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Biocatalytic methylation and demethylation via a shuttle catalysis concept involving corrinoid proteins

Judith E. Farnberger[1], Nina Richter[1], Katharina Hiebler[2], Sarah Bierbaumer[2], Mathias Pickl[2], Wolfgang Skibar[3], Ferdinand Zepeck[3] & Wolfgang Kroutil[2]

Synthetically established methods for methylation of phenols and demethylation of methyl phenyl ethers rely in general on hazardous reagents or/and harsh reaction conditions and are irreversible. Consequently, alternative regioselective methods for the reversible formation and breakage of C-O-ether bonds to be performed under mild and sustainable conditions are highly desired. Here we present a biocatalytic shuttle concept making use of corrinoid-dependent methyl transferases from anaerobic bacteria. The two-component enzymatic system consists of a corrinoid protein carrying the cofactor and acting as methyl group shuttle, and a methyltransferase catalyzing both methylation and demethylation in a reversible fashion. Various phenyl methyl ethers are successfully demethylated and serve in addition as sustainable methylating agents for the functionalization of various substituted catechols. Therefore, this methyl transfer approach represents a promising alternative to common chemical protocols and a valuable add-on for the toolbox of available biocatalysts.

[1] Austrian Centre of Industrial Biotechnolgy, ACIB GmbH, c/o University of Graz, Heinrichstrasse 28, 8010 Graz, Austria. [2] Institute of Chemistry, University of Graz, NAWI Graz, BioTechMed Graz, Heinrichstrasse 28, 8010 Graz, Austria. [3] Sandoz GmbH, Biocatalysis Lab, Biochemiestrasse 10, 6250 Kundl, Austria. These authors contributed equally: Judith E. Farnberger, Nina Richter. Correspondence and requests for materials should be addressed to W.K. (email: wolfgang.kroutil@uni-graz.at)

From a synthetic point of view formation as well as cleavage of alkyl aryl ethers are valuable transformations contributing to the structural diversity of pharmaceuticals and natural products[1–3]. Beyond that, *O*-methylated phenol derivatives are highly demanded building blocks for the manufacture of antioxidants, flavoring agents, fragrances, dyes, agrochemicals, and fine chemicals[4,5]. Although numerous chemical procedures[6] for methylation and demethylation are available (Fig. 1) and have been broadly applied, none of these protocols allows for reversible C-O-ether bond formation and breakage on synthetic scale. Conventionally, the addition of methyl moieties onto heteroatoms is accomplished with methyl iodide or dimethyl sulfide as methylating agent together with a strong base[7,8]. Besides toxicity and corrosiveness of the reagents needed in excess and the sizable amounts of inorganic salts formed as by-products, these traditional procedures are in general neither chemo- nor regioselective in case substrates possess more than one hydroxyl group or other reactive functional moieties (e.g., amine). Attempts to improve or even replace the classical Williamson ether synthesis led to the development of methods making use of less hazardous alkylating agents such as dimethyl carbonate[9,10] or methanol[11,12] which, however, still require high temperatures for reaction. Due to the high stability of *O*-methyl ethers against hydrolysis also their cleavage usually involves harsh reaction conditions and reagents such as strong Bronsted[13,14] or Lewis acids[15,16]. Alternatively, the use of metal catalysts[17,18] or nucleophilic methods employing for instance sodium thiolates[19,20] belong to the most commonly applied chemical techniques.

In the last decades, biocatalysis has gained increasing attention as promising research field at the interface of organic chemistry and biotechnology, complementing or even replacing conventional organic synthesis methods by more selective, mild and sustainable procedures[21–23]. Due to nature's manifold source of enzymes supported by the rapid development in molecular biology and gene technology, there is no limitation in accessing novel biocatalysts and thus, the repertoire of biocatalytic reactions is constantly increasing and broadening[24–26]. Since the transfer of methyl groups is an indispensable reaction in many aspects of life, methyl transferases (MTases) are ubiquitously distributed in nature[27,28] and according to the required cofactor two main enzyme classes have evolved (Fig. 2). The most prominent one relies on S-adenosyl-L-methionine[29,30] and typically catalyzes the methylation of *C*-, *O*-, *N*-, or *S*-nucleophiles through a S_N2 mechanism by simultaneously generating S-adenosyl-homocystein (SAH) as by-product. Alternatively, methylation can take place at non-nucleophilic carbons involving a radical-based mechanism[31,32]. Even if well-understood and providing exceptional access to a range of methylated compounds, SAM-dependent methyl transfer occurs in an irreversible fashion and thus, the cofactor is required in stoichiometric amounts hindering its feasibility in synthetic processes. In spite of the substantial progress that has been made in this research area[1,33–35] including the use of artificial SAM-analogs[36] and an in-vitro SAM-regeneration system[37], there is still need for a scalable alternative. Much less investigated is the class of corrinoid-dependent MTases[38,39]. Corrinoids share the structural motif of a corrin skeleton, a heterocyclic system composed of four pyrrole rings. The most prominent members of this group are cobalamins[40,41] (B_{12}-derivatives, Fig. 2b), in which the unique central cobalt-carbon bond is key to enable chemically challenging biotransformations[42]. Methyl cobalamin (Me-Cbl)-dependent MTases have been described to mediate the transfer of a methyl cation received upon heterolytic cleavage of the Co-C bond to nucleophilic substrates. The corrinoid cofactor cycles thereby between the Co^{III} and the highly reactive Co^{I} state[43] and thus functions as methyl group shuttle (Fig. 3a). Nature exploits this methyl transfer mainly for *O*-demethylation, playing an essential role in the amino acid metabolism of eukaryotes as well as in the energy generation of anaerobic organisms[39,44,45]. For instance, several acetogenic bacteria have been described to utilize methyl phenyl ethers as carbon and energy source, amongst others *Acetobacterium dehalogenans*[46], *Acetobacterium woodii*[47], *Moorella thermoacetica*[48], and *Desulfitobacterium hafniense*[49,50]. These organisms encode a complex *O*-demethylation machinery constituted of four enzymes (Fig. 3b), mediating a methyl group transfer from a substrate onto tetrahydrofolate (FH_4) via two half-reactions. In the first half reaction, MTase I binds the substrate and mediates both ether cleavage and transfer of the methyl group onto Co^{I} of the super-reduced cofactor bound to the corrinoid protein (CP). Then, a second MTase (MTase II) transfers the methyl group from Co^{III}-CP onto FH_4, being further metabolized by the organism. The fourth protein acts as an activating enzyme (AE) to catalyze the ATP-driven reduction of the oxidized cofactor in the inactive Co^{II} redox state to the catalytically active Co^{I} species. While most of the MTase systems described in the literature have primarily been studied with respect to their physiological role using crude cell extracts of wild-type organisms, just a few of them[51,52] have been recombinantly produced and characterized from a biochemical point of view. Even though they displayed promising demethylation properties, their feasibility as biocatalysts for synthetic purposes has not been evaluated yet.

