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Research Article

Oxygen-Free Regioselective Biocatalytic Demethylation of Methylphenyl Ethers via Methyltransfer Employing Veratrol-*O*-demethylase

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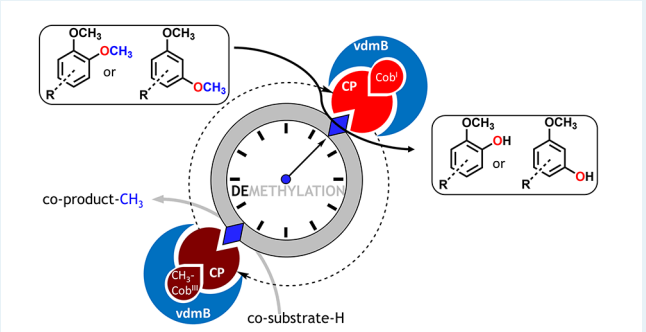
ABSTRACT: The cleavage of aryl methyl ethers is a common reaction in chemistry requiring rather harsh conditions; consequently, it is prone to undesired reactions and lacks regioselectivity. Nevertheless, *O*-demethylation of aryl methyl ethers is a tool to valorize natural and pharmaceutical compounds by deprotecting reactive hydroxyl moieties. Various oxidative enzymes are known to catalyze this reaction at the expense of molecular oxygen, which may lead in the case of phenols/catechols to undesired side reactions (e.g., oxidation, polymerization). Here an oxygen-independent demethylation via methyl transfer is presented employing a cobalamin-dependent veratrol-*O*-demethylase (vdmB). The biocatalytic demethylation transforms a variety of aryl methyl ethers with two functional methoxy moieties either in 1,2-position or in 1,3-position. Biocatalytic reactions enabled, for instance, the regioselective monodemethylation of substituted 3,4-dimethoxy phenol as well as the monodemethylation of 1,3,5-trimethoxybenzene. The methyltransferase vdmB was also successfully applied for the regioselective demethylation of natural compounds such as papaverine and *rac*-yatein. The approach presented here represents an alternative to chemical and enzymatic demethylation concepts and allows performing regioselective demethylation in the absence of oxygen under mild conditions, representing a valuable extension of the synthetic repertoire to modify pharmaceuticals and diversify natural products.



KEYWORDS: *biocatalysis, biotransformation, ether cleavage, demethylation, methyltransferases, veratrol-O-demethylase*

INTRODUCTION

The demethylation of aryl methyl ethers[1] is a common transformation in organic chemistry to demask the phenol functionality.[2−8] Several phenolic compounds represent valuable pharmaceutical and natural products.[3,9] In general, chemical methods for ether cleavage require harsh reagents such as strong acids and bases[3,4] but may also be performed with metal catalysts[5,6] and sodium thiolates.[7,8] Nevertheless, alternative approaches toward milder and selective demethylation are demanded.[10,11] *O*-Demethylation of methyl aryl ethers may also be achieved using biocatalysts:[11−14] For example, oxidative enzymes[15−18] such as di- and monooxygenases[19−24] and fungal peroxygenases[25−27] catalyze the *O*-demethylation by using molecular oxygen or hydrogen peroxide as reagents. These enzymes are known for the detoxification of organic compounds,[25] degradation of lignin,[21] and the biosynthesis of secondary metabolites. However, for applications, the oxidative *O*-demethylation of protected phenols at the expense of molecular oxygen may lead to unwanted side products, for example, via hydroxylation at the aromatic ring or the spontaneous formation of polymerized products.[28] An alternative option avoiding molecular oxygen involves the use of cobalamin-dependent methyltransferases (MTases)[29−31] or *O*-demethylases originating from anaerobic bacteria which utilize aryl methyl ethers as carbon source.[32,33] A specific methyltransferase dhaf4610 from *Desulfitobacterium hafniense* (*D. hafniense*) has been recently employed for the methylation of catechols and demethylation of guaiacol derivatives.[34−36] Unlike the well-investigated *S*-adenosyl methionine (SAM)-dependent methyltransferases,[37−39] which are restricted toward methylation only, this MTase[29,40] has been shown to enable both methylation and demethylation in a reversible manner.[34,35] Thereby, the MTase transfers the

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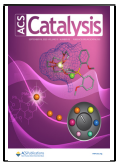

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methyl group from the cofactor methylcobalamin (Me-Cob^{III})[41−44] to the substrate giving cobalt in the oxidation state I (Cob^{I}). Methylcobalamin was then regenerated by the same MTase using a methyl donor like guaiacol **2a** as cosubstrate. Thus, for an overall methyl transfer reaction, a methyl donor and a methyl acceptor are required. By using an excess of one compound, the reaction may be shifted toward demethylation or methylation. It is worthwhile to note that the cobalamin is bound to a carrier protein, the corrinoid protein (CP), which was in the case above the corrinoid protein dhaf4611 originating from the same organism.

