Advanced Synthesis & Catalysis

Accepted Article

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To be cited as: *Adv. Synth. Catal.* 10.1002/adsc.201801590

Link to VoR: http://dx.doi.org/10.1002/adsc.201801590

10.1002/adsc.201801590

UPDATE

DOI: 10.1002/adsc.201((will be filled in by the editorial staff))

Biocatalytic Methyl Ether Cleavage: Characterization of the Corrinoid-Dependent Methyl Transfer System from *Desulfitobacterium hafniense*

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Received: ((will be filled in by the editorial staff))

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201######.((Please delete if not appropriate))

Abstract. The ether functionality represents a very common motif in organic chemistry and especially the methyl ether is commonly found in natural products. Its formation and cleavage can be achieved via countless chemical procedures. Nevertheless, since in particular the cleavage often involves harsh reaction conditions, milder alternatives are highly demanded. Very recently, we have reported on a biocatalytic shuttle catalysis concept for reversible cleavage and formation of phenolic *O*-methyl ethers employing a corrinoid-dependent methyl transferase system from the anaerobic organism *Desulfitobacterium hafniense*. Here we report the technical study of this system, focusing on the demethylation of guaiacol as model reaction. The optimal buffer-, pH-, temperature- and cofactor-preferences were determined as well as the influence of organic co-solvents. Beside methyl cobalamin also hydroxocobalamin turned out to be a suitable cofactor species, although the latter required activation. Various *O*-methyl phenyl ethers were successfully demethylated with conversions up to 82% at 10 mM substrate concentration.

Keywords: Biocatalysis, Biotransformations, Ether Cleavage, Demethylation, Methyl transferases, Corrinoids

Introduction

Ether cleavage[1] is a valuable integral group transformation in synthetic organic chemistry, frequently used as a deprotection step to unmask hydroxyl moieties.[2-3] Since *O*-methyl groups are rather stable and thus more resistant to hydrolysis/cleavage, classical procedures for the cleavage of C-O-bonds of methyl ethers usually involve the presence of strong Bronsted[4-7] or Lewis acids.[8-10] Alternatively, the use of metal catalysts[11-12] or nucleophilic methods employing sodium thiolates[13-15] belong to the most commonly applied chemical techniques. Ether cleavage is also prevalent in nature, where *O*-demethylation is part of many aspects of life.[16-17] Particularly, oxidative enzymes[18-21] such as fungal peroxygenases[22-23] and bacterial monooxygenases[24-25] including P450s[26-27] are known to catalyze *O*-dealkylation reactions. They are primarily involved in the biodegradation of lignocellulosic compounds, detoxification of organic chemicals and alkaloid biosynthesis. Apart from that, specific *O*-demethylases have been described to occur in various anaerobic organisms[28-30] utilizing methyl aryl ethers as carbon and energy source. Considering this vast number of biocatalysts capable of performing ether cleavage, it is astonishing that with exception of a few examples[31-33] hardly any of them have been applied for synthetic purposes, yet. This is in contrast to SAM-dependent methyltransferases which are broadly investigated, however exclusively for alkylation.[34-36] An alternative group of enzymes which may be considered for ether cleavage are corrinoid-dependent methyltransferases (MTases)[37-38] which have recently been used for both *O*-methylation as well as demethylation.[39] The reaction involves a two-component methyl transfer system consisting of a corrinoid protein (CP) carrying the cobalamin cofactor[40-42] and a MTase from *Desulfitobacterium hafniense* (*D. hafniense*).[43] This system allows performing both demethylation of a donor molecule and subsequent methylation of an acceptor in a reversible fashion. The CP with the cobalt containing cofactor acts as shuttle for the methyl group with cobalt cycling between the nucleophilic Co^{I} and the Co^{III} state. By using an excess of the methyl acceptor as co-substrate, demethylation of phenyl ethers can be performed. Here, we present now the characterization of the recently published MTase system from *D. hafniense*,[39] reporting on the influence of various parameters on the reaction and

