conjugates and subsequent preclinical evaluation studies.

CONCLUSIONS AND PROSPECTUS

This review points to notable advances in bioorthogonal chemoselective reaction and enzyme reagent (enzymes and non-native substrates) development to support emerging transferase-mediated bioorthogonal platforms. While limited proof of concept examples for cell-based applications of MT-enabled bioorthogonal labeling/conjugation exist, three key barriers to broader use remain. First, unlike many of the commercially available kits/reagents for bioorthogonal chemoselective conjugation, the reagents (enzymes and non-native substrates) for MT-enabled platforms are highly specialized and largely inaccessible to the broader research community. Improved access to non-native AdoMet analogues via commercial sources and/or user-friendly production methods is expected to present new opportunities and unlock new discoveries. Second, the stability, PK, biodistribution and cellular uptake of non-native AdoMet analogues remain substantive barriers to cell-based and in vivo applications. Strategies to stabilize and/or 'deliver' AdoMet analogues as well as improved methods for in situ (cell and/or tissue-specific) production are anticipated to help circumvent these roadblocks. Finally, MT reaction rates and bioorthogonality (*i.e.*, selectivity for non-native substrates) fall far short of the corresponding rates or selectivities of the best bioorthogonal chemoselective reactions. The ongoing discovery and evolution/engineering of improved catalysts is expected to continue to narrow this gap. Cumulatively, such platform improvements are expected to usher in broader application and impact including, but not limited to, lead discovery or development efforts.

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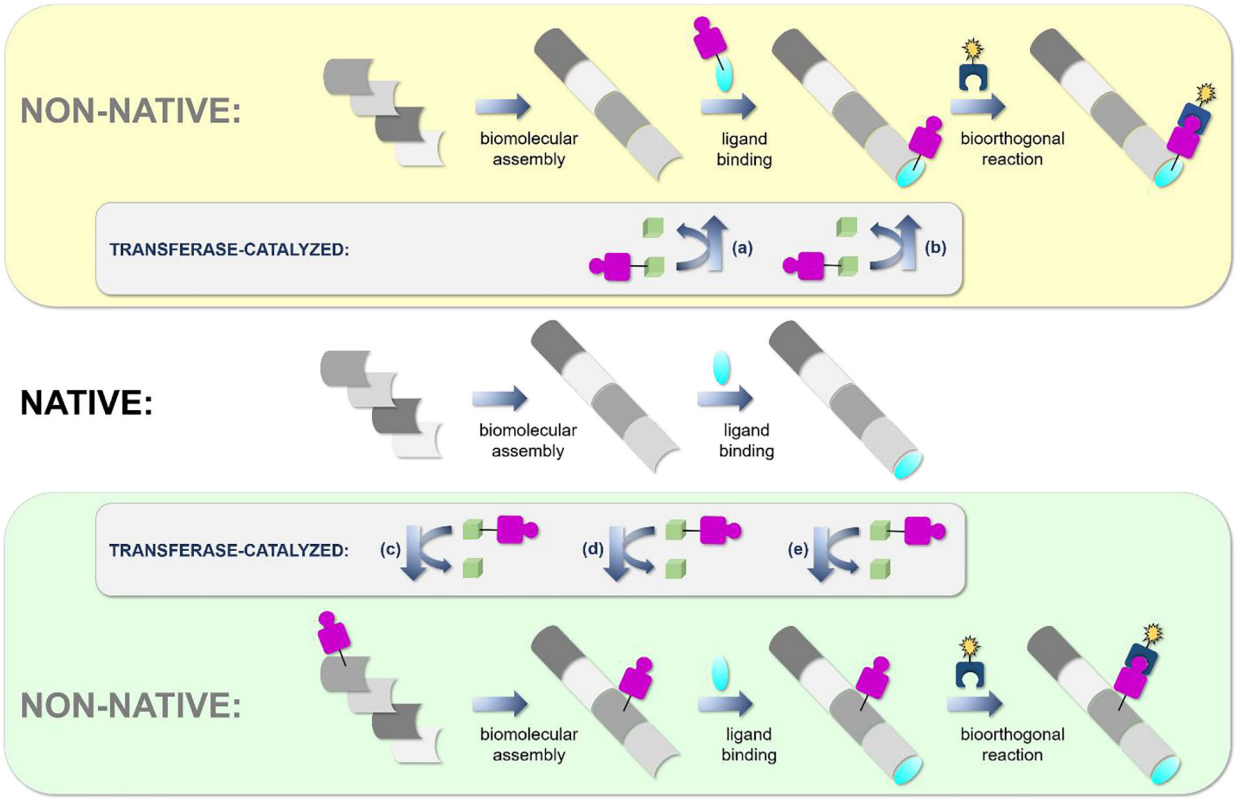

Figure 1. Overview of bioorthogonal strategies used in chemical biology research.