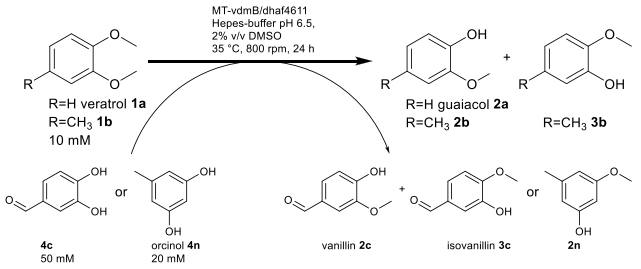
Unfortunately, the MTase from *D. hafniense* is limited to guaiacol derivatives as substrates for demethylation (i.e., methyl donor) and catechol derivatives as methyl acceptor. Thus, in a typical reaction, the methyl transfer occurred from the substrate guaiacol **2a** as methyl donor to 3,4-dihydroxybenzoic acid **4c** as methyl acceptor.[31,34,35]

Since many natural and pharmaceutical compounds[45,46] contain more than one protected methoxy group in close proximity and due to the limitation of the substrate pattern of the MTase from *D. hafniense*, alternative enzymes transforming substrates with fewer restrictions are needed.

RESULTS AND DISCUSSION

As a starting point, we chose the methyltransferase veratrol-*O*-demethylase (MT-vdmB)[32,47−49] and the carrier protein vdmA both originating from the same organism, namely the anaerobic bacterium *Acetobacterium dehalogenans*.[50,51] The methyltransferase MT-vdmB is described to demethylate veratrol **1a** to guaiacol **2a** (Scheme 1); thus, here two methoxy groups are present in 1,2-position. This type of substrate was not accepted by the previously described MTase from *D. hafniense*.[31,34−36] However, when MT-vdmB was used in combination with the carrier protein vdmA from the same organism as described in literature, only very low conversion for demethylation of veratrol **1a** (10 mM substrate concentration) was observed (3%) when using a 5-fold molar excess of 3,4-dihydroxybenzaldehyde **4c** as a methyl acceptor (Scheme 1). The acceptor was converted to the regioisomers vanillin **2c** and isovanillin **3c** in a 3:1 ratio. Since optimization studies did not improve the conversion, an alternative carrier protein was considered. Aligning carrier proteins from other hosts with vdmA, the carrier protein dhaf4611 from *Desulfitobacterium hafniense* showed the highest sequence identity with 72% (EMBOSS needle). Remarkably, testing now the combination of MT-vdmB with dhaf4611 led to a higher conversion of veratrol **1a** (10% conv.) compared with the reaction with the "natural" carrier protein vdmA from the same organism (3% conv.).

Scheme 1. Biocatalytic Demethylation of the Model Substrates 1a and 1b



In a next step, various pairs of methyl donors and acceptors were analyzed to get a first idea of the substrate scope. Thereby, the 1,3-phenyldiol orcinol **4n** unexpectedly stood out as methyl acceptor (Scheme 1, for not accepted methyl donors see Supporting Information, Figure S7). This was a surprise since the substitution pattern is not related to veratrol or its demethylated product guaiacol **2a**. Orcinol **4n** was methylated to 3-methoxy-5-methylphenol **2n**. Furthermore, 3,4-dimethoxytoluene **1b** differing to veratrol **1a** by one methyl group, was demethylated with better conversion compared to veratrol (Scheme 1).

The methyltransfer reaction employing MT-vdmB/dhaf4611 was optimized regarding the type of buffer salt, pH, the concentration of Zn^{2+}, temperature, cosolvents, and the ratio between MT-vdmB and the carrier protein dhaf4611. Best conversions were obtained at pH 6.5 in 50 mM HEPES, MES, or MOPS buffer (Supporting Information, Figure S1). Nevertheless, the biocatalyst also tolerated basic conditions in CHES buffer (pH 9.5 and 10, Supporting Information, Figure S2). According to literature,[47] the MT-vdmB is probably Zn^{2+} dependent due to a unique zinc-binding motif D-X_{27}-C-X_{39}-C. Indeed, the highest conversion was obtained in the presence of 20 μM of $ZnCl_2$, which was about twice as much as in the absence of Zn^{2+}; higher concentrations ($\geq$50 μM Zn^{2+}) led to less conversion (Supporting Information, Figure S3). Furthermore, the optimal temperature was found to be 35 °C (Supporting Information, Figure S4).