In the present study, we report the use of a cobalamin-dependent methyltransferase system for a shuttle catalysis concept enabling both methylation and demethylation reactions in a reversible manner. To the best of our knowledge, such a system has not previously been investigated for synthetic biocatalytic applications and may represent an alternative to SAM-dependent enzymes as well as related chemical protocols.

Results

Concept of reversible methyl transfer. For the design of an alternative biocatalytic demethylation and methylation approach inspired by the corrinoid-dependent methyltransferase system from anaerobic bacteria, we envisioned a simpler concept than shown in Fig. 3b. It relies on the use of just one methyltransferase being the only biocatalyst together with a corrinoid protein (CP) carrying the cobalamin prosthetic group and acting as methyl group shuttle. In nature MTase I is described to transform more than one substrate, whereas MTase II is restricted to the methylation of tetrahydrofolate for the purpose of energy generation. Therefore, due to the reversibility of catalysis we propose to employ MTase I both for demethylation and methylation, coupling the cleavage of a methyl phenyl ether with the methylation of unnatural acceptor molecules in shuttle catalysis like fashion[53–55] (Fig. 4). Thus, MTase I catalyzes the demethylation of a methyl donor molecule transferring the methyl group onto the CP bound cobalamin; in the subsequent methylation step the methyl group is transferred from the cofactor to the methyl acceptor again mediated by MTase I to yield the methylated acceptor.

Employing a single enzyme (MTase I) for both half reactions offers considerable benefits as the number of required enzymes is reduced. Furthermore, the system can be employed for the demethylation as well as the methylation of a substrate. An undesired oxidation of Co^{I} to Co^{II} by molecular oxygen and thus an inactivation of the catalytic shuttle system is prevented by working in an inert atmosphere (glove-box).

Enzyme selection and production. The methyltransferase MTase I and the corrinoid protein CP from *Desulfitobacterium*

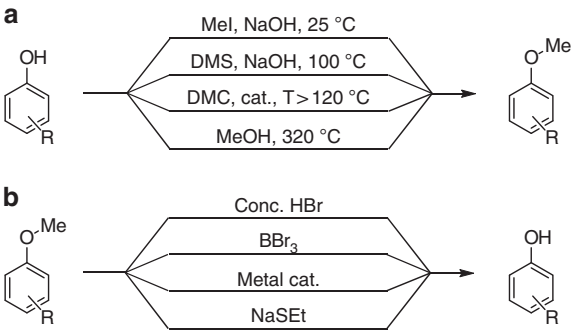

Fig. 1 Conventional procedures for *O*-methylation and -demethylation. **a** Toxic methyl iodide (MeI) or dimethylsulfide (DMS) are commonly used methylating agents under basic conditions. Alternatives are dimethylcarbonate (DCM) or methanol (MeOH), which however require an elevated temperature. **b** Strong Bronsted- or Lewis acids such as hydrobromic acid (HBr) or boron tribromide (BBr_3), respectively, as well as sodium ethanethiolate (NaSEt) are the traditional reagents for demethylation

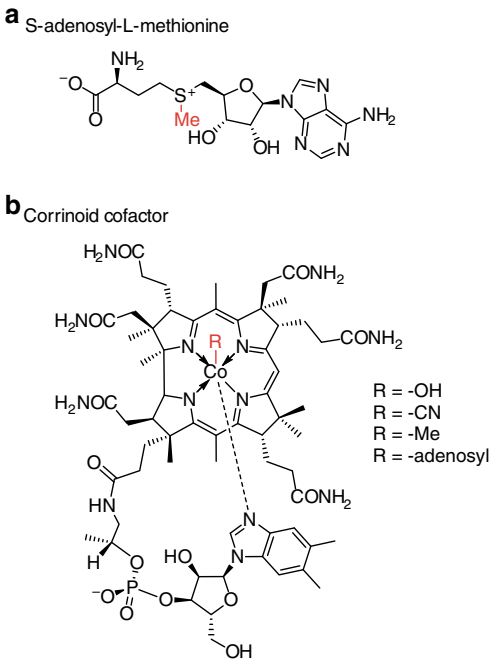

Fig. 2 Nature's cofactors for enzymatic (de)methylation reactions. **a** S-adenosyl-L-methionine (SAM) is nature's methylating agent and donates a methyl group while irreversibly forming S-adenosyl-homo-cystein (SAH) as by-product. **b** General structure of cobalamins, being the most relevant representatives of corrinoid cofactors: hydroxocobalamin (OH-Cbl, R=OH), vitamin B_{12} (CN-Cbl, R=CN), mehylcobalamin (Me-Cbl, R=Me), coenzyme B_{12} (Ado-Cbl, R=5′-deoxyadenosyl)