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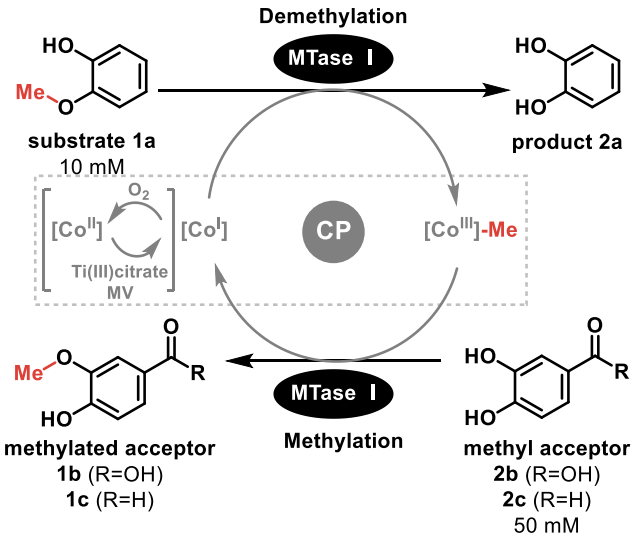
its unique properties focusing here especially on demethylation.

Results and Discussion

The transfer of a methyl group from the model substrate guaiacol **1a** (Scheme 1) onto a non-natural methyl acceptor such as 3,4-dihydroxybenzoic acid **2b** has been successfully proven in a reversible shuttle catalysis concept[39] using a corrinoid-dependent MTase system from the anaerobic bacterium *D. hafniense*. Alternatively, aldehyde **2c** was used as methyl accepting co-substrate in 5-fold molar excess in order to push the reaction equilibrium towards the product side. Since the used corrinoid cofactor is known to be highly oxygen-sensitive, biotransformations were performed under inert atmosphere. An inadvertently formed Co^{II} was reduced back to the active Co^{I} species using a chemical reactivation system composed of titanium^{III} citrate and methyl viologen (MV).

As a first step the catalytic system was characterized with regard to preferred buffer and pH conditions, enzyme amount as well as its optimal temperature range (Figure 1 and Figure S1). While the enzymes showed to have their pH optimum at neutral pH, high conversions (up to 75%) were still achieved under slight basic conditions (pH 8) when using MOPS-KOH buffer.



Scheme 1. Model reaction for characterization of biocatalytic corrinoid-dependent methyl transfer. MTase I and CP from *D. hafniense* catalyze the demethylation of guaiacol by simultaneously transferring the methyl group onto the co-substrate (3,4-dihydroxy-benzoic acid or related aldehyde) acting as methyl acceptor. While MTase I catalyzes both methyl transfers, CP resembles a molecular shuttle for the methyl group, switching between the super-reduced Co^{I} and the Co^{III} state. In order to reactivate an inadvertently formed Co^{II} species a chemical activation system based on titanium^{III} citrate and methyl viologen (MV) was used. Both demethylation and methylation are reversible reactions but for the sake of clarity for this study the arrows just point in one direction.

