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Cobalamin-Dependent Apparent Intramolecular Methyl Transfer for Biocatalytic Constitutional Isomerization of Catechol Monomethyl Ethers

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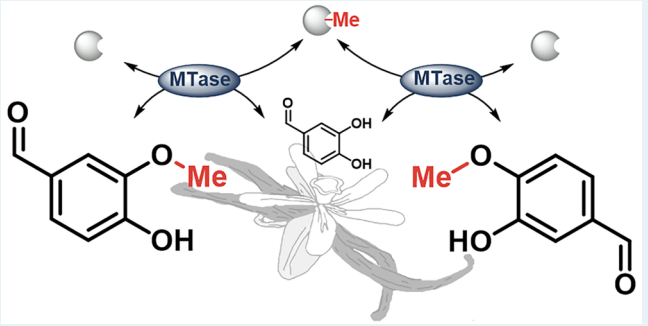
Supporting Information

ABSTRACT: Isomerization is a fundamental reaction in chemistry. However, isomerization of phenyl methyl ethers has not been described yet. Using a cobalamin-dependent methyl transferase, a reversible shuttle concept was investigated for isomerization of catechol monomethyl ethers. The methyl ether of substituted catechol derivatives was successfully transferred onto the adjacent hydroxy moiety. For instance, the cobalamin-dependent biocatalyst transformed isovanillin to its regioisomer vanillin with significant regioisomeric excess (68% vanillin). To the best of our knowledge, isomerization by methyl transfer employing a methyl transferase has not been reported before.



KEYWORDS: *biocatalysis, biotransformations, methyltransferases, corrinoids, isomerization, intramolecular*

INTRODUCTION

Isomerization reactions are fundamental transformations in both a biological as well as a chemical sense, in which a substrate undergoes intramolecular rearrangement of bonds or functional groups resulting in a structurally varied product without changing its molecular formula, and thus, it can be considered a constitutional isomer.[1−5] While established synthetic methods involving such interconversions mainly deal with allylic rearrangements catalyzed by metal catalysts,[6−9] nature offers a vast source of enzyme-catalyzed transformations[10−13] including isomerizations[14−20] which have already emerged as an attractive alternative for many requests.[21−23] Various isomerization reactions in nature are enabled by cobalamin cofactors,[24−26] which belong to the most complex organometallic molecules found in nature and offer remarkable options for catalysis. They all share the structural motif of a corrin skeleton with a cobalt−carbon bond in the center, being key to their unique reactivity.[27] Cobalamin-dependent enzymes predominantly depend on the cofactor with the Co atom bound either to a methyl moiety (Me-Cbl) or to 5′-deoxyadenosine (Ado-Cbl), and accordingly, they perform different reactions (Scheme 1b).[28,29] Ado-Cbl-dependent enzymes involve homolytic cleavage of the Co−C bond and mainly catalyze isomerization reactions via a radical rearrangement mechanism.[15]

In contrast, the reactivity of Me-Cbl-dependent enzymes relies on heterolytic cleavage of the organometallic Co−C bond, allowing for methyl group transfer via nucleophilic substitution (Scheme 1B). Members of this enzyme class are methyl transferases (MTases),[30−32] which play an important role both in eukaryotes for amino acid metabolism as well as in anaerobes where they are involved in one-carbon metabolism and CO_2-fixation. As part of a complex multienzymatic system, they catalyze the transfer of methyl groups from a donor molecule to a methyl acceptor via two half reactions. This "ping-pong" reaction cycle has been applied in a biocatalytic shuttle catalysis concept[33] for reversible demethylation and methylation, representing a sustainable alternative to chemistry.[34−36] The cobalamin-dependent methyl transfer machinery originating from the anaerobic organism *Desulfito-bacterium hafniense*[37−39] was used for a simplified in vitro approach, consisting of one MTase as the only biocatalyst and a corrinoid protein (CP) transporting the cobalamin cofactor and acting as methyl group shuttle. In contrast to the use of SAM-dependent methyltransferases,[40−43] the presented approach allows methylation as well as demethylation devoid of cofactor recycling issues.[42]

