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Alkylating enzymes

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Chemospecific and regiospecific modifications of natural products by methyl, prenyl, or *C*-glycosyl moieties are a challenging and cumbersome task in organic synthesis. Because of the availability of an increasing number of stable and selective transferases and cofactor regeneration processes, enzyme-assisted strategies turn out to be promising alternatives to classical synthesis. Two categories of alkylating enzymes become increasingly relevant for applications: firstly prenyltransferases and terpene synthases (including terpene cyclases), which are used in the production of terpenoids such as artemisinin, or meroterpenoids like alkylated phenolics and indoles, and secondly methyltransferases, which modify flavonoids and alkaloids to yield products with a specific methylation pattern such as 7-*O*-methylaromadendrin and scopolamine.

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Introduction

Mostly hydrolases but also some oxidoreductases are well established as biocatalytic tools in synthesis since decades, whereas transferases — especially alkylating enzymes — have been applied in this field for only a few years. However, alkylation reactions are essential steps in the biosynthesis of a multiplicity of natural products, where classical chemistry would apply Friedel-Crafts and Prins type alkylation, or Williamson ether synthesis, to name a few [1]. In fact, the attachment of methyl, prenyl, or *C*-glycosyl groups accounts for the crucial diversity of metabolites which are generated from a limited number of biosynthetic precursors.

For example, the ca. 70.000 terpenes and (mero-) terpenoids occurring in nature mainly originate from only five mono- and oligo-(isoprenyl)diphosphates [2]. In the conversion of these activated donors by prenyltransferases (including terpene synthases/terpene cyclases), product formation is initiated by cleaving off the diphosphate unit as leaving group by (Lewis) acid activation. The resulting (enzyme stabilized) carbocation reacts in a crucial product formation step with an internal carbon nucleophile (intramolecular prenyl transfer) or a corresponding nucleophilic center provided by a second reaction partner (intermolecular prenyl transfer) (Figure 1a). Eventually, the alkylation reaction is completed by deprotonation or scavenging by a nucleophile, for example water, of the product carbocation. Because of the possibility of different regiospecific attacks, cascade reactions, rearrangements and isomerizations of the intermediary carbocation, a broad range of products can obtained with different prenyltransferase enzymes, even from only a handful of prenyl diphosphate precursors [3].

A second type of alkylation reaction important in natural product biosyntheses is the transfer of methyl groups. Methyltransferases, which are especially abundant in plant metabolic pathways, lead to such important compound groups as lignins, flavonoids, stilbenes or alkaloids. They are almost exclusively relying on the cofactor *S*-adenosylmethionine (SAM or AdoMet). According to their strict specificity toward the nucleophilic substrate, these enzymes are classified as *O*-methyltransferases, *N*-methyltransferases, *S*-methyltransferases and *C*-methyltransferases (Figure 1b) [4]. As many methyltransferases are additionally highly regiospecific, they are metabolic tools to mediate 'hydrophobic masking' of a certain substructures or peripheric areas within their acceptor molecule. In contrast to these 'pure' alkylations, introduction of glycosyl residues (Figure 1c) often mediates hydrophilicity to metabolites which are poorly soluble in water. However, as glycosylation at heteroatoms is usually not regarded as an alkyl transfer, only glycosyltransferases catalyzing the formation of carbon–carbon bonds are included in this review.

As described above, regiospecific alkylation of a natural product alters its physicochemical properties and often results in a strongly increased affinity toward, for example, membranes and receptors. For example, the dietary flavonoid naringenin does not show any hormone-like properties, whereas its derivative 8-prenylnaringenin is the most active natural phytoestrogen known yet [5]. However, many alkylated natural products, for example terpenoids, flavonoids or alkaloids (Figure 2), cannot be obtained from natural sources in sufficient amounts [6–9].