To improve the bioavailability of less water-soluble substrates in buffer, DMSO was investigated as cosolvent at different concentrations (0−10%). Interestingly, best conversions were obtained without addition of cosolvent (18% conv.) when using substrate **1b** (10 mM) and methyl acceptor **4n** (20 mM); although solubility is not an issue for these compounds at the concentration used, 2% v/v DMSO was chosen as suitable value for the investigation of less soluble substrates (17% conv., Supporting Information, Figure S5). In comparison to other cosolvents such as MeOH, EtOH, 1,4-dioxane and THF, DMSO performed the best (Supporting Information, Figure S6). Finally, the optimal ratio of the methyltransferase and the carrier protein was investigated. Applying a 1:20 ratio of the MT-vdmB/carrier protein led to highest conversion (30% conv., Supporting Information, Table S1).

Subsequently, the optimized conditions were applied for the demethylation of veratrol **1a** and a range of 1,2-dimethoxy substituted substrates **1b-f** using a 2-fold molar excess of orcinol **4n** as methyl acceptor. At these conditions, veratrol **1a** was preferentially monodemethylated to guaiacol **2a** (54% conv.), giving at this stage of the reaction a tiny amount of the didemethylated product catechol **4a** (1%, Table 1, entry 1). Veratrol derivatives were efficiently demethylated with up to 77% conversion as observed for the 4-methyl substituted substrate **1b** (Table 1, entries 2−6).

For selected substrates mono- as well as didemethylation was observed, like for **1a-c** and **1f**. On the other hand, for substrates **1d** and **1e** exclusively monodemethylated products were observed at the analyzed stage of conversion; to perform exclusively monodemethylation is challenging if not impossible by chemically means. Especially 3,4-dimethoxyphenol **1d** is worthwhile to mention, since the monodemethylation occurred with perfect regioselectivity, namely in *meta*-position to the phenolic OH of the substrate **1d** leading exclusively to 4-methoxyresorcinol **3d** with >99% regioselectivity. So far,

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Table 1. Biodemethylation of Substituted Veratrol Derivatives[a]

	substrate [10 mM]	T [°C]	conv. [%]	mono-demethylation		di-demethylation
1	 1a	35	55	 2a (95%)		 4a (5%)
2	 1b	25	77	 2b (52%)	 3b (35%)	 4b (13%)
3	 1c	35	59	 2c (81%)	 3c (11%)	 4c (8%)
4	 1d	35	30		 3d (>99%)	
5	 1e	35	53	 2e (53%)	 3e (47%)	
6	 1f	35	53	 2f (81%)		 4f (19%)

[a]Reaction conditions: substrate **1a-f** (10 mM), methyl acceptor **4n** (20 mM, 2.5 mg/mL), vdmB (40 mg/mL cell-free extract, ≡ 0.077 mM vdmB), CP (31 mg/mL or 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 μM $ZnCl_2$) at either 25 or 35 °C, 800 rpm in Eppendorf Thermomixer (1.5 mL), 24 h. Total volume 500 μL. The conversions were analyzed on HPLC-UV using calibration curves.

compound **3d** was until now chemically accessible either via (i) the oxidation of isovanillin **3c** by the Dakin reaction,[52] (ii) a multistep synthesis,[53] and (iii) by the oxidation of 5-hydroxy-2-methoxyphenyl acetate using a Cu^{2+}-ascorbic acid O_2 system.[54] It was also obtained by hydroxylation of guaiacol **2a** with a toluene 4-monooxygenase with 87% conv. at 1 mM substrate concentration.[55] The here presented enzymatic reaction opens an alternative one-step procedure to convert a commercially available substrate to a more valuable product.

To analyze the regioselectivity of the demethylation in more detail, the transformation of **1b** was followed over time (Figure 1). Initially, the monodemethylated regioisomer in *para*-position to the methyl group **2b** (green squares) was formed about 1.6 times faster than the corresponding regioisomer **3b** (blue dots). After 6 h, the didemethylated product **4b** started to be formed reaching 23% after 20 h (purple triangles). From the graph it can also be concluded that the second demethylation step to give **4b** occurs from both possible precursors **2b** and **3b**, whereby it seems that **2b** is preferred, as the difference between **2b** and **3b** gets smaller over time. Thus, the monodemethylation of **1b** gives compound **2b** with a slight preference, which is also the preferred substrate for the second demethylation step. A similar regioselectivity as for **1b** was observed for the benzaldehyde derivative **1c** (81% *para* **2c**, Table 1).