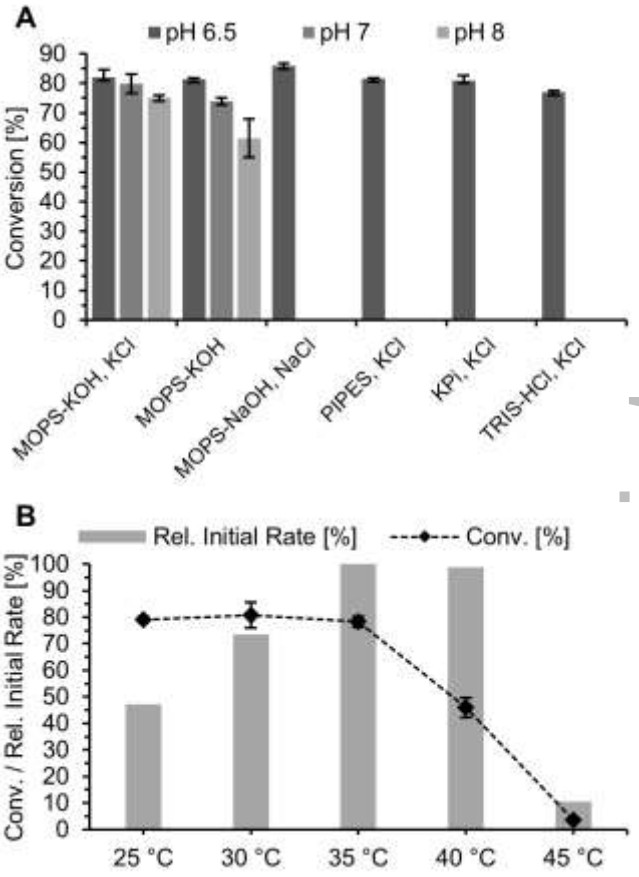


Figure 1. Characterization studies addressing reaction conditions. (**A**) Effect of varied buffers, salts and pH values on the conversion of guaiacol. (**B**) Temperature profile of the reaction giving relative initial rates (bars) and conversions after 24 h (-♦-). Reaction conditions if not indicated otherwise in respective studies: substrate **1a** (10 mM, 1.2 mg/mL), methyl acceptor **2b** (50 mM, 7.7 mg/mL), MTase I (40 mg/mL lyophilized cell-free crude extract CFE), CP (400 µL/mL reconstituted holo-CP solution), activation system (4.19 mM Ti^{III} citrate and 0.3 mM methyl viologen) in MOPS buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm in in Eppendorf Orbital Shaker (1.5 mL) for 24 h. Conversions were determined after 24 h by HPLC-UV from areas of **1a** and **2a** using calibration curves. Reaction rates were determined from the linear slopes within 0-2 hours of the reaction time courses and calculated relatively to the highest one obtained in each case.

With respect to the temperature optimum, increased temperatures of up to 40 °C significantly enhanced the initial reaction rate, however, enzyme inactivation over time led to reduced overall conversion at 40 °C and to a loss of activity at 45 °C. The methyl transfer

reaction is depending on the MTase catalyzing the demethylation itself as well as on the CP carrying the prosthetic group and shuttling the methyl group between substrate and co-substrate. Since CP is produced by *E. coli* as *apo*-protein devoid of its cofactor, because *E. coli* does not produce cobalamin, a reconstitution with Me-Cbl is required after expression to obtain active *holo*-CP. Various reconstitution protocols were examined (see Figure S2), showing that reconstitution occurs rather fast (no extended incubation time needed) probably because the cobalamin-binding domain is located on the surface of CP.[44-45] In addition to methyl cobalamin (Me-Cbl), other cobalamin derivatives with varied β-axial ligands at the central cobalt atom were examined (Figure 2). Since all of them bear cobalt in oxidation state three, the chemical reducing agent Ti^{III} is required together with MV for activation in order to get cobalt in the demanded oxidation state (I). While cyano- (CN-Cbl) and 5´-deoxyadenosyl cobalamin (Ado-Cbl) led to a significantly reduced demethylation activity, hydroxocobalamin (OH-Cbl) was equally well accepted as Me-Cbl with a comparable conversion of 80% and a relative initial reaction rate of 93% (Figure 2). While Me-Cbl can directly enter the catalytic cycle as methyl donor to yield the reduced species during the first methyl group transfer onto the acceptor molecule, a two-electron-reduction of the Co^{III} has to precede in case of OH-Cbl before it can act as nucleophile to pick up the first methyl group. In-vitro activation of the CP-bound cofactor is promoted by Ti^{III} citrate in combination with the redox mediator MV, which actuates also as O_2-scavenger system and reduces inadvertently oxidized cofactor.[46] Suitable concentrations of both compounds have been determined for Me-Cbl-mediated reactions (Table S1) and subsequently the requirements of Ti^{III}/MV were compared with the OH-Cbl-mediated demethylation of guaiacol (Figure 3). The experiments employing Me-Cbl clearly show that Ti^{III}/MV is not required in this case as long as the transformation can be performed under strictly oxygen free conditions (Figure 3, entry 1-2). For OH-Cbl mediated reactions, almost no activity was observed in the absence of Ti^{III} (entry 5-6), thus Ti^{III} turned out to be the crucial reagent needed for CP activation. MV seemed to have a supporting function since in presence of both compounds (entry 3) the reaction rate almost reached the same level as observed with Me-Cbl-CP (entry 1). Interestingly, despite the significant differing initial rates observed depending on the presence and absence of Ti^{III} and MV, comparable conversions in the range of 74-84 % were obtained in all cases when using a crude enzyme preparation. In contrast, when performing the OH-Cbl mediated reaction with purified enzyme (entry 8-9), activity was only observed in the presence of the chemical activation system reaching a comparable initial rate as obtained with Me-Cbl (entry 7). Therefore, it can be assumed that there are alternative pathways for OH-Cbl activation in crude enzyme preparations originating from *E. coli* (cell free extract). Since the reaction employing Me-Cbl worked well under inert atmosphere even in the absence of Ti^{III}/MV, we investigated whether each step of the reaction had to be handled under strictly anaerobic conditions. Thus, the sensitivity of the reaction mixture towards oxygen/air was examined as well as the use of Ti^{III}/MV for reactivation of reactions that had been exposed to air. For this purpose, a series of identical biotransformation reactions were prepared under inert conditions and then treated differently with respect to incubation, oxygen input and reactivation (Figure 4). While more than 30% of substrate has been converted after 2 hours under inert conditions (entry 1), conversion tremendously decreased (2%) when the reaction mixture was oxygenated right after its preparation (entry 2), most likely due to oxidation of the catalytic Co^{I} species. Nevertheless, it can be assumed that the enzymatic system was not completely inactivated since some demethylation product (16%) was still observed after 24 h despite of occurred oxygen-input and incubation under aerobic conditions. Interestingly, reactions which were incubated on air but only ventilated after 2 h (entry 3-6) gave comparable conversions (27-29%) as the oxygen-free counterpart, indicating that almost no oxygen entered the reaction tubes without actively opening and mixing them. The effects of O_2-input turned out to be severe though, since it entirely stopped the demethylation reaction, no matter if incubation afterwards was performed on air (entry 3) or in the glove-box (entry 4). However, a repeated addition of O_2-scavenger system (entry 5-6) showed to actuate the reaction again and increased conversions up to 41-42%. These results demonstrate the option to reactivate oxidized cofactor by chemical reduction, even though the highest efficacy of the enzymatic system could not be regained.