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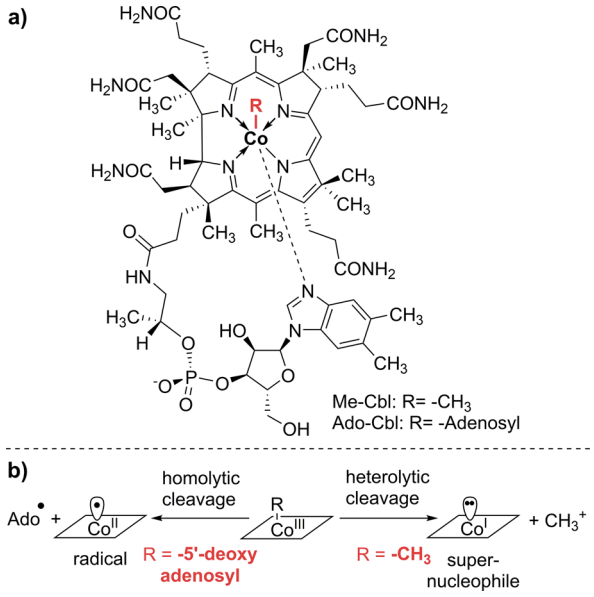
Scheme 1. (a) Cobalamin (Cbl) Cofactors with the Unique Co−C Bond in the Center of the Corrin Skeleton and (b) the Reactivity They Offer According to the Type of α Ligand Present at the Co Atom



Initial options have already been demonstrated for the formation as well as cleavage of various phenyl methyl ethers.

In the present study, we report an apparent intramolecular methyl group transfer resulting in constitutional substrate isomerization employing the cobalamin-dependent MTase from *D. hafniense*. The apparent intramolecular migration of methyl groups has been hardly investigated despite its important role in organic synthesis involving homogeneous and heterogeneous catalysis.[44−48] To the best of our knowledge, Me-Cbl-dependent enzymes have never been described before to catalyze an isomerization reaction; these reactions are usually encountered in the repertoire of their Ado-Cbl-dependent analogue.

RESULTS AND DISCUSSION

The MTase from *D. hafniense* has been reported to break heterolytically the methyl-*O*-ether bond in a substrate such as guaiacol and transfer the methyl group onto an acceptor molecule such as 3,4-dihydroxybenzoic acid.[33] Because of the structural similarity of the methyl donor and acceptor substrate containing a catechol backbone, the possibility of an intramolecular methyl transfer seemed feasible. Thus, the transfer of a methyl group attached to one hydroxy group in substituted guaicol derivatives **1a−d** to the other hydroxy moiety was investigated resulting in the formation of the constitutional isomer (Scheme 2, *meta* and *para* refer to the position of the introduced methyl group in relation to the additional substituent at the catechol core). Formally, the demethylated intermediate **2a−d** has to be formed.

For a first approach to a formal intramolecular migration of the methyl group the demethylation of equimolar mixtures of vanillic/isovanillic acid **1a** (*meta*/*para* = 50/50) as well as vanillin/isovanillin **1b** was investigated (Table 1). As methyl acceptor, the demethylated counterparts of the substrates (**2a** and **2b**, respectively) were employed in excess (5 equiv) to ensure a sufficiently fast reaction. Indeed, the equimolar *m*/*p* mixture of substrate **1a** employed at the beginning of the

Scheme 2. Concept of Apparent Intramolecular Methyl Transfer Catalyzed by the Corrinoid-Dependent MTase from *D. hafniense* Resulting in Constitutional Isomerization