The temperature did not have a significant influence on the regioselectivity when comparing biotransformations at 25 and 35 °C (Supporting Information, Table S2 and S3); thus, the preference remained comparable. Nevertheless, the temperature effected the amount of product formation leading in most cases to higher conversion at higher temperature and, if a second demethylation occurred, a higher amount of the didemethylated product (**4b**, **4c**, and **4g**, see Supporting Information, Table S2 and S3). The only exception was observed in the case of the formation of **4f** which was highest at 25 °C (Supporting Information, Table S2).

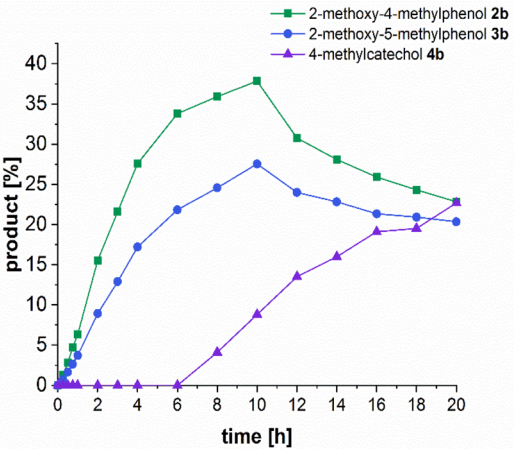
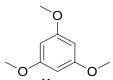
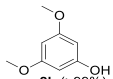
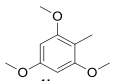
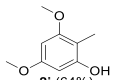
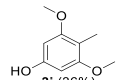


Figure 1. Time course of the mono- and didemethylation of 3,4-dimethoxytoluene **1b**. Reaction conditions: substrate **1b** (10 mM, 1.5 mg/mL), methyl acceptor **4n** (20 mM, 2.5 mg/mL), vdmB (40 mg/mL cell-free extract, ≡ 0.077 mM vdmB), CP (31 mg/mL or 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH6.5, 150 mM KCl, 20 μM $ZnCl_2$) at 35 °C, 800 rpm in Eppendorf Thermomixer (1.5 mL) for 24 h. Total volume 120 μL. The conversions were analyzed on HPLC-UV using calibration curves.

Since all methyl donor substrates investigated so far contained the 1,2-dimethoxy motif, the spectrum was extended to the 1,2,3-trimethoxy compound **1g** (Table 2, entry 1). This

Table 2. Product Formation of 1,3-Dimethoxybenzene Derivatives[a]

	substrate [10 mM]	T [°C]	conv. [%]	mono-demethylation		di-demethylation
1	 1g	35	47	 2g (31%)	 3g (13%)	 4g (56%)
2	 3g	35	61	 4g (>99%)		
3	 1h	25	50	 2h (>99%)		
4	 1i	35	56	 2i (64%)	 3i (36%)	

[a]Reaction conditions: substrate **1g-i** (10 mM), methyl acceptor **4n** (20 mM, 2.5 mg/mL), vdmB (40 mg/mL cell-free extract, ≡ 0.077 mM vdmB), CP (31 mg/mL or 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 μM $ZnCl_2$) at either 25 or 35 °C, 800 rpm in Eppendorf Thermomixer (1.5 mL) for 24 h. Total volume 500 μL. The conversions were analyzed on HPLC-UV using calibration curves.

motif was demethylated about 2 times faster at position 1 compared with position 2, which corresponds to the statistically expected value. Nevertheless, after 24 h, the didemethylated product **4g** was the main compound formed under these conditions. Since in this experiment a phenol was formed bearing two methoxy groups in *ortho*-position (compound **3g**), it was investigated whether **3g** is demethylated as well to give **4g**. Indeed, the experiment showed that

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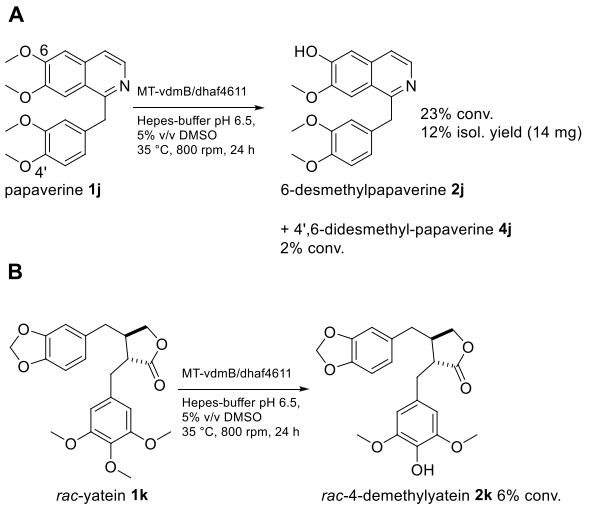
demethylation of **3g** leads exclusively to compound **4g** (Entry 2), thus leaving one single methoxy group intact.