hafniense[52] *(D. hafniense)* were selected as model proteins for investigation of the suggested concept. The proteins were heterologously expressed in *E. coli* BL21 (DE3) pLysS using synthetic codon-optimized genes cloned into the pASK-IBA3plus vector. The use of this vector enabled the production of C-terminal *Strep*-tag®-fusion-proteins, which could be purified by affinity chromatography. Similarly, MTase II from *D. hafniense* was recombinantly produced in *E. coli* in order to verify the natural reaction in vitro. For a detailed description of cloning strategy and expression results, see Supplementary Fig. 1–3 and Supplementary Table 1. Since *E. coli* is lacking the ability to synthesize

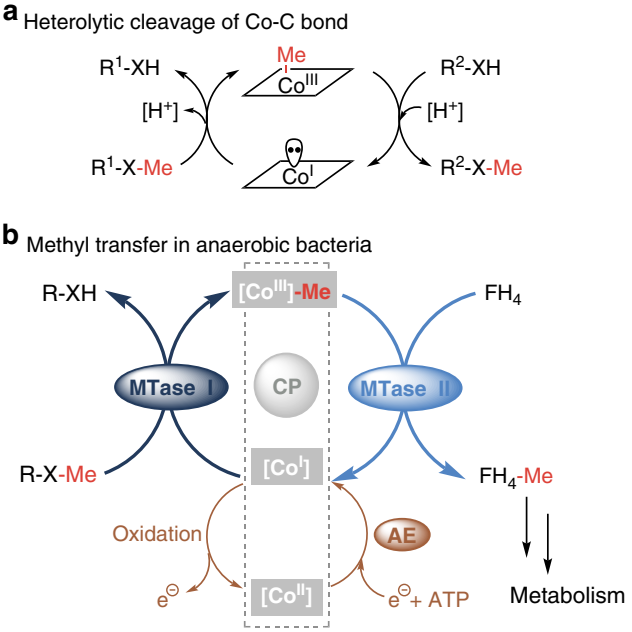

Fig. 3 Natural cobalamin-dependent methyl transfer. **a** Heterolytic cleavage of the central cobalt-carbon bond in methylcobalamin. **b** *O*-demethylation machinery in anaerobic organisms consisting of two MTases I and II, a corrinoid protein (CP) with bound cobalamin and an activating enzyme (AE). The methyl group is transferred from a substrate onto tetrahydrofolate (FH_4) via two half reactions with the cofactor switching between the super-reduced Co^{I} and the Co^{III} state and functioning as molecular shuttle

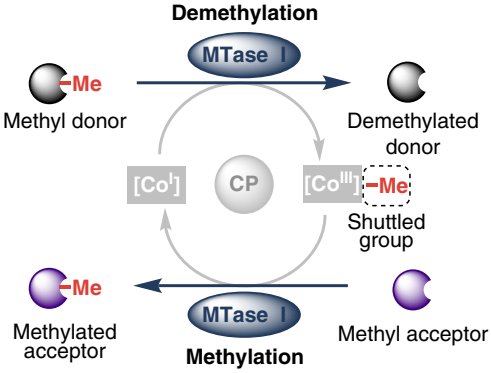

Fig. 4 Envisioned concept for biocatalytic methyl transfer via reversible shuttle catalysis. The cobalamin-dependent methyl transfer system consists of MTase I and CP catalyzing the methyl group shuttle between a donor and an acceptor and can be exploited for either demethylation or methylation of a substrate

any of the cobalamin derivatives[56], the corrinoid cofactor was incorporated into the *apo*-protein after expression in order to yield active *holo*-CP[51].

Characterization of the MTase system from *Desulfitobacterium hafniense*. Having the required enzymes in hand the MTase system from *D. hafniense* was tested for its applicability for biocatalytic methyl transfer. Based on the reported demethylation properties of MTase I[52], guaiacol **1a** was chosen as model methyl donor. Since it is obtainable from renewable resources[57,58] it represents an attractive substrate to demonstrate the concept and serves as a cheap and sustainable methylating agent. Due to the oxygen and light-sensitive cobalt cofactor all biotransformations

were performed under inert atmosphere and light exposure was kept to a minimum. Initially, titanium(III) citrate and methyl viologen were used to reduce inadvertently formed Co^{II} to the active Co^{I} species. However, in an oxygen-free environment the use of this O_2-scavenger system became obsolete and was omitted if not indicated otherwise.