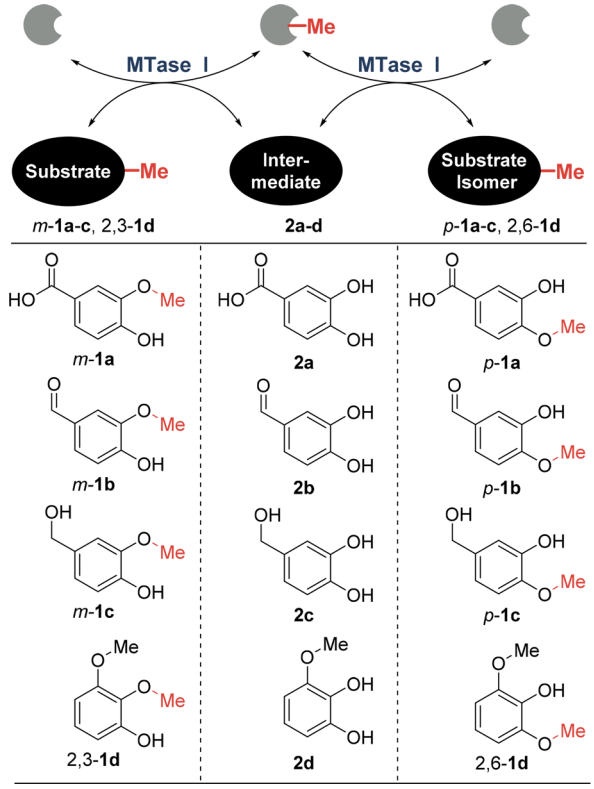


Table 1. Change of *m*/*p* Ratio of Substrates 1a and 1b in the Presence of an Excess of Acceptor Molecules 2a/b with and without Methyltransferase[a]

			ratio *m*-1/*p*-1[c]	
entry	substrate	acceptor	24 h	48 h
1	*m*-**1a**/*p*-**1a** = 50/50	**2a**	57/43	65/35
2[b]	*m*-**1a**/*p*-**1a** = 50/50	**2a**	51/49	51/49
3	*m*-**1b**/*p*-**1b** = 53/47	**2b**	70/30	70/30
4[b]	*m*-**1b**/*p*-**1b** = 53/47	**2b**	54/46	53/47

[a]Reaction conditions: substrate **1a**/**1b** (10 mM), methyl acceptor **2a**/**2b** (50 mM), MTase I (freeze-dried cell-free extract CFE, 40 mg/mL), CP (reconstituted *holo*-CP solution, 400 μL/mL, 67 mg/mL freeze-dried CFE), activation system (4.19 mM Ti^{III} citrate and 0.3 mM methyl viologen) in MOPS buffer (50 mM, 150 mM KCl, pH 6.5,) at 30 °C, 800 rpm in an Eppendorf Orbital Shaker (1.5 mL) in an inert atmosphere. [b]In the absence of enzyme. [c]After indicated incubation time product ratios were determined by HPLC-UV from areas of *m*-**1a** and *p*-**1a** and *m*-**1b** and *p*-**1b**, respectively, using calibration curves. The sum of concentrations of all species corresponded to the initial amount

biotransformation turned into a surplus of the *meta*-isomer over time, ending up with an *m*/*p* ratio of 65/35 after 48 h (Table 1, entry 1). An even more significant and also faster change of the *m*/*p* ratio was observed with the regioisomeric mixture of **1b**, ending up with 70% of vanillin *m*-**1b** already after 24 h, which did not change anymore even after a prolonged incubation time. Interestingly, both final isomer ratios corresponded to the regioselectivities obtained in case of a performed methylation of compounds **2a−b**. From these results, it can be deduced that methylation of the substrates investigated takes place preferen-

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tially at the hydroxy group in *meta*-position, whereas the *para*-methoxy group is preferred to be demethylated. No change of the *m*/*p* ratio was observed in the absence of enzyme (Table 1, entry 2 and 4).

In a next step, the isomerization of substrate **1b** was investigated in more detail using only catalytic amounts of the intermediate methyl acceptor **2b** (1 mM) (Table 2). Besides

Table 2. Results for the Isomerization Study over Time for 1b using Catalytic Amounts of Methyl Acceptor[a]

		ratio *m*-**1b**/*p*-**1b**[b]		
entry	substrate	24 h	48 h	7 days
1	*m*-**1b**/*p*-**1b** = 51/49	59/41	65/35	71/29
2	*m*-**1b**/*p*-**1b** = 73/27	77/23	76/24	75/25
3	*m*-**1b**	100/0	97/3	85/15
4	*p*-**1b**	0/100	35/65	68/32

[a]Reaction conditions: substrate **1b** (10 mM, 1.5 mg/mL), methyl acceptor **2b** (1 mM, 0.14 mg/mL), MTase I (13 mg/mL freeze-dried pure enzyme), CP (400 μL/mL reconstituted solution, 22 mg/mL freeze-dried pure enzyme), activation system (4.19 mM Ti^{III} citrate and 0.3 mM methyl viologen) in MOPS buffer (50 mM, 150 mM KCl, pH 6.5) at 30 °C, 800 rpm in an Eppendorf Orbital Shaker (1.5 mL) in an inert atmosphere. [b]Product ratios were determined after indicated time points by HPLC-UV from areas of *m*-**1c** and *p*-**1c** using calibration curves. The sum of concentrations of all species corresponded to the initial amount.