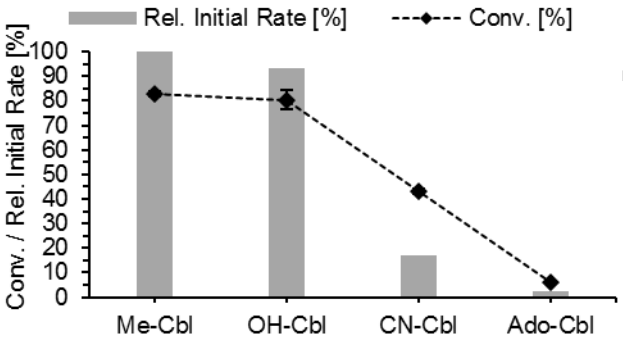


Figure 2. Examination of the cofactor preference of CP. Reconstitution: Incubation of *apo*-CP (100 mg/mL lyophile. crude extract) in 1 mL TRIS-HCl buffer (50 mM, pH 7, 0.5 mM DTT and 0.1 mM PMSF) with 2 mM of the respective cobalamin species and 3 M betaine, followed by a desalting step into MOPS buffer. Reaction conditions: **1a** (10 mM, 1.2 mg/mL), **2b** (50 mM, 7.7 mg/mL), MTase I and CP (40 mg/mL lyophilized crude extract of MTase I and 400 µL/mL reconstituted holo-CP solution), activation system (4.19 mM Ti^{III} citrate and 0.3 mM methyl viologen) in MOPS buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C,

800 rpm in in Eppendorf Orbital Shaker (1.5 mL) for 24 h. Conversions were determined after 24 h by HPLC-UV from areas of **1a** and **2a** using calibration curves. Error bars represent the standard error of the mean (s.e.m., $n = 3$). Reaction rates were determined from the linear slopes within 0-2 hours of the reaction time courses and calculated relatively to the highest one obtained in each case.

