Supporting Information

Oxygen-free Regioselective Biocatalytic Demethylation of Methyl-phenyl Ethers via Methyltransfer Employing Veratrol-O-demethylase

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Optimization of the demethylation of the vdmB system

The first parameter investigated was the buffer composition and the pH. Four buffers (HEPES, MOPS, MES and PIPES) were analyzed in a pH range from 6.5 to 8 (Figure S1). The reaction worked best in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, dark blue, Figure S1). Additionally, vdmB and CP also tolerated higher pH values with the CHES buffer (50 mM, pH 9.5 and 10, 150 mM KCl, Figure S2).

![Bar graph showing the optimization study of different buffer components with pH values ranging from 6.5 to 8. Reaction conditions: substrate 1a (10 mM, 1.4 mg/mL), methyl acceptor 1m (50 mM, 6.9 mg/mL), vdmB (40 mg/mL cell-free extract, ≈ 0.077 mM vdmB), CP (21 mg/mL or 1 mM pure CP loaded with methyl cobalamin) in 50 mM buffer HEPES (dark blue), MOPS (blue), MES (light blue) or PIPES (green) supplemented with 150 mM KCl at 30 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 120 µL. All reactions were quenched by the addition of MeCN (60 vol. % final conc.) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV]
Figure S2. Optimization study of CHES-buffer with higher pH values ranging from 8.5 to 10. Reaction conditions: substrate 1a (10 mM, 1.4 mg/mL), methyl acceptor 1m (50 mM, 6.9 mg/mL), vdmB (40 mg/mL cell-free extract, ≡ 0.077 mM vdmB), CP (21 mg/mL, 1 mM pure CP loaded with methyl cobalamin) in CHES buffer (50 mM, 150 mM KCl) at 30 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 120 µL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.

The methyltransferase vdmB possesses zinc binding motifs according to literature. A concentration of 100 µM zinc was reported as most beneficial for the demethylation reaction whereas our optimization study showed that a 20 µM zinc concentration was sufficient (Figure S3).

Figure S3. Optimization study of the addition of different zinc concentrations. Reaction conditions: substrate 1a (10 mM, 1.4 mg/mL), methyl acceptor 1m (50 mM, 6.9 mg/mL), vdmB (40 mg/mL cell-free extract, ≡ 0.077 mM vdmB), CP (21 mg/mL, 1 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl) supplemented with different concentrations of ZnCl2 at 30 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 120 µL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.
Best conversion was achieved at a temperature of 35 °C (Figure S4). At higher temperatures like 40 °C, the conversion diminished significantly.

![Graph showing conversion vs temperature](image)

**Figure S4.** Optimization study of the temperature profile ranging from 25 to 40 °C. Reaction conditions: substrate **1a** (10 mM, 1.4 mg/mL), methyl acceptor **1n** (20 mM, 2.5 mg/mL), vdmB (40 mg/mL cell-free extract, ≈ 0.077 mM vdmB), CP (21 mg/mL, 1 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) at 25, 30, 35 or 40 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 120 µL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.

In order to improve the bioavailability of apolar substrates, varied concentrations of DMSO (up to 10% v/v) were tested with vdmB. A concentration of 2% v/v DMSO led to the best product formation (Figure S5). Other co-solvents such as MeOH and EtOH (2% v/v) were screened for better acceptance, but none was as good as DMSO (Figure S6).
Figure S5. Study of varied DMSO concentrations. Reaction conditions: substrate 1b (10 mM, 1.5 mg/mL), methyl acceptor 1n (20 mM, 2.5 mg/mL), vdmB (40 mg/mL cell-free extract, ≈ 0.077 mM vdmB), CP (21 mg/mL, 1 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) and different DMSO concentrations (0-10% v/v) at 30 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 120 µL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.