As a first step the natural reaction involving both MTases (I and II) and CP was successfully reconstituted in vitro by transferring the methyl group from **1a** onto the natural substrate tetrahydrofolic acid (see Supplementary Fig. 4 and Supplementary Table 2). Then, MTase I was investigated to act as the only biocatalyst mediating both demethylation and methylation in a reversible fashion. For this purpose 3,4-dihydroxybenzoic acid **2b** was chosen as non-natural model substrate for accepting the methyl group (acceptor). Detection of the demethylated product catechol **2a** as well as the mono-methylated carboxylic acid **1b** (regioisomeric mixture *meta*/*para* = 2/1, see Supplementary Fig. 5) proofed the feasibility of employing MTase I as the only biocatalyst for both methylation and demethylation. Initial conversions of 2–3% were improved by optimizing various parameters such as temperature, enzyme and substrate concentration (see Supplementary Fig. 6–9). An excess of the acceptor molecule instead of equimolar amounts pushed the demethylation of the model substrate **1a** to conversions of up to 80%. Employing 3,4-dihydroxybenzaldehyde **2c** as alternative model acceptor gave identical results, yielding vanillin and isovanillin **1c** in a ratio of 3/1. The significant increase of conversion obtained by using an excess of one substrate indicated an equilibrium between demethylation and methylation caused by the reversible nature of the reaction (Fig. 5a). It can be assumed that the via demethylation of guaiacol **1a** obtained product (catechol **2a**) functions subsequently as methyl acceptor for a methylation in the reverse direction consuming the methyl group from the methylated product (vanillin/isovanillin **1c**). Following mentioned transformations in both directions over time using equimolar amounts of substrates (Fig. 5b) proved this hypothesis. After having a fast methyl transfer at the beginning of the reaction, it rapidly reached an equilibrium after 4–6 h, ending up with a comparable ratio of substrates and products in any case. In contrast, employing a 5-fold molar excess of methyl acceptor **2c** significantly pushed the demethylation of **1a** by diminishing the reverse reaction at the same time (Fig. 5c). Hence, in order to exploit cobalamin-dependent methyl transfer either for demethylation or methylation of a substrate in an efficient way, pushing the reaction equilibrium toward either side is crucial.

Substrate examples for demethylation and methylation. To demonstrate the feasibility of the concept we focused at first on demethylation. Compounds turning out to be easily demethylated might then serve as suitable methyl donors for the methylation reaction in a second step. For initial experiments with MTase I from *D. hafniense* catechol **2a** and derivatives **2b**-**2c** were