starting from an equimolar as well as a 73/27 mixture of *m*- and *p*-**1b**, each single isomer was subjected to intramolecular methyl transfer reactions as well. It has to be noted that when a crude enzyme preparation of MTase and CP (cell-free extract) was used with substrate **1b**, reduction of the aldehyde moiety of **1b** to the corresponding alcohol was observed catalyzed by the *E. coli* background (see supporting Table S1). In order to circumvent this unwanted side reaction, experiments were performed with purified enzymes instead. The presence of just catalytic amounts of **2b** proved to be sufficient to facilitate formal constitutional isomerization, resulting in comparable ratios as observed before when an excess of acceptor was employed. After an extended reaction time of 7 days, isomerization seemed to reach an equilibrium at an isomer ratio of about 3:1 (*m*/*p* = 75/25). This became especially evident in the case where the 73/27 ratio was used as the starting point; in this case, the ratio remained at a comparable level throughout the reaction course (Table 2, entry 2). Isomerization was observed best for the *para*-isomer *p*-**1b** which was isomerized to 68% of the *meta*-isomer *m*-**1b** over time, whereas isomerization of pure *m*-**1b** was significantly slower, reaching a final ratio of *m*/*p* = 85/15. These results and the fact that no isomerization at all occurred in the absence of enzyme (supporting Table S2) unambiguously confirmed an apparent intramolecular methyl transfer catalyzed by the corrinoid-dependent MTase system from *D. hafniense*.

Interestingly, the benzyl alcohol **1c** obtained by reduction of **1b** during isomerization with crude enzyme was present as an equimolar mixture of isomers (*meta*/*para* = 48/52), independent of the kind of aldehyde substrate employed (*m*-**1b** or *p*-**1b**, see supporting Table S1). In order to shed light onto the possible isomerization of **1c** as well as on the isomerization during aldehyde reduction, all isomers of substrates **1b**–**c** were subjected individually and in the absence of any methyl acceptor to corrinoid-dependent isomerization using crude enzyme preparations (Figure 1). Interestingly, both alcohol isomers *m*-**1c** and *p*-**1c** were interconverted leading approximately to a 1:1

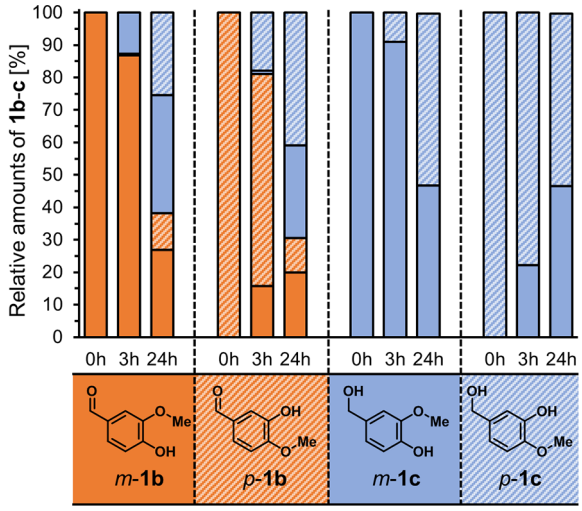

Figure 1. Relative amounts of **1b**-**c** during isomerization with CFE preparations of MTase I and CP in the absence of an acceptor molecule. Reaction conditions: substrate (10 mM), MTase I (freeze-dried CFE, 40 mg/mL), CP (400 μL/mL reconstituted solution, 67 mg/mL CFE) in MOPS/KOH buffer (50 mM, 150 mM KCl, pH 6.5) at 800 rpm, 30 °C for 24 h. Product ratios were determined via HPLC using calibration curves. The sum of concentrations of all species corresponded to the initial amount.

mixture in the absence of any methyl acceptor present using a crude enzyme preparation. However, when performing the same reaction with purified enzymes, isomerization occurred only very slowly for *m*-**1b** and *m*-**1c** (1–3%, supporting Table S3, entry 1–4). Slightly higher amounts of the corresponding regioisomer were formed in the case of the *para*-methoxy substrates *p*-**1b** and *p*-**1c** (9% and 3%, respectively). Although isomerization can be envisioned without any methyl acceptor added according to Scheme 2, a faster isomerization was observed in the presence of a crude enzyme preparation.