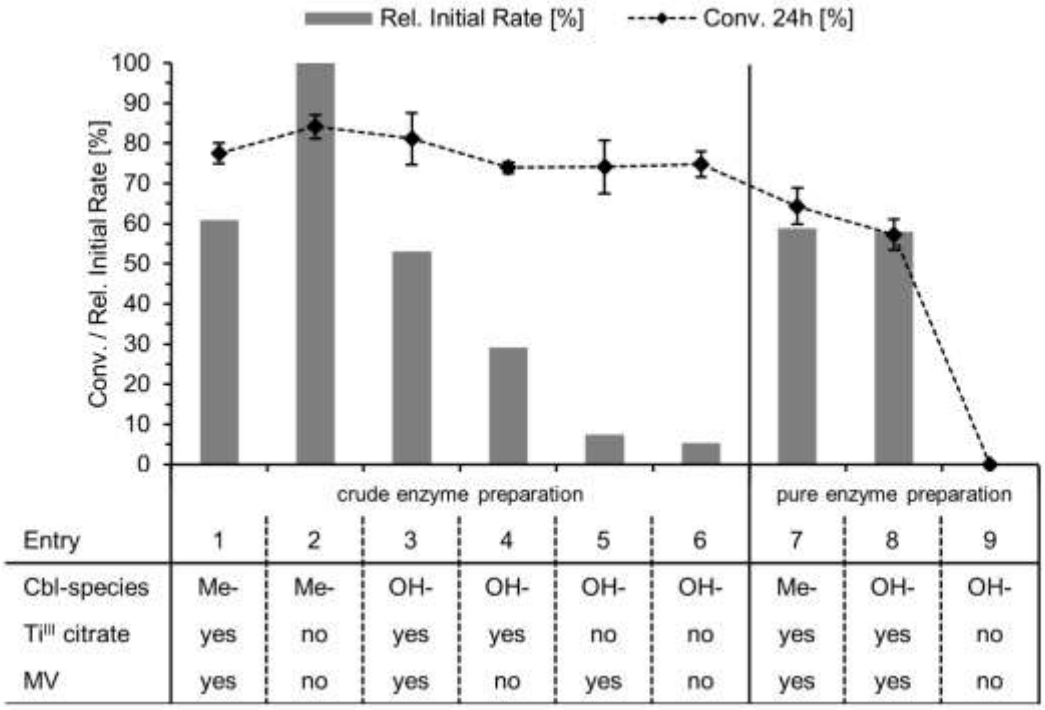


Figure 3. Characterization study addressing the use of Ti^{III} citrate and methyl viologen (MV) for in-vitro activation of OH-Cbl to the superreduced Co^{I} species. Reaction conditions: substrate **1a** (10 mM, 1.2 mg/mL), methyl acceptor **2b** (50 mM, 7.7 mg/mL), MTase I (40 mg/mL freeze-dried CFE or 13 mg/mL freeze-dried pure enzyme ~0.36 mM, respectively), CP (400 µL/mL holo-CP solution; 100 mg/mL freeze-dried CFE or 33 mg/mL pure *apo*-CP reconstituted either with Me-Cbl or OH-Cbl, respectively), chemical activation system composed of Ti^{III} citrate (as indicated, 1x refers to 4.19 mM) and MV (as indicated, 1x refers to 0.3 mM) in MOPS buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm in in Eppendorf Orbital Shaker (1.5 mL). Conversions were determined after 24 h by HPLC-UV from areas of **1a** and **2a** using calibration curves. Error bars represent the standard error of the mean (s.e.m., $n = 3$). Initial reaction rates were determined from the linear slopes within 0-2 hours of the reaction time courses (for examples see Figure S4) and calculated relatively to the highest one obtained in each case.

It is worth to mention that the proteins on their own (without Cbl) can be stored at 4°C at least for 12 months since the activity of the methyltransferase system is afterwards still unchanged.

Finally, the effect of co-solvent addition was investigated since for substrates that are barely soluble in water, the addition of organic co-solvent might be helpful to increase their solubility. First experiments testing the model reaction in presence of dimethyl sulfoxide (DMSO) showed that up to 20% *v/v* of co-solvent are tolerated by the enzymatic system from *D. hafniense* without significant loss of activity (data not shown). Besides DMSO also methanol (MeOH) was tolerated equally well with conversions identical to the co-solvent free counterpart (Table 1, entry 1). Furthermore, the addition of dimethylformamide (DMF), ethanol (EtOH) and 2-propanol (*i*-PrOH) has proven to be possible leading to slightly decreased conversions in the range of 33 to 56%. Finally, the optimized conditions involving the presence of 10 % *v/v* co-solvent (DMSO or MeOH, respectively) were applied for the transformation of a selection of *O*-methyl phenyl ethers (Table 1, entry 2-6). Vanillyl alcohol as well as its regioisomer isovanillyl alcohol (compounds *m*- and *p*-**1d**, entry 2-3, *m* = *meta*, *p* = *para* refers to the position of the methyl group in relation to the substituent at the catechol chore) were demethylated with conversions up to 82%. Comparable results were obtained for the bis-methyl substrates 2,3-dimethoxyphenol 2,3-**1e** (up to 71%; entry 4) and 2,6-**1e** (up to 81%, entry 5). In case of the latter substrates a demethylation reaction can take place at two sites; a single methyl transfer leads to the formation of 3-methoxycatechol **2e** which is further demethylated to 1,2,3-trihodroxybenzene **3e**. Therefore, a product mixture was obtained in both cases, with a slight surplus of **3e**.