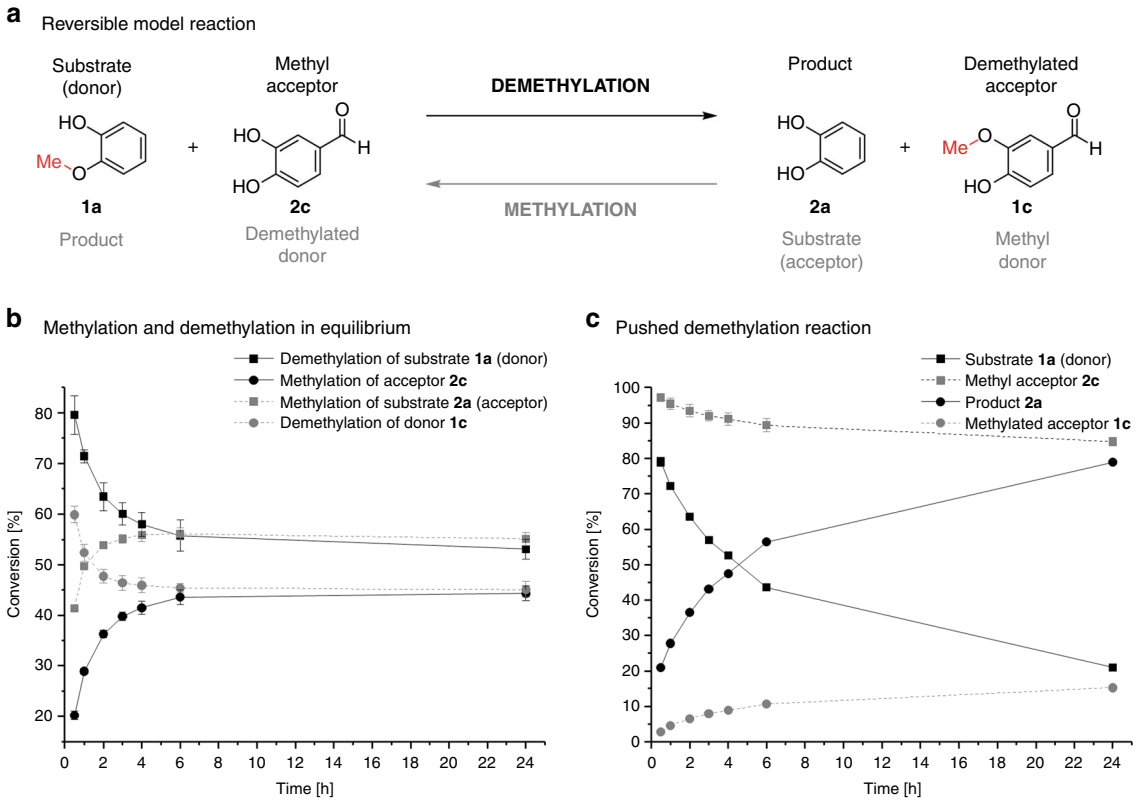

Fig. 5 Reversibility of corrinoid-dependent methyl transfer catalyzed by MTase I and CP. **a** General reaction scheme of the reversible model reaction, in which product and demethylated acceptor obtained by demethylation (black) serve again as substrate and methyl donor for a reverse methylation (gray). **b** Time course of forward and reverse reaction using equimolar amounts of substrates. **c** Time course of demethylation using an excess amount of acceptor. Conversions at indicated time points determined by HPLC-UV from respective areas using calibration curves. Error bars represent the standard error of the mean (s.e.m., $n = 3$). Reaction conditions in panel (**b**): substrate **1a** (10 mM) and methyl acceptor **2c** (10 mM) in case of demethylation; substrate **2a** (10 mM) and methyl donor *m*-**1c** (10 mM) in case of methylation. MTase I (freeze-dried pure enzyme, 13 mg mL^{-1}), CP (400 µL mL^{-1} reconstituted solution, 22 mg mL^{-1} pure CP) in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm for 24 h. Reaction conditions in panel (**c**): substrate **1a** (10 mM) and methyl acceptor **2c** (50 mM), MTase I (freeze-dried cell-free extract CFE, 40 mg mL^{-1}), CP (reconstituted solution, 400 µL mL^{-1}) in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm for 24 h

employed in excess as methyl acceptors. In addition to the model substrate **1a** various other aromatic compounds (Fig. 6) fitting the catechol pattern of published natural substrates[52] as well as a series of aromatic and aliphatic compounds with alternative substrate pattern such as veratrol derivatives as well as 2-(methylamino)- and 2-(methylthio)phenol were tested for demethylation (Supplementary Table 3). Aromatic substrates with a hydroxy moiety in *ortho*-position to the methoxy were efficiently demethylated. Furthermore, a number of anisol derivatives (Fig. 6, compounds **1a-h**, **2h**) bearing various substituents such as an amine-, hydroxymethyl-, aldehyde-, or acid-group in *ortho*-position were converted as well. Remarkably, *o*-anidisine **1d** was the only substrate which was demethylated with higher conversions when the acceptor was present in equimolar amounts instead of excess (Supplementary Table 4). This indicated a reaction equilibrium lying on the demethylation reaction, most likely because the formed product 2-aminophenol **2d** turned out to be not a suitable substrate for methylation. Generally, the choice of the proper acceptor is an important parameter to reach high conversion; interestingly, the most suitable acceptor seemed to vary depending on the substrate. Vanillin and vanillic acid as well their regioisomers (compounds **1b-c**) were demethylated with moderate conversions ranging from 25–45%. Substrates such as guaiacol **1a**, 3-methoxycatechol 3-**2h** and 2-methoxyresorcinol 2–**2h** were demethylated with up to 80% conversion depending on the used co-substrate. Even higher conversions than for **1a** were achieved for 2,3-dimethoxyphenol 2,3-**1h** (up to 93%) and 2,6-**1h** (up to 97%). Featuring two methoxy moieties, 2,3-**1h** and 2,6-**1h** represent particularly useful methyl sources for a possible methylation reaction.

Transfer of one methyl moiety leads to the formation of 3-methoxycatechol 3-**2h**, which is further converted to pyrogallol **3h** upon twofold demethylation. Therefore, they were employed together with **1a** as methyl-donating co-substrates for exploration of corrinoid-dependent methylation in the next step (Fig. 7).

Besides catechol **2a**, 3,4-dihydroxybenzoic acid **2b** and related aldehyde **2c**, also 3,4-dihydroxybenzyl alcohol **2i** and caffeic acid **2j** were successfully methylated. Although a number of aliphatic, cyclic and aromatic compounds including also *S*-,*N*-, and *C*-nucleophiles (see supplementary Table 5) turned out to be non-substrates, numerous enzyme engineering techniques[59] that have emerged in recent years might tune the MTase I from *D. hafniense* to convert an extended range of different substrates with high activity and selectivity. When using the mono-methyl donor **1a** as co-substrate, it had to be employed in excess to achieve an efficient reaction. For instance, the amount of methylated product doubled in case of **2b**, **2i**, and **2j** by using a 5-fold excess of donor, reaching up to 84% conversion. Interestingly, when bis-methyl donors capable of donating two methyl groups such as dimethoxyphenols 2,3-**1h** and 2,6-**1h** were used as methylating reagent, conversions up to 75% were already achieved with equimolar amounts of donor, indicating their