Figure S6. Study of different co-solvents (2% v/v). Reaction conditions: substrate 1b (10 mM, 1.5 mg/mL), methyl acceptor 1n (20 mM, 2.5 mg/mL), vdmB (40 mg/mL cell-free extract, ≈ 0.077 mM vdmB), CP (21 mg/mL, 1 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) combined with the co-solvent [2% v/v, dimethylsulfoxide (DMSO), methanol (MeOH), ethanol (EtOH), acetone or 1,4-dioxane or tetrahydrofuran (THF)] at 30 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 120 µL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.
The last parameter to be optimized was the ratio of vdmB to CP whereby for the experiment crude protein preparations were used, of which the content of pure vdmB and CP was known (Table S1). The best results were obtained by employing at least a two-fold excess of CP over vdmB concerning the amount of crude preparations (Entries 2, 3 and 5). Looking at the corresponding ratio of pure proteins, most product was formed at a 14-21 fold excess of CP in comparison to vdmB (Entries 2, 3 and 5). By incorporating vdmB in a two-fold excess over CP within crude preparations, the reaction produced half as much for the two demethylated products compared to the optimized conditions (Entries 6 and 8). Concluding, the carrier protein was the limiting factor for the demethylation reaction and the use of an excess was beneficial. Therefore, it was used for all subsequent experiments.

Table S1. Optimization of ratio vdmB to CP.

| Entry | ratio CFE preparations vdmB:CP | ratio corresponding to pure protein vdmB:CP | product formation [%] |
|-------|--------------------------|------------------------------------------|---------------------|
|       |                          |                                          | 2-methoxy-4-methylenol 2b [%] | 2-methoxy-5-methylenol 3b [%] |
| 1     | 1:1                      | 1:13                                     | 9.0±0.3              | 4.7±0.1              |
| 2     | 1:2                      | 1:19                                     | 18±0.1               | 9.7±0.3              |
| 3     | 1:3                      | 1:21                                     | 18.1±1.1             | 10.0±0.8             |
| 4     | 1.5:1.5                  | 1:10                                     | 10.6±0.3             | 5.5±0.2              |
| 5     | 1.5:3                    | 1:14                                     | 19.4±0.3             | 10.9±0.4             |
| 6     | 2:1                      | 1:6                                      | 8.7±0.1              | 4.4±0.2              |
| 7     | 2:2                      | 1:10                                     | 17.9±0.6             | 9.7±0.2              |
| 8     | 3:1.5                    | 1:5                                      | 8.9±0.5              | 4.7±0.4              |

Reaction conditions: substrate 1b (10 mM, 1.5 mg/mL), methyl acceptor 1n (20 mM, 2.5 mg/mL), CP (“1” = 21 mg/mL, = 1 mM pure CP with MeCob; “1.5” = 26 mg/mL, = 1.2 mM pure CP with MeCob; “2” = 31 mg/mL, = 1.5 mM pure CP with MeCob; “3” = 33 mg/mL, = 1.6 mM pure CP with MeCob) and vdmB (“1” = 40 mg/mL CFE, = 0.077 mM pure vdmB, “1.5” = 60 mg/mL CFE, = 0.12 mM pure vdmB, “2” = 80 mg/mL CFE, = 0.154 mM pure vdmB, “3” = 120 mg/mL CFE, = 0.231 mM pure vdmB) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 μM ZnCl2) at 30 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. All reactions were quenched by the addition of MeCN (60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.
Effect of temperature (25 and 35 °C)

Table S2. Product formation of veratrol derivatives at 25 and 35 °C.

| substrate [10 mM] | T [°C] | conv. [%] | mono-demethylation | di-demethylation |
|------------------|--------|-----------|--------------------|-----------------|
| 1                | 25     | 55<sup>a</sup> | 2a (96%, 95%)<sup>b</sup> | 4a (4%, 5%)<sup>b</sup> |
| 2                | 35     | 55<sup>b</sup> |                     |                 |
| 1b               | 25     | 77<sup>a</sup> | 2b (52%, 35%)<sup>b</sup> | 3b (35%, 26%)<sup>b</sup> |
| 3                | 35     | 62<sup>b</sup> |                     |                 |
| 1c               | 25     | 52<sup>a</sup> | 2c (81%, 68%)<sup>b</sup> | 3c (11%, 13%)<sup>b</sup> |
| 2                | 35     | 59<sup>b</sup> |                     |                 |
| 3                | 25     | 52<sup>a</sup> | 2d (>99%)<sup>a,b</sup> |                 |
| 4                | 35     | 30<sup>b</sup> |                     |                 |
| 5                | 25     | 42<sup>a</sup> | 2e (55%, 53%)<sup>b</sup> | 3e (45%, 47%)<sup>b</sup> |
| 6                | 35     | 44<sup>a</sup> | 2f (75%, 81%)<sup>b</sup> | 4f (25%, 19%)<sup>b</sup> |