Conclusion

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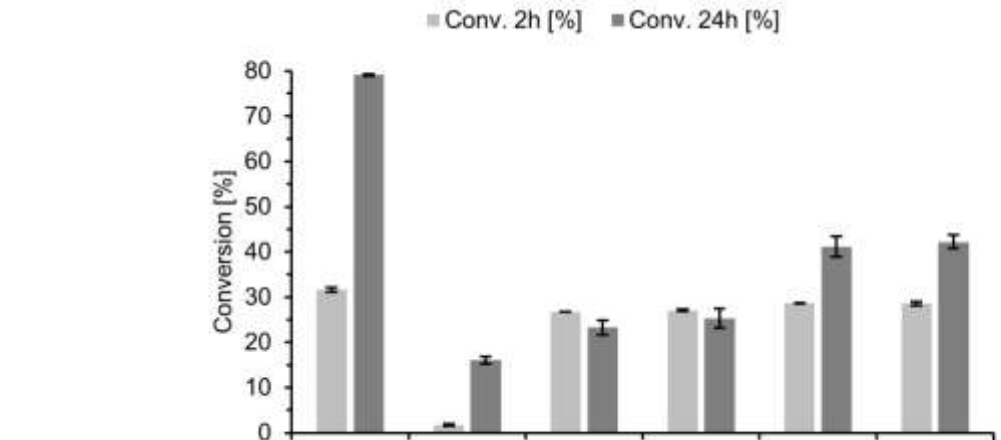
A corrinoid-dependent methyl transfer system from *D. hafniense* was shown to be applicable for the demethylation of selected methyl phenyl ethers. The reaction works in a broad operational window concerning pH (pH 6.5-8) and temperature (25-40 °C), whereby ideally the reaction is performed at neutral pH and 30-35 °C. Reconstitution of CP with the corrinoid cofactor has been shown to occur spontaneously and to be crucial for an efficient reaction. Methyl- and hydroxocobalamin (Me-Cbl, OH-Cbl) turned out to be the suitable cofactor species. In an inert atmosphere the addition of a chemical activation system for reducing inadvertently oxidized cobalamin (Co^{II}) can be omitted. The reaction can be performed in presence of various organic co-solvents such as DMSO and MeOH without loss of activity at 10% *v/v*. The conditions reported in this study were successfully employed for the demethylation of a range of methyl phenyl ethers indicating that this transformation may become a suitable reaction to be considered for biocatalytic retrosynthesis.[47-50]



Entry	1	2	3	4	5	6
Inert set-up	yes	yes	yes	yes	yes	yes
Incubation before O_2-input	inert	aerobic	aerobic	aerobic	aerobic	aerobic
O_2-input after x hours	no	0h	2h	2h	2h	2h
Incubation after O_2-input	inert	aerobic	aerobic	inert	inert	inert
Reactivation with Ti^{III}	no	no	no	no	1x	2x

Figure 4. Reactivation of reactions exposed to air. A series of identical biotransformation reactions was prepared under inert conditions. Reaction no.1 was entirely kept under an oxygen-free atmosphere as a control, whereas no.2-6 were incubated for at least 2 h under aerobic conditions (closed vials on air). Samples were actively aerated either directly (0 h, no. 3) or after 2 h incubation time (no. 3-6) by repeatedly opening the respective vials on air and mixing. While incubation was continued under aerobic conditions in case of reaction no.3, reactions no. 4-6 were again transferred back into the glove-box and incubated under inert atmosphere for the remaining 22 h. The O_2-scavenger system was added to reaction no.5 (1x) and no.6 (2x). Reaction conditions: substrate **1a** (10 mM, 1.2 mg/mL), methyl acceptor **2b** (50 mM, 7.7 mg/mL), MTase I and CP (40 mg/mL lyophilized crude extract of MTase I and 400 µL/mL reconstituted holo-CP solution), O_2-scavenger system (1x relates to 4.19 mM Ti^{III} citrate and 0.3 mM methyl viologen) in MOPS buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm in in Eppendorf Orbital Shaker (1.5 mL) for 24 h. Conversions were determined after 2 h and 24 h by HPLC-UV from areas of **1a** and **2a** using calibration curves. Error bars represent the standard error of the mean (s.e.m., $n = 3$).