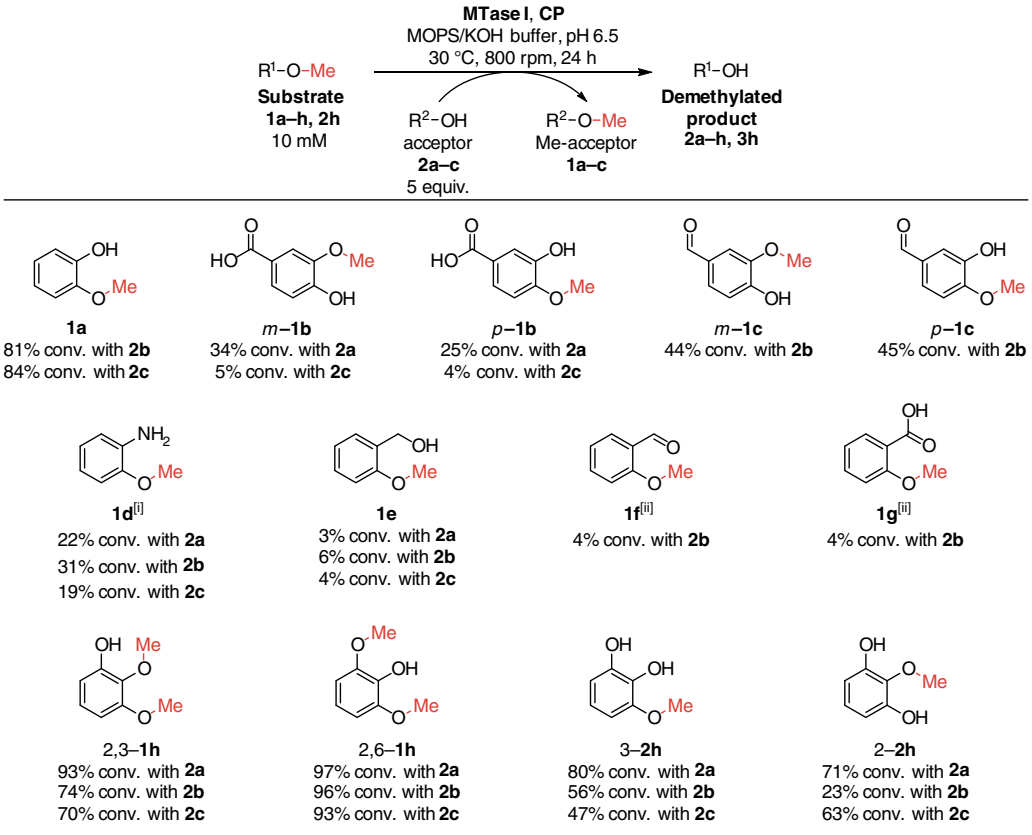

Fig. 6 Biocatalytic demethylation of various methyl phenyl ethers employing cobalamin-dependent MTase I from *D. hafniense*. Reaction conditions: substrate (10 mM), methyl acceptor **2a-c** (50 mM, if not indicated otherwise), MTase I (freeze-dried CFE, 40 mg mL^{-1}), CP (400 µL mL^{-1} reconstituted solution) in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm for 24 h. Conversions determined by HPLC-UV from areas for substrate and demethylated product, if not indicated otherwise, using calibration curves. Since the reaction has no by-products, thus the chemoselectivity was >99%, the conversion corresponds to the amount of product formed. Deviation values (s.e.m., $n = 3$) lie between 0.1 and 3.3 % for the substrates tested. [i] Reaction conditions as described but using methyl acceptor **2a-c** at 10 mM concentration. [ii] Conversion determined by HPLC-UV from areas for the methylated acceptor using calibration curves and related to the limiting substrate concentration. Deviation values (s.e.m., $n = 3$) lie between 0.2 and 0.4% for the substrates tested