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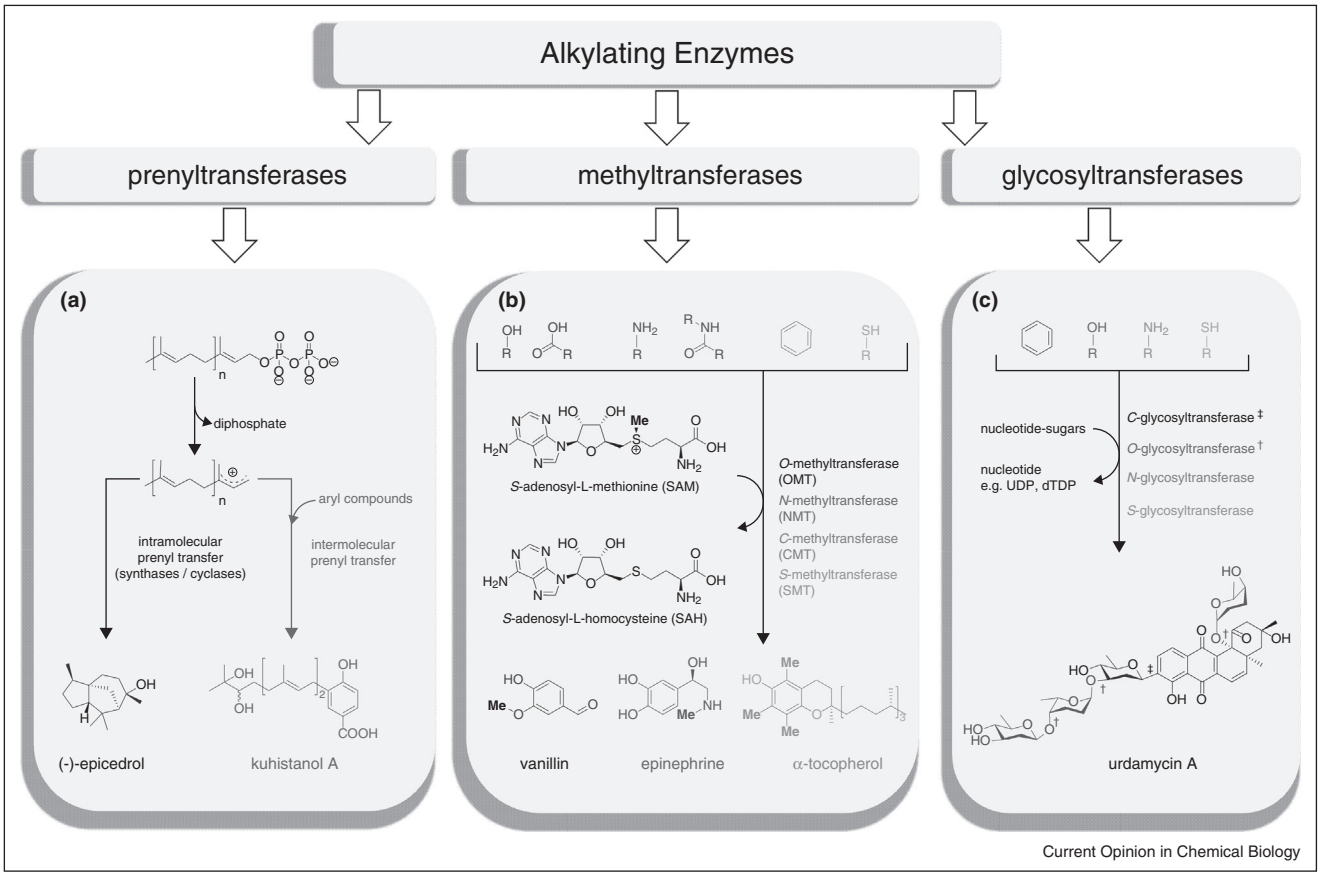
Figure 1



Scheme of reactions catalyzed by alkylating enzymes. The residues of prenyl **(a)**, methyl **(b)** or glycosyl transfer **(c)** are shown in different shades of gray.

Moreover, classical chemical syntheses of these compounds require multistep reactions, making some processes costly and time-consuming. In other cases, legislation or consumer preferences require natural-like production processes based on enzymes. Thus, the commonly mild conditions of biotransformations combined with high selectivity and sufficient yields (as exemplified for the compounds in Figure 2) accommodate the constantly growing demand for 'greener' (and cleaner) chemistry. On the other hand, most prenylated or methylated products of commercial interest are plant derived. Plant biosynthesis and plant enzymes are often not as well behaved in research and applications as their microbial counterparts, but currently significant advances are being made in view of bioeconomic processes required to substitute for petroleum based production. In this short review, emphasis is placed on selected recent applications of prenyl- and methyltransferases which are the most important alkylating enzymes in the modification of mostly plant natural products, but also of artificial analogs.