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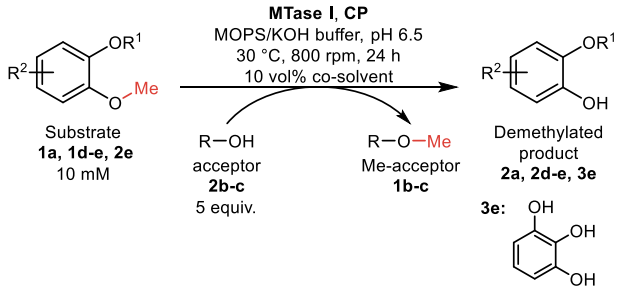
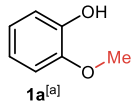
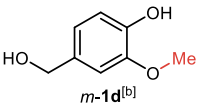
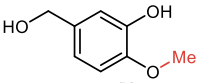
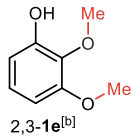
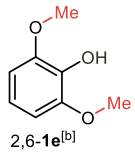
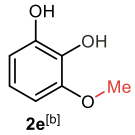
Table 1. Effect of organic co-solvents on the corrinoid-dependent demethylation of selected substrates.



Entry	Substrate	Co-solvent	Conversion [%][c]
1	 **1a**[a]	DMSO MeOH EtOH DMF *i*-PrOH *n*-PrOH	79 78 56 39 33 5
2	 *m*-**1d**[b]	DMSO MeOH	80 72
3	 *p*-**1d**[b]	DMSO MeOH	82 80
4	 2,3-**1e**[b]	DMSO MeOH	71 (**2e**/**3e**=40/60) 70 (**2e**/**3e**=41/59)
5	 2,6-**1e**[b]	DMSO MeOH	81 (**2e**/**3e**=45/55) 78 (**2e**/**3e**=47/53)
6	 **2e**[b]	DMSO MeOH	40 38

[a] Reaction conditions: substrate (10 mM), methyl acceptor **2c** (50 mM), MTase I (freeze-dried CFE, 40 mg mL^{-1}), CP (400 µL mL^{-1} reconstituted solution), organic co-solvent (10% *v/v*, as indicated) in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl) at 30 °C, 800 rpm for 24 h. [b] Reaction conditions as in a) but with methyl acceptor **2b**. [c] Conversions determined after 24 h by HPLC-UV from areas for substrate and demethylated product using calibration curves. Deviation values (s.e.m., $n = 3$) lie between 0.1 and 3.3 % for the substrates tested.

Experimental Section

All chemicals and solvents were obtained from commercial suppliers (Sigma Aldrich/Fluka, VWR International/Merck, Roth) and used as such unless stated otherwise. Cloning of genes and recombinant expression in *E. coli* was performed as described before.[39] If necessary, enzymes were purified by affinity chromatography, using Strep-Tactin® technology (IBA Lifesciences) according to the manual provided by the supplier (see SDS gel of overexpressed and purified enzymes in Figure S3). Biocatalysts were used as freeze-dried cell free extracts (CFE) or purified preparations and biotransformations were performed at least in triplicates in 1.5 mL Eppendorf tubes using an Eppendorf Thermomixer comfort. Due to the oxygen-sensitive cofactor, biocatalytic reactions were performed in degassed buffers under inert atmosphere (N_2 5.0) in a MBraun LABstar glove box equipped with a MB-OX-EC O_2-sensor. For detailed analytical methods and NMR-spectra see electronic Supplementary Information.