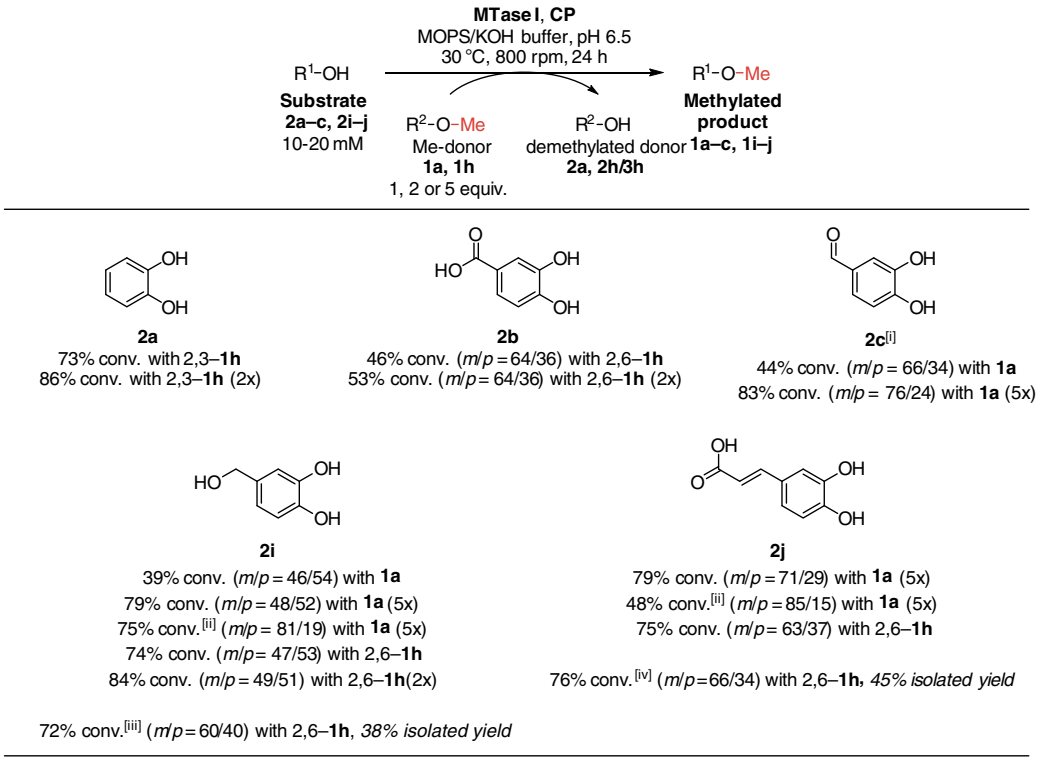

Fig. 7 Biocatalytic methylation of substituted catechols employing cobalamin-dependent MTase I from *D. hafniense*. Reaction conditions: substrate (10 mM) and methyl donor **1a**/2,3-**1h**/2,6-1**h** (2× = 2 equivalents, 5× = 5 equivalents), MTase I (freeze-dried CFE, 40 mg mL^{-1}), CP (reconstituted solution, 400 µL mL^{-1}) in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm for 24 h. Conversions determined by HPLC-UV from areas for substrate and methylated product using calibration curves (mono-methylation; *m* = *meta*, *p* = *para* refers to the position of the introduced methyl group in relation to the substituent at the catechol chore). Since the reaction has no by-products, thus the chemoselectivity was >99%, the conversion corresponds to the amount of product formed. Deviation values (s.e.m., $n = 3$) lie between 0.1 and 2.7% for the substrates tested. [i] Reaction conditions as described but with MTase I (freeze-dried pure enzyme, 13 mg mL^{-1}) and CP (400 µL mL^{-1} reconstituted solution, 22 mg mL^{-1} pure CP) in pure form. [ii] Addition of 10% (v/v) EtOH. [iii] Preparative scale: substrate (50 mM), methyl donor 2,6-**1h** (50 mM). [iv] Preparative scale: substrate (20 mM), methyl donor 2,6-**1h** (20 mM)

potential as efficient donors. It seemed that the availability of two transferable methyl groups in a single molecule had a similar effect on the reaction as using excess of donor. It is worth mentioning, that exclusive mono-methylation was observed for all substrates investigated. For those substrates where the formation of regioisomers was possible, product mixtures of *meta*- and *para*-methylated isomers were identified. Generally, *meta*-preference was observed for substrates **2b**, **2c**, and **2j** with a regioisomeric excess of approximately = 65/35 or higher. Therefore, the corrinoid-dependent MTase I from *D. hafniense* displayed a similar regioselectivity than observed for various SAM-dependent catechol *O*-MTases (COMT)[1,60,61]. One exception was 3,4-dihydroxybenzyl alcohol **2i** for which almost an 1/1 mixture of isomers with marginal *p*-preference was obtained. Since related compound **2c** on the contrary was methylated with reasonable regioselectivity (*m*/*p* up to 76/24) it can be assumed that the side chain at the aromatic catechol chore has an effect on substrate binding within the enzyme and thus on discrimination of regioisomeric groups[1,44]. However, the regioselectivity of MTase I could be increased in favor of the *m*-methylated product by medium engineering. For instance, in the presence of an organic co-solvent such as ethanol (EtOH, 10% v/v) the ratio of ferulic/isoferulic acid obtained from substrate **2j** was significantly increased from 71/29 to 85/15. An even more pronounced effect of EtOH addition on the regioselectivity was observed in case of substrate **2i**. Here, the minimal preference for the *p*-methylated product (*m*/*p* = 48/52) turned into a surplus formation of the *meta*-isomer (*m*/*p* = 81/19) without any loss of activity. Finally, the corrinoid-dependent methylation was performed in semi-preparative reactions (24 mL). Both 3,4-dihydroxybenzyl alcohol **2i** and caffeic acid **2j** were methylated employing equimolar amounts of 2,6-**2h** giving the methylated products with 72% conversion (38% isol. yield) and 76% conv. (45% isol. yield), respectively.

Discussion

This study demonstrates the feasibility of a methyl transfer concept based on corrinoid-mediated shuttle catalysis. Being sustainable, safe, scalable and not restricted by cofactor recycling issues, this protocol may lay the base for an alternative method for methylation as well as demethylation. With the substrate scope identified, the applicability and possible future impact has already been demonstrated. While the concept might enable (i) tailoring of natural products, (ii) valorization of waste materials or biomass (e.g. lignin), and (iii) deprotection by demethylation on the one hand, methylation on the other hand will allow the preparation of value-added products. The substrate scope can be expanded by common rational engineering techniques and furthermore, other MTase systems with different substrate specificities can be chosen from nature's vast supply. Therefore, corrinoid-dependent MTases may represent a valuable and promising add-on to the enzymatic toolbox applicable for organic synthesis.

Methods

General information. For DNA- and amino acid sequences of the enzymes used see Supplementary Note 1. Biocatalysts were recombinantly expressed in *E. coli* and

used as lyophilized cell-free extract (CFE) or purified preparation. Since *E. coli* is not able to synthesize cobalamins[56], the corrinoid protein (CP) was reconstituted with Me-Cbl prior to use in order to yield active *holo*-CP. General material and detailed experimental procedures related to protein expression and cofactor reconstitution are given in Supplementary Methods. Biocatalytic reactions were performed at least in triplicates in degassed buffers under inert atmosphere, using a Labstar MB10-compact Glove-box (MBraun, Germany) equipped with a special O_2-sensor (MB-OX-EC) to be used with aqueous systems.

Determination of enzyme activity. See Supplementary Methods and Supplementary Fig. 10–12.

Representative biotransformation procedure. Analytical biotransformation reactions were carried out on 120 µL scale as follows: MTase I (10–20 mU, 40 mg mL^{-1} CFE or 13 mg mL^{-1} pure enzyme, respectively) was dissolved in *holo*-CP solution (400 µL mL^{-1}, containing 67 mg mL^{-1} CFE or 22 mg mL^{-1} pure enzyme, respectively). Then appropriate amounts of substrate (10–50 mM final concentration) and co-substrate (10–50 mM final concentration) dissolved in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl) were added and the reaction samples were shaken at 30 °C and 800 rpm. See Supplementary Note 2 for details with respect to the use of an O_2-scavenger system during initial experiments. After 24 h an aliquot of the reaction was withdrawn and diluted (1:10) with MeCN (6 parts) and deionized water (3 parts). Precipitated protein was removed by centrifugation (14,000 rpm, 15 min) and the clear supernatant was filtered and directly analyzed by reversed-phase HLPC.