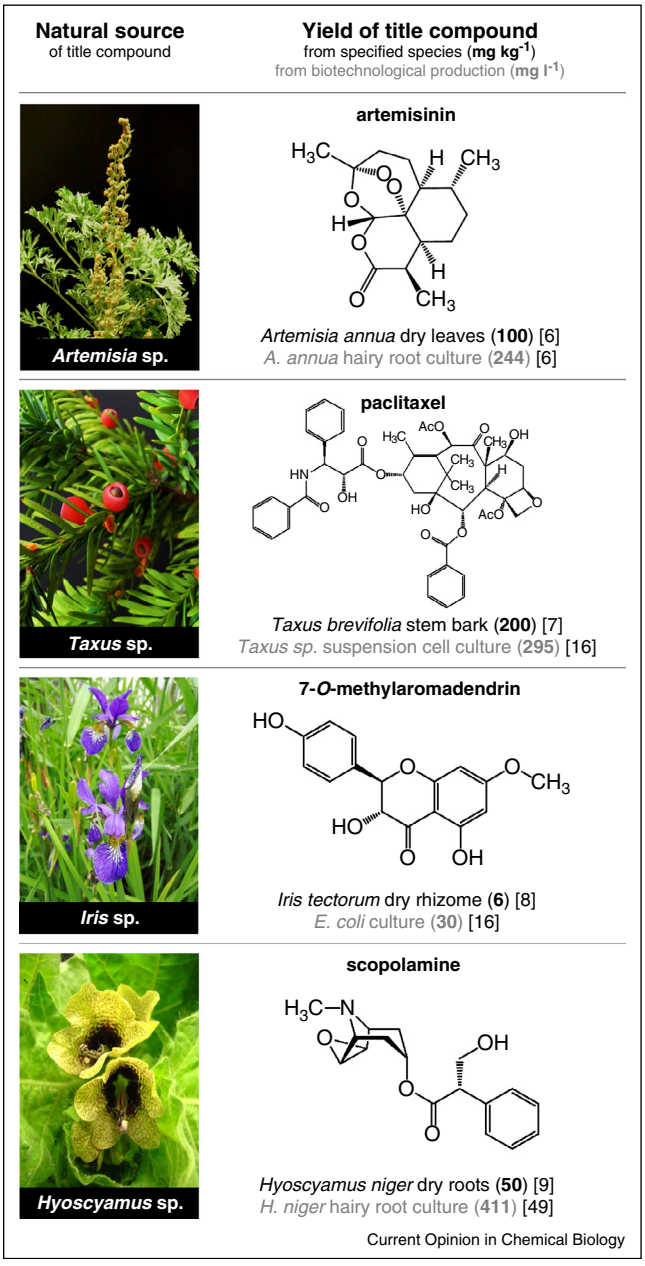
Prenylating enzymes

Intramolecular prenylation (terpene synthases/terpene cyclases)

Intramolecular prenylation reactions convert geranyl diphosphate (GDP) and higher oligomers into structurally diverse mono-terpenes, sesqui-terpenes, di-terpenes or triterpenes, which are high value components of pharmaceutical products, cosmetics, fragrances, flavors and foods. Hence, cyclase reactions offer significant potential to substitute traditional chemical syntheses or isolation from often limited natural resources of these compounds.

In an exploratory study, purified α-pinene synthase was used to transform GDP into α-pinene, which was oxidized in a subsequent reaction step with $Cr(CO)_6$/*tert*-butylperoxide leading to the attractant pheromone (+)-verbenone (Figure 3a) in high enantiomeric purity (>98% ee) and yield (150 mg l^{-1}) [10]. The diterpene sclareol could be produced from geranylgeranyl diphosphate by an enzymatic two-step process involving two terpene cyclases from *Salvia sclarea* [11]. Owing to the acceptance of

Figure 2



Production of valuable terpenoids and alkaloids by the source plants [4–7] and by biotechnological processes involving alkylating enzymes [4,13,34,42].

artificial substrates, germacrene synthases were even applied in the synthesis of several non-natural terpene products [12].