As further substrates the bis-methyl donors 2,3- and 2,6-dimethoxyphenol (2,3-**1d** and 2,6-**1d**) were investigated, being also constitutional isomers. Theoretically, in either case, both methyl groups are accessible for transfer onto an acceptor molecule. However, MTase I preferentially catalyzes the demethylation of a methoxy group in *ortho*-position to a hydroxy group. Since 2,3-**1d** exhibits only a single methoxy group matching this pattern, a stepwise demethylation was anticipated being different from the demethylation route involving 2,6-**1d** as substrate, where both methoxy groups are in *ortho*-position to a hydroxy group. Indeed, following as an initial experiment, the demethylation of 2,3-**1d** over time with an excess of acceptor **2b** (Figure 2a) showed that at first the methyl group next to the hydroxy function is cleaved, resulting in the accumulation of 3-methoxycatechol 3-**2d**. Displaying now the beneficial *ortho*-OH pattern, 3-**2d** is able to donate the last methyl group efficiently as well, ending up in pyrogallol **3d**. A demethylation in 3-position of 2,3-**1d**, which would result in 2-methoxyresorcinol 2-**2d**, was not observed at all. Interestingly, with proceeding reaction also the formation of the regioisomeric 2,6-**1d** (3%) became evident, suggesting an apparent intramolecular methyl transfer resulting in isomerization. Similarly, also in the case of the demethylation of 2,6-**1d** (Figure 2b) minor amounts of the respective isomer were formed after 48 h.

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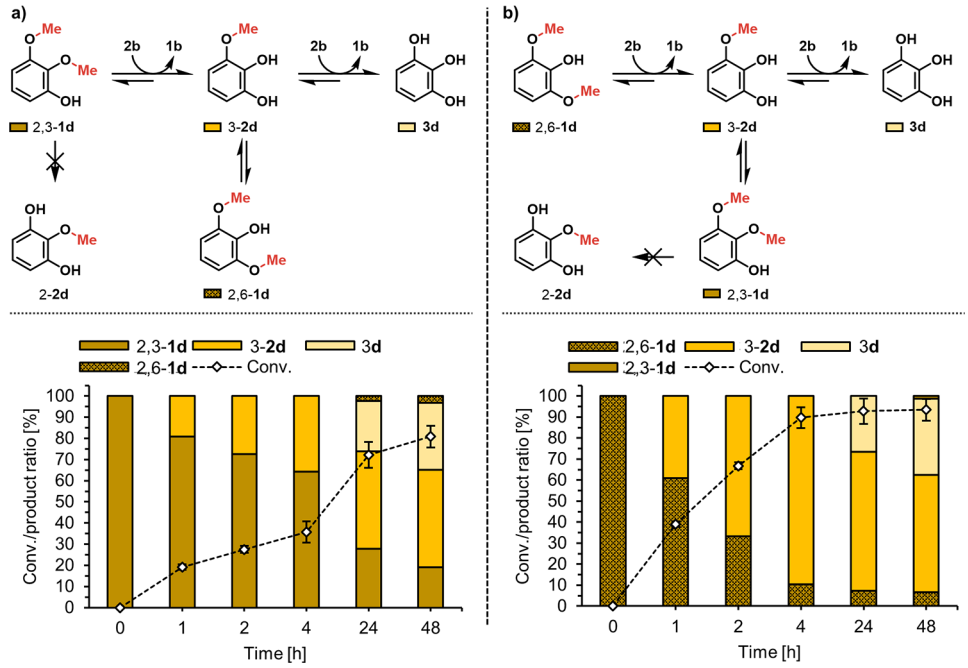

Figure 2. Stepwise methyl transfer in the (a) demethylation of bis-methyl donor 2,3-**1d** and (b) demethylation of 2,6-**1d** using **2b** as methyl acceptor. Reaction conditions: substrate **1d** (10 mM, 1.5 mg/mL), methyl acceptor **2b** (50 mM, 6.9 mg/mL), MTase I (40 mg/mL freeze-dried CFE), CP (400 μL/mL reconstituted holo-CP solution, 67 mg/mL freeze-dried CFE), activation system (0.3 mM methyl viologen and 4.19 mM Ti^{III} citrate) in MOPS buffer (50 mM, 150 mM KCl, pH 6.5) at 30 °C, 800 rpm in an Eppendorf Orbital Shaker (1.5 mL) in an inert atmosphere. Conversions of substrates and relative product amounts were determined after indicated time points by HPLC-UV from areas of **1d**, **2d**, and **3d** using calibration curves.