<sup>a</sup> product formation at 25 °C
<sup>b</sup> product formation at 35 °C

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, 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) at either 25 or 35 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 500 µL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.
Table S3. Product formation of 1,3-dimethoxybenzene derivatives at 25 and 35 °C.

| substrate [10 mM] | T [°C] | conv. [%] | mono-demethylation | di-demethylation |
|-------------------|--------|-----------|--------------------|------------------|
| 1                  |        |           |                    |                  |
| 1g                | 25     | 52        | 2g (44%, 31%)      | 3g (12%, 13%)    |
|                   | 35     | 61        |                    | 4g (44%, 56%)    |
| 2                  |        |           |                    |                  |
| 3g                | 25     | 32        | 4g (>99%)          |                  |
|                   | 35     | 47        |                    |                  |
| 3                  |        |           |                    |                  |
| 1h                | 25     | 50        | 2h (>99%)          |                  |
|                   | 35     | 32        |                    |                  |
| 4                  |        |           |                    |                  |
| 1i                | 25     | 45        | 2i (62%, 64%)      | 3i (38%, 36%)    |
|                   | 35     | 56        |                    |                  |

* product formation at 25 °C  
*b product formation at 35 °C

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, 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 μM ZnCl2) at either 25 or 35 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 500 μL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.
Substrates which were not accepted by the vdmB methyltransferase

Figure S7. Aromatic and non-aromatic substrates which were not demethylated by the vdmB methyltransferase. Reaction conditions: substrates S1a-S27a (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, 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) at 35 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume: 120 µL. All reactions were quenched by the addition of MeCN (final conc. 60 vol. %) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV.
Materials

All starting materials were obtained from Sigma-Aldrich, Alfa Aesar or TCI-Chemicals and used as received unless stated otherwise. Yatein 1k was chemically synthesized according to literature.2

The following compounds were obtained by the mentioned suppliers and used as corresponding references for HPLC-UV qualification: dimethoxybenzol (veratrol, 1a), guaiacol (2a), catechol (4a), 3,4-dimethoxymethane (1b), 2-methoxy-4-methylphenol (2b), 2-methoxy-5-methylphenol (3b), 3,4-dimethoxybenzaldehyde (4c), 2,3-dimethoxyphenol (1d), 4-methoxyresorcinol (3d), 3,4-dimethoxybenzyl alcohol (1e), vanillyl alcohol (2e), isovanillyl alcohol (3e), 2,3-dimethoxynaphthalene (1f), 3-methoxy-2-naphthol (2f), 2,3-dihydroxynaphthalene (4f), 1,2,3-trimethoxybenzene (1g), 2,3-dimethoxyphenol (2g), 2,6-dimethoxyphenol (3g), 3-methoxyacetate (4g), 1,3,5-trimethoxybenzene (1h), 3,5-dimethoxyphenol (2h), 2,4,6-trimethoxytoluene (1i), papaverine (1j), orcinol (4n), 3-methoxy-5-methylaniline (2n), anisole (S1a), 2-chloroanisole (S2a), 2-methoxyphenylamine (S3a), 2-methoxypropyridine (S4a), 3-methoxyphenol (S5a), m-anisidine (S6a), 3,4-dimethoxybenzoic acid (S7a), 2,3-dimethoxyphenol (S8a), 3,4-methoxyccinnamic acid (S9a), 3,4-dimethoxybenzylamine (S10a), 3,4-dimethoxythiophenol (S11a), 2,3-dimethoxyquinol (S12a), 2,3-dimethoxy-1,4-benzoquinone (S14a), 2',4',5',5'-tetramethoxymethane (S24a), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (S15a), N,N'-dimethoxy-1,2-phenylenediamine (S16a), 3,5-dimethoxyamine (S17a), 2-(3,4-dimethoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaboralone (S18a), 2-(3,4,5-trimethoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (S19a), 3,4,5-trimethoxyphenol (S20a), retusine (S21a), colchicine (S22a), carbonic acid dimethyl ester (S23a), tetramethoxymethane (S24a), trimethyl phosphate (S25a), L-methionine (S26a), 3-(methylsulfonyl)propionic acid (S27a).