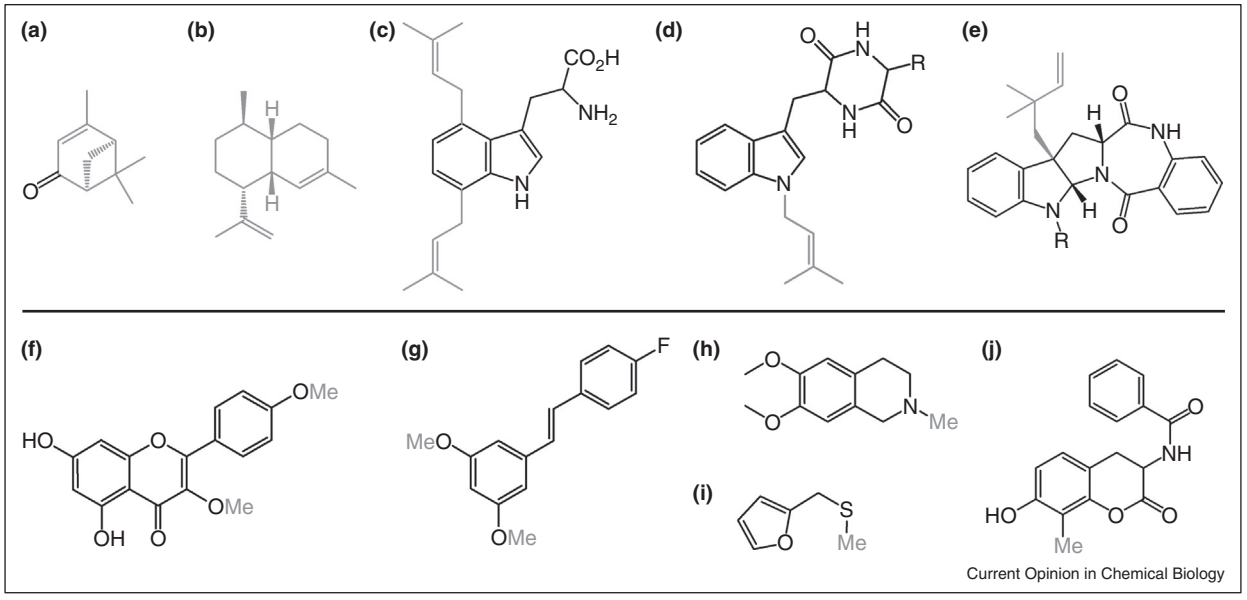
Jackson *et al.* described the production of fragrances in a genetically modified *Saccharomyces cerevisiae* strain [13]. This yeast was modified in its capacity to generate increased levels of isoprene diphosphates originating from an engineered mevalonate pathway. Upon heterologous expression of *epi*-cedrol synthase from *Artemisia annua*, intracellular synthesis of *epi*-cedrol (Figure 1a) was achieved. The same concept was successfully applied in the biosynthesis of amorpha-4,11-diene (Figure 3b) or artemisinic acid, which could be produced in yields as high as 40 g l^{-1} or 0.1 g l^{-1} in yeast [14•,15]. Both compounds are important intermediates in the production of artemisinin (Figure 2), which is the most effective therapeutic for the treatment of malaria. Because of the high demand for this drug, biotechnological production of the precursors assists the current production of artemisinin from the plant source *A. annua* or from cell cultures thereof [6]. Similarly, the limited natural occurrence of the potent anti-cancer agent paclitaxel (Figure 2, commercially known as Taxol®) in Pacific yew (*Taxus brevifolia*) necessitates its biotechnological production, for example, by plant cell cultures [16].

Intermolecular prenylation — prenyltransferases

From the multitude of enzymes involved in prenyl transfer to aromatic compounds or isoprenoids (e.g. those from the pathways leading to ubiquinones, alkaloids, or carotenoids), proteins catalyzing the attachment of dimethylallyl groups are best understood. Especially the substrate specificity of several dimethylallyltryptophan synthases (DMATS) from the fungus *Aspergillus fumigatus* was the subject of extensive studies. DMATS4 was shown to be selective for position 4 [17] in the isoprenylation of artificial indole derivatives or 2,5-diketopiperazines [18], whereas a second isoform modifies the C7 atom [19]. Tandem incubation of the indole substrates with both enzymes has been used for the synthesis of diprenylated products (Figure 3c) [20•]. Interestingly, a further protein recently cloned from *A. fumigatus* alkylates the non-aromatic α-carbon of 3-indolylbutenone [21].