Furthermore, the demethylation experiment indicates that the methyl group is transferred from the substrate onto the cobalamin cofactor followed by dissociation of the substrate from the enzyme, which enables another substrate molecule to bind again and take the methyl group. This dissociation and transfer to another molecule has previously been exploited for trans-methylation.[33] Isomer 2,6-**1d** possesses two equal methyl groups in *ortho*-position to a hydroxy moiety, which are both available for methyl transfer leading to the formation 3-**2d** in either case. Upon accumulation, 3-**2d** also served as a methyl donor and was converted to **3d**. Nevertheless, small amounts of the other regiosiomer 2,3-**1d** produced indicated an internal methyl transfer via 3-**2d**. Presumably, the present excess of methyl acceptor **2b** pushed the reaction equilibrium toward the second demethylation.

Indeed, subjecting both dimethoxyphenols 2,3-**1d** and 2,6-**1d** to corrinoid-dependent methyl transfer in the absence of a methyl acceptor using cell-free extracts clearly demonstrated isomerization (Figure 3). Formation of 3-**2d** (e.g., 8−10% after 24 h) was observed for both substrates 2,3- and 2,6-**1d**, whereby 3-**2d** represents the demethylated intermediate required for the interconversion of isomers. It is worthwhile to note that in this isomerization experiment neither the formation of the 2-fold demethylated product **3d** nor formation of 2-methoxyresorcinol 2-**2d** was observed. When the experiment was performed with purified enzymes only, no formation of 3-**2d** was observed, and isomerization was significantly decelerated (supporting Table S4). In the experiment with CFE, a final product ratio of 3-**2d**/2,3-**1d**/2,6-**1d** = 15/20/65 was obtained independent from the substrate used (2,3-**1d** or 2,6-**1d**), suggesting an equilibrium between the regioisomers.

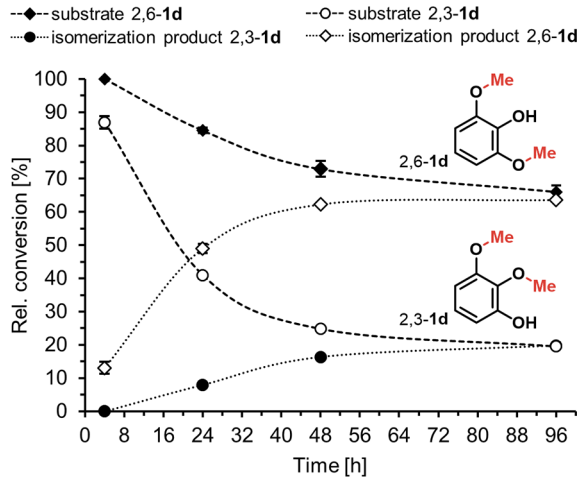

Figure 3. Isomerization of substrate 2,3-**1d** (open symbols) and 2,6-**1d** (filled symbols) in the absence of a methyl acceptor over time. 100% corresponds to the sum of 2,6-**1d**, 2,3-**1d**, and 3-**2d**. Reactions conditions: substrate **1d** (10 mM, 1.5 mg/mL), MTase I (40 mg/mL freeze-dried CFE), CP (400 μL/mL reconstituted holo-CP solution, 67 mg/mL freeze-dried CFE), activation system (4.19 mM Ti^{III} citrate and 0.3 mM methyl viologen) in MOPS buffer (50 mM, 150 mM KCl, pH 6.5) at 30 °C, 800 rpm in an Eppendorf Orbital Shaker (1.5 mL) in an inert atmosphere. Relative amounts were determined after indicated time points by HPLC-UV from respective areas using calibration curves.

CONCLUSIONS

This study demonstrates the constitutional isomerization of catechol methyl ethers via an apparent intramolecular methyl transfer employing cobalamin-dependent methyl transferases. In contrast to radical-based reactions for rearrangements, which

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have been reported for a different class of cobalamin-dependent enzymes, apparent intramolecular transmethylation is described here which occurs via nucleophilic reactions involving the Co^{I} cobalamin species as supernucleophile. Substrates sharing the structural motif of a catechol core, involving both protocatechuate derivatives as well as dimethoxyphenols were successfully isomerized to the corresponding regioisomers. To the best of our knowledge, isomerization by methyl transfer has never been reported for other methyl transferases before, making cobalamin-dependent MTases especially valuable and promising enzymes for further research in the field of biocatalysis allowing to extend the toolbox[49−51] of biocatalytic reactions.