The center of the figure reflects key steps in native biological systems. Specifically, in the *biomolecular assembly step* metabolic building blocks (*e.g.*, nucleotides, amino acids, acetate, isoprenes, etc.; represented by grey cylinders) are commonly used to generate functional biopolymers (*e.g.*, RNA, DNA, proteins, lipids, etc.). In the *ligand binding step*, certain biopolymers (e.g., proteins, RNA or DNA complexes) are able to selectively bind ligands (*e.g.*, hormones, small molecules, metabolies, drugs, etc.; highlighted in light blue). The *upper panel (yellow)* reflects an application of a ligand analogue (light blue) bearing a bioorthogonal chemoselective 'tag' (purple puzzle shape). This ligand can be tracked in situ via a chemoselective bioorthogonal reaction with a partner reagent (dark blue puzzle piece) commonly appended with a fluorescent reporter or affinity capture ligand (yellow burst). The *lower panel (green)* reflects an application of a metabolite building block analogue bearing a bioorthogonal chemoselective functional group (purple puzzle shape). This building block can be tracked in situ via a similar chemoselective labeling strategy using a partner reagent (dark blue puzzle piece) commonly appended with a fluorescent reporter or affinity capture ligand (yellow burst). Transferases are able to introduce bioorthogonal chemoselective functional groups (purple puzzle shape) at many different stages including: modification of the ligand prior to **(a)** or after **(b)** binding to it's molecular target as well as modification of metabolic building blocks **(c)** or biopolymers prior to **(d)** or after **(e)** ligand binding. *Transferase reactions (grey panels)* typically utilize a co-substrate comprised of the transfer group (*e.g.*, alkyl, glycosyl, acyl, etc.) and 'activating group' (*e.g.*, *S*-adenosylhomocysteine/AdoHcy or SAH, nucleotide diphosphate/NDP, coenzyme A/CoA, etc.; represented by the green cube).

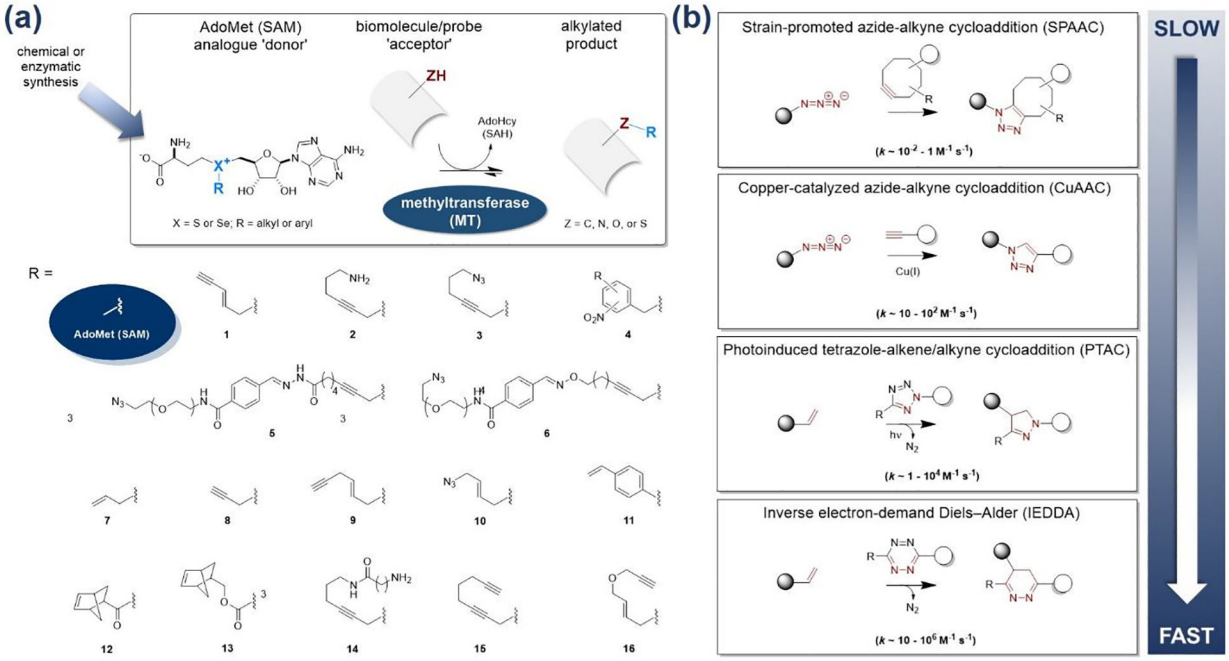

Figure 2.