Besides the chemoselective enzymes mentioned above, several dimethylallyl transferases are new valuable tools in chemoenzymatic synthesis because they promiscuously catalyze *O*-prenylation, *N*-prenylation and *C*-, or even 'reverse' prenylation (v.i.). The enzyme CdpNMT which was used for the modification of 2,5-diketopiperazines to the *N*1-dimethylallylated derivatives (Figure 3d) [22] also mediates *C*-prenylation of phenolics such as 1,6-dihydroxynaphthalene [23]. The prenyltransferase SirD from *Leptosphaeria maculans* alkylates the *p*-position in phenylalanines, tyrosines or 4-amino-phenylalanine derivatives [24•]. Introduction of a reverse β-configured C3-dimethylallyl moiety was observed in conversion of pyrrolo[2,3-b]indole derivatives by CdpC3PT from *Neosartorya fisheri* [25]. This enzyme was applied in the one-step transformation of the non-prenylated substrates into four enantiomerically pure aszonalenin (Figure 3e) derivatives reaching conversion rates of 85–100% [26].

Figure 3



Natural products synthesized with the assistance of alkylating enzymes. The residues introduced by prenylation (upper panel) or methylation (below) are shown in gray. **(a)** (+)-verbenone, **(b)** amorpha-4,11-diene, **(c)** 4,7-bis(dimethylallyl)tryptophan, **(d)** *N*1-dimethylallylated derivative of 6-(3-indolylmethyl)-2,5-diketopiperazine, **(e)** *epi*-aszonalenin, **(f)** ermanin, **(g)** 3,5-dimethoxy-4′-fluorostilbene, **(h)** *N*-methyl-6,7-dimethoxy-tetrahydroisoquinoline, **(i)** furfuryl-methyl-sulfide, **(j)** 3-(benzoylamino)-8-methylumbelliferone.

The first prenyltransferase used in chemistry for the biocatalytic prenylation of aromatics was UbiA, a broad-spectrum GDP-dependent enzyme from *Escherichia coli* [27•]. UbiA-mediated coupling of an artificial hydroxylated geranyl moiety to *p*-hydroxybenzoic acid yielded Kuhistanol A (Figure 1a) [28], an antibacterial agent active against multi-resistant *Staphylococcus aureus* strains. Further promiscuous enzymes which might have great potential in biotechnological application are Fnq26 from *Streptomyces cinnamonensis*, which catalyzes regular and reverse C–C- and C–O-prenylations of polyketides and flavonoids [29,30], and oligoprenyl diphosphate synthases, which show activity toward a wide range of natural and artificial prenyl donors [31]. A prominent example for the application of long-chain prenyltransferases is the production of coenzyme Q_{10} by whole-cell biotransformation [32]. Although problems in intracellular storage capacity have to be solved yet, recent developments in this field focus on heterologous systems enabling access to carotenoids [33,34].

Methyltransferases

O-methyltransferases (OMTs)

Because of the presence of vanilloid motifs in many bioactive phenylpropanois, especially in flavoring substances, regiospecific *O*-methylation of catechols is an indispensible tool in the synthesis of such natural products. Thus, first examples for the application of OMTs in chemoenzymatic synthesis were focused on flavor compounds, like the world's number one flavor vanillin (Figure 1b). More than a decade ago, Li and Frost [35] presented an environmentally friendly method to produce vanillin from glucose by genetically modified *E. coli* cells. During the fermentation, methyl transfer was achieved by recombinantly expressed catechol-*O*-methyltransferase.

Recent developments mainly focus on the synthesis of structurally more complex and highly valuable compounds — especially on flavonoids because of their manifold biological effects. For example, ermanin (Figure 3f) with anti-inflammatory and antiviral activity claimed, can be synthesized from its inactive precursor kaempferol (5,7-dihydroxy-3,4′-dimethoxyflavone) by whole-cell biotransformation [36]. Sequential introduction of the two methyl groups was performed by OMT-9 from rice (ROMT-9) and OMT-2 from soybean and resulted in almost quantitative conversion of the substrate. Similarly, transformation of kaempferol and quercetin by an engineered variant of the OMT-7 from *Populus deltoides* gave the 3,7-di-*O*-methyl products in 58% and 70% yield, respectively [37]. The conversion of naringenin (4′,5,7-trihydroxyflavanone) to 3-*O*-methylkaempferol was performed as an enzymatic two-step process involving initial oxidation by flavonol synthase and methylation of the intermediate by ROMT-9 [38•].

The most promising approach for the chemoenzymatic production of flavonoids is the *de novo* synthesis from inexpensive biosynthetic precursors such as *p*-coumaric acid, which is initially processed into non-methylated flavonoids. Subsequently, OMT reactions give rise to individual methylation patterns. In this way, 7-*O*-methylaromadendrin (Figure 2) and the corresponding flavone genkwanin were obtained from recombinant *E. coli* in yields of 2.7 and 0.2 mg l^{-1}, containing OMT enzymes from *Streptomyces avermitilis* and peppermint, respectively [39•,40].