EXPERIMENTAL SECTION

General Remarks. All chemicals and solvents were obtained from commercial sources (VWR International/Merck, Roth, Sigma-Aldrich/Fluka) and used as obtained unless stated otherwise. Biocatalysts were produced in *E. coli* as described before[33] and used as frieze-dried cell free extracts (CFE). Alternatively, enzymes were purified by affinity chromatography (Strep-Tactin technology, IBA Lifesciences) according to the manual provided by the supplier and used as freeze-dried pure preparation. Due to the oxygen-sensitivity displayed by the corrinoid cofactor, biocatalytic reactions were performed in degassed buffers under inert atmosphere (N2 5.0) in a MBraun LABstar glovebox, which was equipped with a MB-OX-EC O_2-sensor and an Eppendorf Thermomixer comfort. For detailed analytical methods see electronic Supporting Information.

General Procedure for Preparation of *holo*-CP. Since the heterologous *E. coli* host used for recombinant enzyme production does not synthesize methylcobalamin,[52] the CP was reconstituted with exogenous cofactor under inert atmosphere. A reconstitution buffer was prepared by dissolving methyl cobalamin (2 mM) in the presence of DTT (2 mM) and betaine (3 M) in TRIS/HCl buffer (50 mM, pH 7, 0.1 mM PMSF, 0.5 mM DTT). Then, freeze-dried CP (100 mg/mL CFE or 33 mg/mL purified enzyme, respectively) was added to the reconstitution buffer (1 mL) and incubated for 2 h at 4 °C. Then, unbound cobalamin and salts were removed using a PD MidiTrap G-25 column (GE Healthcare), and *holo*-CP was eluted with MOPS/KOH buffer (100 mM, 150 mM KCl, pH 6.5). Successful in vitro reconstitution was verified by the bright red color of obtained protein fraction based on the characteristic absorbance of the Co^{III} in methyl cobalamin. The CP-solution (containing 22 mg/mL pure enzyme or 66.7 mg/mL CFE, respectively) was stored at 4 °C until further use.

General Procedure for Analytical Biotransformation. Biotransformations were carried out at least in triplicates in 1.5 mL Eppendorf tubes on 180 μL scale as follows: freeze-dried MTase I (40 mg/mL CFE or 13 mg/mL pure enzyme, respectively) was rehydrated in *holo*-CP solution (400 μL/mL). Then, appropriate amounts of substrate (final concentration 10 mM) and methyl acceptor (as indicated) were added as stock solutions in MOPS/KOH buffer (50 mM, pH 6.5, 150 mM KCl). If not indicated otherwise, a chemical activation system consisting of methyl viologen (0.3 mM) and Ti^{III} chloride (4.15 mM) was added for reducing undesired Co^{II}. The latter one was added as a stock solution in MOPS/KOH (1 M, 333 mM sodium citrate, pH 7.9). Reaction samples were shaken at 800 rpm and 30 °C for 24 h. The MTase system employed as described above (involving MTase I and CP) displayed an activity in the range 10−20 mU. Initial rates were determined as described before[33] and enzyme activity (mU) was defined as the amount of enzyme that catalyzes the conversion of 1 nanomole of substrate per minute.

Determination of Conversion. After the indicated reaction time, an aliquot (30 μL) was withdrawn, quenched by adding MeCN (180 μL), incubated (room temperature, 30 min), and diluted with deionized water (90 μL). Denatured protein was removed by centrifugation (14 000 rpm, 15 min), the supernatant was filtered and analyzed by HLPC (Agilent 1260 Infinity system, UV detector) using an achiral C18 column (Phenomenex, Luna, C18 100c, 250 × 4.6 mm, 5 mm). Eluent: H_2O/MeCN (containing 0.1% TFA), flow rate: 1 mL/min. Compounds were detected by UV-absorption, and conversions were calculated according to calibrations curves.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.8b05072.

Supporting figures, tables, and discussion as well as detailed analytical methods including HPLC chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

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