Representative procedure for preparation of *holo*-CP. The corrinoid protein was reconstituted with exogenous cofactor under inert atmosphere in order to obtain functional *holo*-CP. For this purpose, the respective cobalamin species (2 mM) was dissolved in the presence of betaine (3 M) and DTT (2mM) in TRIS/HCl buffer (50 mM, pH 7, 0.5 mM DTT, 0.1 mM PMSF). Then, freeze-dried CP (100 mg/mL CFE or 33 mg/mL purified enzyme, respectively) was dissolved in the prepared reconstitution buffer (1 mL) and incubated for 2 h at 4 °C to allow incorporation of the cofactor. Afterwards salts and unbound cobalamin were removed using a PD MidiTrap™ G-25 column (GE Healthcare) or PD 10™ G-25 column (GE Healthcare) according to the manual provided by the manufacturer. Finally, reconstituted *holo*-CP was eluted with MOPS/KOH buffer (100 mM, pH 6.5, 150 mM KCl) yielding a red colored protein fraction (containing 66.7 mg/mL CFE or 22 mg/mL pure enzyme, respectively) which was stored at 4 °C until further use. The procedure was adapted according to the performed investigations (e.g. incubation times, cofactor species).

Representative procedure for biocatalytic demethylation. Analytical biotransformation reactions were carried out on 180 µL or 1 mL scale as follows: freeze-dried MTase I (10-20 mU or 56-112 mU, 40 mg/mL CFE or 13.3 mg/mL pure enzyme, respectively; ratio MTase I to substrate: 1:30) was rehydrated in *holo*-CP solution (400 µL/mL). The reaction was started by adding appropriate amounts of **1a** (final concentration 10 mM) and methyl acceptor **2b**/**2c** (final concentration 50 mM) as stock solutions in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl). In case the addition of an activation system was required, Ti^{III} chloride (final concentration 4.15 mM) was added as a stock solution in MOPS/KOH (1M, pH 7.9, 333 mM sodium citrate) to the reaction mixture as well as MV (0.3 mM). Reaction samples were shaken at 30 °C and 800 rpm for 24 hours. The procedure was adapted according to the parameters investigated (e.g. enzyme amount, buffer composition and pH, temperature, co-solvent addition etc.).

Biotransformations for NMR analytics. Biotransformations were started on 1 mL scale as described above 32 times in parallel. After 24 h incubation, the biotransformation was centrifuged for 10 min at 14680 rpm. All supernatants were pooled in a centricon and basified to pH 8.0 with a saturated aqueous $NaHCO_3$ solution. The aqueous phase was extracted with EtOAc (5 x 15 mL). The phases were separated by centrifugation at 9000 rpm for 20 min. The combined organic phase was dried (Na_2SO_4) and the solvent was evaporated under reduced pressure. The products of the methyl donors **1a, *p*-1d and 2,3-1e** were purified via preparative HPLC (**1a, *p*-1d** Method A, see table S2; **2,3-1e** Method B, see table S2). (In cases using **2b** as acceptor, excess of **2b** may be removed by SPE; not performed here). Catechol **2a** (10 mg, 28%), 3,4-dihydroxybenzyl alcohol **2d** (9.4 mg, 19%) and 3-methoxycatechol **2e** (6 mg, 12%) were isolated by this procedure.

Determination of conversions. After specified time points (e.g. 30 min, 1 h, 2 h, 24 h) a reaction aliquot (30 µL) was withdrawn, quenched by the addition of MeCN (180 µL), incubated at room temperature (30 min) and diluted with

deionized water (90 µL). Precipitated protein was removed by centrifugation (14000 rpm, 15 min) and the clear supernatant was filtered and directly analysed by HLPC (Agilent 1260 Infinity system equipped with an UV detector) using an achiral C18 column (Phenomenex, Luna, C18 100c, 250x4.6mm, 5mm). H_2O/MeCN (0.1% TFA) was used as eluent with a flow rate of 1 mL/min. Compounds were detected by UV-absorption and conversions were calculated based on calibrations curves.

Determination of initial rates. In order to enable a better comparison of different characterization studies, initial rates were determined for most conditions investigated. For this purpose the demethylation of guaiacol was performed under respective conditions according to the procedure described above and assayed over the first time period of 1-2 h (representing the linear range of the reaction, for examples see Figure S4). The amount of product formed after specified time points was plotted against the reaction time and the slope of linear correlation was used to calculate the initial reaction rate. Enzyme activity (mU) was defined as the amount of enzyme that catalyzes the conversion of 1 nanomole of substrate per minute.

Acknowledgements

This study was financed by the Austrian FFG, BMWFJ, BMVIT, SFG, Standortagentur Tirol and ZIT through the Austrian FFG-COMET- Funding Program (Austrian Centre of Industrial Biotechnology). Financial support by the Austrian Science fund is gratefully acknowledged (P30920-B21).

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10.1002/adsc.201801590

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