Plasmids used in this study

Table S4. Empty plasmids and plasmids with genes encoding for enzymes from *Desulfitobacterium hafniense* and *Acetobacterium dehalogenans*.

| Plasmidsa | Origin (GeneBank ID) | Description/Comments |
|-----------|----------------------|----------------------|
| pASK-IBA3plus | IBA-Lifescience | *Pre*, *Amp*, *ColE1ori*, C-terminal StrepTag |
| pEG459 | Trends Biotechnol.3, Biotechnol. Commun. Chem.4, Adv. Synth. Catal.5, ACS Catal.6 | dhaf4611 (CP) from *Desulfitobacterium hafniense*, codon optimized gene cloned into pASK-IBA3plus, EcoRI & HindIII |
| pET21a(+) | Novagen | *Pre*la, *Amp*, *pBR322ori*, N- and/or C-terminal HisTag |
| pEG462 | this study | vdmB-MT (veratrol O-demethylase) from *Acetobacterium dehalogenans*, wild-type gene cloned into pET21a(+), Ndel & Xhol |
| pEG464 | this study | vdmA (CP) from *Acetobacterium dehalogenans*, wild-type gene cloned into pET21a(+), Ndel & Xhol |

a pEG number is an internal numbering of plasmids (stands for plasmid of the Elk Group)
DNA and amino acid sequences of used genes

Dhaf4611 (CP):

**DNA sequence**

ATGAGCAAAAATCGCCGAAGTTAAGCAATGGAAGTAAAGCAATGGTTGCAGCTGGCTGAGAGGCAATGGGT
TTCAAGAGGGACTGATCAGTGAAGTAAAGCAATGGTTGAGAAGCAGGTAAAGCAAAACTGGTTCCGGGTCTGG
TTCAAGAGGCACTGGATGTAAGTAAAGCAATGGTTGAGAAGCAGGTAAAGCAAAACTGGTTCCGG

**Amino acid sequence**

MSKIAEVKAMVEAKLVPVLQVQALDAGAAGIDLAGMIDSMGVVGDKFSAGELFVPEMLMAAKAM
SKGVDVLKPHLTGESATLSGTVAGDLDHIDIKNLVAMMLVESGFGNVDLGVDVSAEKFDVADVREND
NVKIVACCGTTLTTPAMKEVQLNKSGLTFKVIYGGAPVSQAMADEIGAGDFAPDAGAAVAR

vdmB-MT (veratrol O-demethylase):

**DNA sequence**

ATGAAATCACAGAGAGAAATTTTTTGCGATGATCGAGGGAAAGAAACCGGAGTTTATCCCGAATTCAT
GGAGGTTTTATAAATCTGTTGATGGAACAAAGATATATAGACAGTCTTTTCAAGGGGAATTAGATG
CTATGTTGTCATTTGAAATGGCAACCCAAAGAGGAATCAATCTTGAATTTTCAATGTTGATACACTGGGGA
TAAGAAGAGCTATGTTGAAATGGGTAATTAGTAAAAATGTGGTTTTGTTTTGTTTTGTTTTGTTTTG

**Amino acid sequence**

MNQRENFFAMIEGKKPEFIPNSMEVYKTCVMATSIIDSPFQGGLDAYGVNWIATKEGSIPEPNKFMNDITD
WKDHVKFNPVNTLGIIEAAAMELAVKRDMEVINVFASACGLFERMAAAMFGFENTLCLSVLEDPSREFFE
AFADFRIEDCHNRIRIDAYQPDVITYFDDLATANGLFMSPKVRYEIKPAHQRJAEAVTSRGVFQSHTCGKCEE
vdmA (CP):