(a) The native function of a methyltransferase (MT) is to transfer a methyl group from an *S*-activated AdoMet donor to an acceptor *C*-, *N*-, *O*- or *S*-nucleophile (upper panel). As described in this review, MTs are capable of also using non-native *S*- or *Se*-activated alkyl or aryl (light blue) AdoMet analogues and thereby catalyze differential alkylation of target biomolecules. The lower panel highlights the structure of the *S*/*Se*-substituent for representative examples highlighted in this review. (b) Chemoselective reactions that fit criteria for transferase-mediated platforms.

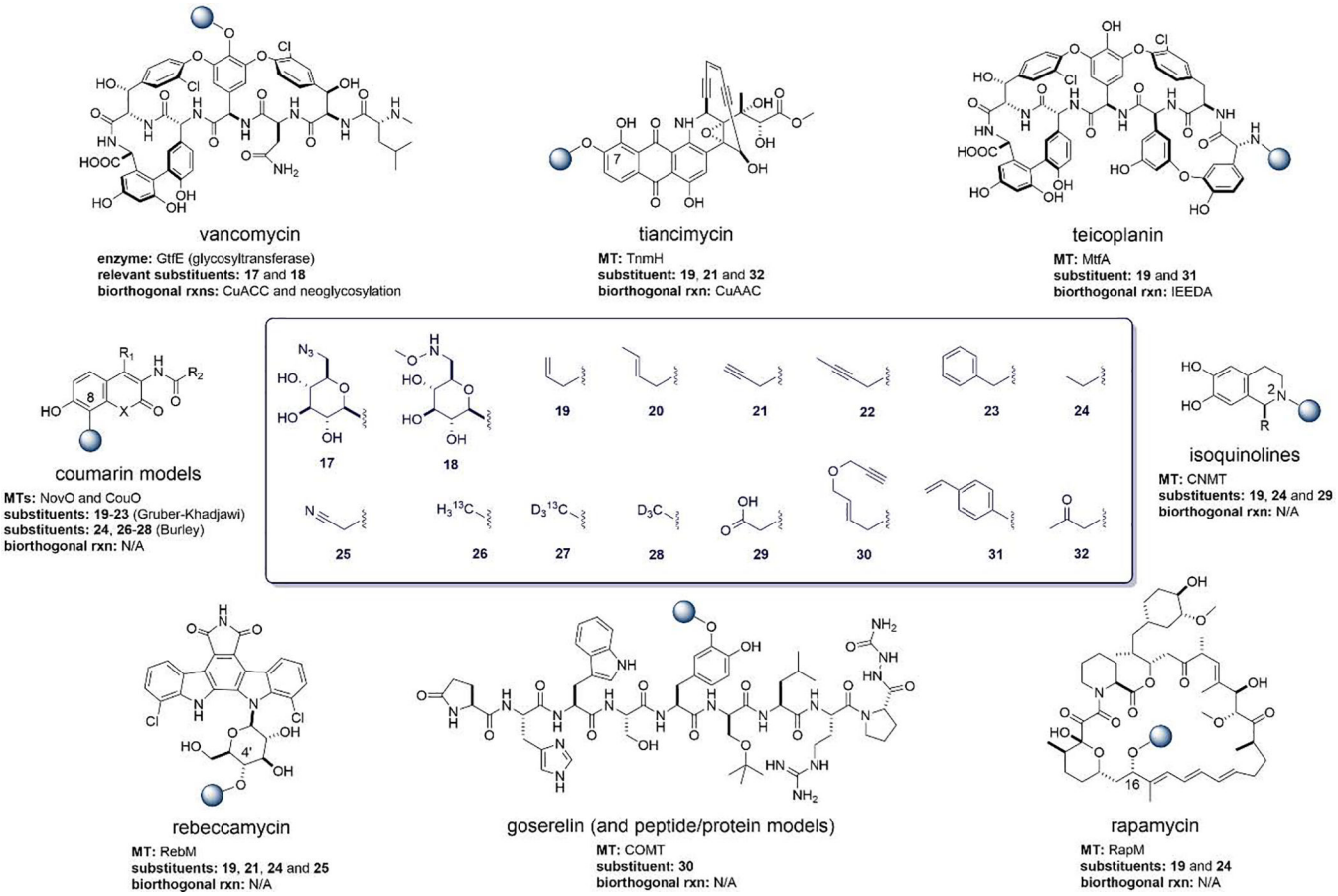

Figure 3.

Natural products for which transferase-enable bioorthogonal chemoselective modification and/or MT-enable non-native alkylation has been reported. The regiochemistry of non-native modification is highlight by the blue ball with corresponding non-native substituents illustrated in the center box.