In a next step to extend the substrate scope even further, compounds were investigated bearing a 1,3-dimethoxy motif instead of the 1,2-dimethoxy motif. Surprisingly, even this type of pattern was accepted as shown for 1,3,5-trimethoxybenzene **1h** (Entry 3). The latter was exclusively monodemethylated to give compound **2h** as a single product. Introducing an additional methyl group as in substrate **1i**, showed that again exclusively monodemethylation occurred, whereby the two regioisomers **2i**/**3i** were formed in a ratio of ∼2:1 corresponding to the statistical value.

As the 1,2-dimethoxy as well as the 1,2,3-trimethoxy are regular motifs found in a plethora of natural products, we turned our attention in a next step to these more complex molecules. The regioselective demethylation of natural products may enable diversification to access other or even new derivatives in a selective manner and may be a tool to valorize natural and pharmaceutical compounds by deprotecting reactive hydroxyl moieties. Since the demethylation is performed in the absence of molecular oxygen, oxidation-sensitive functionalities present in the substrates and/or products will be preserved. The first natural substrate investigated was papaverine **1j** which is produced by the opium plant *Papaver somniferum*.[56] Papaverine **1j** contains twice the 1,2-dimethoxybenzene motif, thus in total four methoxy groups (Scheme 2A). Papaverine **1j** was preferentially

Scheme 2. Biotransformation of Pharmaceutical Relevant Compounds (A) Papaverine and (B) *Rac*-yatein



monodemethylated regioselectively to 6-desmethylpapaverine **2j** which was isolated and confirmed by 2D-NMR experiments[57] (see Supporting Information for details). Thus, the enzymatic transformation allowed us to demethylate selectively in one out of the four possible positions. The obtained monodemethylated product **2j** was demethylated further to the didemethylated product 4′,6-didesmethylpapaverine **4j**; thus, the second slower demethylation step took place at the second benzene ring of the molecule. Both demethylated products **2j** and **4j** are valuable biomarkers which are used to improve the detection time of the drug heroin.[58] Since papaverine **1j** is used as a drug to relax smooth muscles[45,46] and to inhibit human prostate cancer cell growth,[59] the demethylated derivatives may be tested for similar applications as well. Another natural product is *rac*-yatein **1k**, which is an antimicrobial[60] and antiproliferative compound against cancer cells[61] possessing three methoxy groups (Scheme 2 B). Interestingly, *rac*-yatein **1k** was demethylated regioselectively at position 2 of the 1,2,3-trimethoxy motif leading to *rac*-4-demethylyatein **2k**. This is actually in contrast with the observed regioselectivity in the case of 1,2,3-trimethoxybenzene **1g**, which was preferentially demethylated at position 1. The demethylated product **2k** is found in *T. occidentalis*[62] and may act as a precursor of etoposide and teniposide,[63] which are chemotherapeutical agents and part of the WHO's list of essential medicines.[64]

CONCLUSIONS

The cobalamin-dependent methyltransferase MT-vdmB (veratrol-*O*-demethylase from *A. dehalogenans*) in combination with the cobalamin carrier protein dhaf4611 from *D. hafniense* demethylated 1,2- and 1,3-dimethoxy as well as 1,2,3-trimethoxy derivatives under inert atmosphere via methyl transfer. The MT-vdmB accepts a wide range of aromatic substrates with two methoxy moieties either in close proximity to each other (1,2-position) such as in 3,4-dimethoxytoluene **1b** or in 1,3-position such as in 2,6-dimethoxyphenol **3g**. The MT-vdmB showed clear regioselectivity for the demethylation of the methoxy moiety located in the *para*-position to the substituent like in 3,4-dimethoxytoluene **1b** and 3,4-dimethoxybenzaldehyde **1c**. For other substrates, like 3,4-dimethoxyphenol **1d**, perfect regioselectivity was observed leading exclusively to 4-methoxyresorcinol **3d** with >99% regioselectivity. Natural products such as papaverine **1j** and *rac*-yatein **1k** were demethylated in a regioselective fashion. The demethylation of these natural compounds indicates that the biocatalytic methyltransfer approach paves the way to alternative, environmentally benign, oxygen-free demethylation methods to possible novel bioactive agents/pharmaceuticals.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c02790.

Results of the optimization study, experimental procedures for enzyme expression and purification, set up of biotransformations, and analytical methods (PDF)

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Notes

The authors declare no competing financial interest.

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