**DNA sequence**

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ATGATTTCAAAATTACAGAAGCTCAAAACAATTGTAATGGAAGCAGGAAAGTCCAGAGAGTTGCTGACAGTCCAGGGAAGGAGTCAAGAGATCGGGCCAGCGGTCCAGGGAGACGGAGATGGTGAAATGCTGGTGCGACAACGCCGCTTCACTGGAAACTCCCTGCTCAATGATGATTGAAAGC
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**Amino acid sequence**

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MSKITEVKQLVEAGSKSKIGPAVQELNAGGCCPVEILQPMVDSMSVVDKFSAGEIFVPEMLAAMSKGVDVLRLMAGDNAASLGTGVIWAGDLHDKGLNVLVSMMIESAGFTMVDMGVDPHEKFVAAARENDVTLIACGLLTTTTPALKEAVATIKASGLAGCKVIVVGPVTSEFAAEGADGYAADAGSAAVKAADKDLVK*
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Methods

Protein expression

The plasmids containing the respective genes (Table S3) were transformed into chemically competent *E. coli* BL21/Lemo21 (DE3) cells. The over-night cultures were prepared by picking one colony from an agar plate and suspending it in LB-medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with ampicillin (100 µg/mL) followed by an over-night incubation at 37 °C and 120 rpm. The LB-medium (0.5-1 L, non-baffled flasks) supplemented with the ampicillin was inoculated with the over-night culture and incubated at 37 °C and 140 rpm until an OD$_{600}$ of 0.6-0.8. The protein expression was induced by the appropriate addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) or anhydrotetracycline (AHTC, Table S4). Subsequently, the cell suspension was incubated for 24 h at 25 °C prior to its harvest by centrifugation (5,000 rpm, 4 °C, 10 min). In order to prepare the cell free extract (CFE) cells were resuspended in HEPES-buffer (50 mM, pH 6.5, 150 mM KCl, 7 mL buffer to 1 g wet cells) and disrupted by ultrasonication on ice (40% amplitude, 3x 6 min, pulse 1 sec, pause 2 sec) using a Sonics & Materials Vibra Cell CV26 (13 mm tip, amplitude range 36-240). After the crude cell extract was separated from the cell debris by centrifugation (30 min, 14,000 rpm, 23,519 g), the extract was frozen in liquid nitrogen and lyophilized over-night. The CFE was analyzed on SDS-PAGE (Figure S9) and stored at -20 °C or instantly used for further experiments.

**Table S5.** Expression conditions for vdmB, vdmA and dhaf4611 regarding their expression plasmid, host and inducer. All proteins were expressed at 25 °C and 140 rpm for 24 h.

| Protein  | Plasmid       | Host (DE3) | Inducer          |
|----------|---------------|------------|------------------|
| vdmB     | pET21a        | BL21       | 0.5 mM IPTG$^a$  |
| vdmA     | pET21a        | BL21       | 1 mM IPTG$^a$    |
| dhaf4611 | pASKIBA3plus  | Lemo21     | 0.2 µg/mL AnTc$^b$ |

$^a$ IPTG= isopropyl β-D-1-thiogalactopyranoside  
$^b$ AnTc= anhydrotetracycline
The protein content of the single fraction of vdmB-MT (Figure S9, lane 1, red box) amounted to 0.7 µg which was calculated by densitometry (ImageJ).

![Image](image.png)

Figure S8. SDS-PAGE of the protein expression of vdmB (MT, lane 1, 36 kDa) and dhaf4611 (CP, lane 2, 21 kD). Both proteins were applied with 10 µg protein per lane.