Also stilbene derivatives (Figure 3g) have produced in *E. coli* harboring a reconstructed plant pathway. Katsuyama and co-workers showed that the modified cells transform phenylalanine, tyrosine and several non-native cinnamic acid substrates into novel mono- and dimethylated stilbenoids [41]. They obtained yields up to 37 mg l^{-1} in 60 hours.

For the production of benzylisoquinoline alkaloids, for example, norreticuline [42], specialized OMTs originating from the plant alkaloid biosynthetic machinery were employed. Impressively, (*S*)-reticuline could be synthesized in *E. coli* or *S. cerevisiae* strains harboring the corresponding OMT activity [43,44]. Intriguing is also the possibility to create novel alkaloids by differential combination of genes derived from the rebeccamycin pathway of several actinomycete species [45]. Although *O*-methylation plays only a minor part in the biosynthesis of rebeccamycin, this combinatorial approach gives rise to a variety of structurally highly diverse analogs.

N- and S-methyltransferases

In contrast to the relaxed substrate specificity of many OMTs, most known methyltransferases that catalyze *N*-methylations (NMTs) exclusively act on specific substrates such as alkaloid precursors. The cloning of a number of these highly specialized NMTs provided the basis for the *in vitro*-production of alkaloids with pharmaceutical application, for example, of epinephrine (Figure 1b) or *N*-methyltetrahydroisoquinoline (Figure 3h). Epinephrine was produced from dopamine by Markoglou and Wainer [46], who applied phenolethanolamine-NMT co-immobilized with a β-hydroxylase in a bioreactor. For methylation of tetrahydroisoquinoline by coclaurine-NMT, whole-cell biotransformation has proven to be beneficial [47]. The same is true for the production of scopolamine, which can be obtained in high yield (Figure 2) from transgenic root cultures over-expressing the putrescine-NMT initiating alkaloid biosynthesis [48,49]. Application of NMTs beyond alkaloid chemistry includes enzymes specific for amino acids used for the modification of dimethylallyltryptophan [50] or even peptides [51].

S-methyltransferases (SMTs) — which are widely known for their role in the degradation of xenobiotic thiopurines in mammals [52] — have only scarcely been used in biotransformations. However, a SMT cloned from *Catharanthus roseus* might be an interesting candidate for application in biotechnology because it shows promiscuous activity toward aliphatic and aromatic thiols (Figure 3i) [53].

Enzymes catalyzing methylation and glycosylation of carbon atoms

Enzymes of the *C*-methyltransferase (CMT) and *C*-glycosyltransferase types are especially abundant in actinomycetes (e.g. in the genus *Streptomyces*) or fungi. As they are usually involved in the production of antibacterial or antifungal metabolites, they are potential targets for pathway engineering. Hence, heterologous expression or inactivation of genes coding for CMTs or *C*-glycosyltransferases in streptomycetes gave rise to new antibiotics with modified sugar moieties [54,55]. Similarly, metabolic engineering of plants by introduction of bacterial CMT genes from the tocopherol biosynthetic pathway led to an altered ratio of the three main tocopherol species [56].

Examples for applications of bacterial CMTs in chemoenzymatic syntheses include the methylation of phenolics such as tyrosine [57] and coumarin derivatives (Figure 3j) [58•]. A future perspective is the generation of new enzymes catalyzing carbon alkylation by rational protein design. As demonstrated by Bechthold and coworkers, *C*-glycosyltransferase activity can be engineered into an enzyme usually catalyzing *O*-glycosylation [59].

Conclusions

Alkylating enzymes, mostly transferases, some years ago were considered to be too specific and too difficult for biosynthetic applications. This has changed very much in recent years with availability of suitable enzymes and increased cofactor or co-substrate access. Also increasing knowledge of detailed reaction mechanisms [2,60,61] as a basis for rational protein redesign or focused directed evolution helps to overcome the current problems.

In the future, the discovery of new enzymes with broad substrate spectra but strict regiospecificity will certainly facilitate production of a broad range of valuable natural products. However, for industrial applications of prenyltransferase and methyltransferase in vitro, the limited availability of cofactors and co-substrates is still an important limitation. For cell-free applications, the development of strategies for the large-scale production of prenyl diphosphates and for the regeneration of the expensive cofactor SAM is mandatory.

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- •• of outstanding interest

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