Analytical methods. See Supplementary Methods, Supplementary Table 6–7, and Supplementary Fig. 13. HPLC-spectra are also available (Supplementary Fig. 14–16).

Preparative methylation reactions. Semi-preparative reactions were carried out on a 24 mL scale in accordance to the procedure described above using equimolar amounts of substrate and methyl donor. The reaction mixture was shaken at 30 °C and 120 rpm for 24 h. Work-up, isolation and purification were performed as follows.

Methylation of 3,4-dihydroxybenzyl alcohol. Substrate **2i** (1.2 mmol, 168 mg) was methylated according to the described procedure using 2,6-**1h** (1.2 mmol, 185 mg) as methyl donor. The reaction mixture was quenched by adding MeCN (24 mL) and the precipitated catalyst was removed by centrifugation (4000 rpm, 30 min). Then the supernatant was extracted with EtOAc (2× 25 mL), combined organic phases were dried over Na_2SO_4 and the solvent evaporated under reduced pressure. Purification by column chromatography on silica (eluent: EtOAc/Hexanes = 1/1) afforded the product mixture (vanillyl/isovanillyl alcohol = 60/40) as colorless solid in 38% yield (70 mg, 0.45 mmol). R_f (EtOAc/Hexanes = 1/1) = 0.26.

Vanillyl alcohol *m*-**2i**. ^{1}H-NMR (300 MHz, d_4-MeOH): δ 6.94 (s, 1H), 6.76 (s, 2H), 4.50 (s, 2H), 3.85 (s, 3H); ^{13}C-NMR (75 MHz, d_4-MeOH): δ 148.9. 146.9, 134.2, 121.1, 116.0, 112.1, 65.3, 56.3. NMR spectra are in agreement with literature[62].

Isovanillyl alcohol *p*-**2i**. ^{1}H-NMR (300 MHz, d_4-MeOH): δ 6.88 (s, 1H), 6.86 (s, 1H), 6.83 (s, 1H), 4.46 (s, 2H), 3.83 (s, 3H); ^{13}C-NMR (75 MHz, d_4-MeOH): δ 148.4, 147.5, 135.6, 119.6, 115.4, 112.5, 65.1, 56.4. NMR spectra are in agreement with literature[63].

Methylation of caffeic acid. Substrate **2j** (0.48 mmol, 87 mg) was methylated according to the described procedure using 2,6-**1h** (0.48 mmol, 74 mg) as methyl donor. The reaction mixture was basified with aqueous NaOH (10% w/v) to pH 14 and extracted with EtOAc (2 × 25 mL). After acidifying the aqueous phase with HCl (6N) to pH 1 the mixture was extracted with EtOAc (2 × 25 mL), combined organic phases were dried over Na_2SO_4 and the solvent evaporated. Column chromatography on silica (eluent: EtOAc/Hexanes = 1/3, AcOH 1% v/v) afforded the product mixture (ferulic/isoferulic acid = 60/40) as brownish solid in 45% yield (42 mg, 0.23 mmol). R_f (EtOAc/Hexanes = 1/1, AcOH 1% v/v) = 0.71.

Ferulic acid *m*-**2j**. ^{1}H-NMR (300 MHz, d_4-MeOH): δ_H [ppm] = 7.60 (d, J = 15.9 Hz, 1H), 7.18 (d, J = 1.9 Hz, 1H), 7.06 (dd, J = 8.2, 1.9 Hz, 1H), 6.81 (d, J = 8.2 Hz, 1H), 6.31 (d, J = 15.9 Hz, 1H), 3.89 (s, 3H); ^{13}C-NMR (75 MHz, d_4-MeOH): δ_C [ppm] = 171.0, 150.5, 149.4, 146.9, 127.8, 124.0, 116.4, 115.9, 111.6, 56.4. NMR spectra are in agreement with literature[64].

Isoferulic acid *p*-**2j**. ^{1}H-NMR (300 MHz, d_4-MeOH): δ_H [ppm] = 7.54 (d, J = 15.9 Hz, 1H), 7.05 (d, J = 2.1 Hz, 1H), 6.94 (dd, J = 8.2, 2.1 Hz, 1H), 6.81 (d, J = 8.2 Hz, 1H), 6.27 (d, J = 15.9 Hz, 1H), 3.88 (s, 3H); ^{13}C-NMR (75 MHz, d_4-MeOH): δ_C [ppm] = 170.8, 151.4, 148.0, 146.6, 128.9, 122.7, 116.4, 114.7, 112.5, 56.3. NMR spectra are in agreement with literature[65].

Characterization of products. See Supplementary Fig. 17–20 for NMR-spectra.

Data availability

The data generated and/or analyzed during this study are available within the article and the supplementary information file or obtainable from the corresponding author upon reasonable request.

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Author contributions

W.K. conceived the project idea; J.E.F., N.R., F.Z. and W.K. designed the study; J.E.F., N.R., K.H., S.B. and M.P. performed the experiments; N.R. and J.E.F developed methods, J.E.F. and N.R. analyzed and interpreted the data; J.E.F. and W.K. wrote the manuscript. Funding acquisition: W.K. F.Z. and W.S.; supervision: W. K. All the authors discussed the results and approved the final manuscript.

Additional information

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