**Protein purification of CP**

The carrier protein dhaf4611 (CP) was purified according to the Iba Strep-tag®II protocol and was obtained in almost pure form at 20 kDa (Figure S10, pure CP) with a faint band at 35 kDa as impurity. This purified protein was further used to determine the protein content of pure CP in the crude cell-free extract CFE (CP, see chapter: Determination of the pure holo-CP content in the CFE).
Figure S9. SDS-PAGE of the protein purification of and dhaf4611 (CP). The carrier protein was successfully purified according to the Iba Strep-tag® II purification protocol leading to a protein band at 21 kDa (15 µg protein per lane; FT = flow through fraction, W = washing fraction; pure CP = purified dhaf4611).

Preparation of holo-CP (loading of methylcobalamin)

The loading of the CP with its cofactor methylcobalamin hydrate was performed under inert atmosphere accordingly to the optimized protocol in literature.4–6 As a first step, the reconstitution buffer was prepared consisting of methylcobalamin (6 mM; four times excess to CP) and betaine (3 M) which were dissolved in Tris/HCl buffer (50 mM, pH 7, 2.5 mM DTT, 0.1 mM PMSF). The lyophilized crude CP (133 mg/mL CFE or 31 mg/mL, = 1.5 mM pure CP with MeCob; a lyophilized powder was used to simplify the transfer of the protein to an inert atmosphere) was mixed with the reconstitution buffer (1mL) and incubated for at least 2 h at 4°C. This incubation step was crucial for complete loading of the CP with the methylcobalamin. The removal of salts and unbound cofactor was performed via a desalting step using a PD MidiTrap™ G-25 column (GE Healthcare) or PD 10™ G-25 column (GE Healthcare) according to the manufacturer’s manual. During this step, the buffer was exchanged to HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl2) yielding a red colored protein solution (66.7 mg/mL CFE or 21 mg/mL pure CP). Prior to biocatalytic reactions, this holo-CP solution was stored at 4 °C.

Biocatalyst preparation for demethylation

All biotransformation reactions were performed on a 0.5 or 1 mL scale under inert atmosphere (99.8% N2 gas, 200 bar) in a glove box. The lyophilized vdmB (40 mg/mL final concentration cell-free extract) was dissolved in the holo-CP solution (400 µL/mL, 21 mg/mL pure CP). As a model methyl donor veratrol 1a (10 mM) or 3,4-dimethoxytoluene 1b (10 mM) was used with either 3,4-dihydroxybenzaldehyde 1m (50 mM) or later with orcinol 1n (20 mM) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl2). For all screened substrates, stock solutions were prepared in HEPES buffer containing DMSO (20% for dimethoxy substrates, 50% for compounds with at least
three methoxy groups). All demethylation reaction samples containing the proteins with the methyl donor and acceptor in HEPES buffer were shaken in a vertical position in an Eppendorf Thermomixer® at 35 °C and 800 rpm for 24 h in the glove-box (inert atmosphere). Samples (90 µL) were quenched with acetonitrile (MeCN, 540 µL), mixed thoroughly and incubated for 20 min at room temperature. Afterwards, water (270 µL, HPLC pure) was added and the denatured protein was removed by centrifugation (14,000 rpm, 10 min). The supernatant was filtered through a pipette tip filled with cotton. The samples were analyzed on HPLC using the LUNA C18 column with a mobile phase consisting of water and MeCN with 0.1% trifluoroacetic acid (TFA). The flow rate was set to 1 mL/min. In the standard method the column was rinsed for 2 min with 100% H2O, then a gradient from 0% MeCN to 40% MeCN over 13 min was applied, followed by a gradient to 100% MeCN for within 5 min, which was held for 2 min. All retention times and the corresponding k-values of the methyl donors and acceptors are summarized in Table S5.

Isolation of papaverine and 2,4,6-trimethoxytoluene products for NMR analytics

Several biotransformations were performed with as 0.5 mL reaction samples for papaverine (50 samples, 3.4 mg/sample, 170 mg in total, 0.5 mmol) and 2,4,6-trimethoxytoluene (28 samples, 1.7 mg/sample, 47.6 mg in total, 0.28 mmol) for 24 h at 35 °C and 800 rpm in an Eppendorf Thermomixer® under inert atmosphere in a glove box. Each reaction sample was extracted with EtOAc (3x 500 µL) and the organic phase was dried (Na2SO4). All samples were pooled and the solvent was evaporated to dryness under reduced pressure. The reaction sample was dissolved in H2O/MeCN (1:3, 1 mL) mixture and purified by preparative HPLC (Phenomenex Luna® C18 (2), 100A, 250 x 21.2 mm, 5 µm). The substrate papaverine 1j was recovered (27% isolated yield) as well as the products, namely the mono-demethylated product 2j (14.2 mg, 12% isolated yield) and the di-demethylated product 4j (1 mg, 0.9% isolated yield). Missing amounts in the mass balance are due to impure fractions. Both products were isolated by preparative HPLC (Method A) and analyzed in methanol-d4 by 1H NMR, 13C{1H} NMR and 2D NMRs (1H-1H-COSY, 1H-1H-NOESY, 1H-13C-HSQC, 1H-13C-HMBC). The substrate 2,4,6-trimethoxytoluene 1i was demethylated to 2-hydroxy-4,6-dimethoxytoluene 2i (3.6 mg, 17% isolated yield) and 4-hydroxy-2,6-dimethoxytoluene 3i (2.6 mg, 12% isolated yield). Missing amounts in the mass balance are due to impure fractions. These products were isolated by preparative HPLC (Method B) and analyzed in CDCl3 by 1H NMR, 13C{1H} NMR. All isolated products were calibrated on HPLC to calculate the product formation of the biotransformation.

Semi-preparative scale of yatein for NMR analytics

Special approaches were used for the demethylation of yatein 1k (100 mg, 0.15 mmol, 60% isolated yield) on a 25 mL semi-preparative scale. After all reagents were set up in a 100 mL round bottom flask under inert atmosphere, the flask was sealed by a rubber plug, parafilm and a clamp. The biotransformation was incubated for 24 h at 25 °C and 160 rpm in the incubator shaker (Multitron Infors Ht®) outside the glove box. The reaction was quenched, extracted with EtOAC (3x 25 mL), prepared for preparative HPLC as stated above (Method C) and the product 4-O-demethylyatein 2k (9% isolated yield) analyzed in CDCl3 by 1H NMR, 13C{1H} NMR.
Determination of the pure holo-CP content in the CFE

First, different spectra of methylcobalamin (MeCob, square, dark red) and the pure holo-CP (pure holo-CP, triangle, red, Figure S11) were measured between 350 to 650 nm on the UV-spectrophotometer under inert atmosphere by sealing the UV-cuvettes with parafilm. The HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) was used as a blank for all measurements. Both spectra of methylcobalamin and the pure holo-CP share the same absorption maximum at 520 nm which refers to the [Cob^{III}-Me] state. The unloaded and reduced state [Cob⁻] appears at 375 nm according to literature. The extinction coefficient of pure holo-CP (ε_{520-600nm} = 0.0834 mg⁻¹ mL⁻¹ cm⁻¹) was calculated by different concentrations of this protein (Figure S12). With this extinction coefficient the total amount of pure holo-CP in the CFE could be calculated.

Figure S10. Absorption spectra of methylcobalamin (MeCob, square, dark red) and pure holo-CP (pure holo-CP, triangle, red). The spectra were measured on the UV-spectrophotometer between 350 and 650 nm including the HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) as a blank for each measurement. Two different cobalamin states were observed at 375 nm [Cob⁻] and 520 nm [Cob^{III}-Me].
Figure S11. Extinction coefficient of pure *holo-CP*. Different concentrations of the pure *holo-CP* were measured to calculate the coefficient and to determine the total amount of pure *holo-CP* in CFE.
Analytics

HPLC Method
The product formation and the conversion of the biotransformations were analyzed by reversed phase HPLC-UV (Agilent 1260 Infinity system equipped with a SPD-M20A diode array detector) on a C18 column (Phenomenex Luna® C18, 100c, 250 x 4.6 mm, 5 mm). All samples were injected with 10 µL and eluted with a H2O/MeCN (+0.1% TFA) gradient at 1 mL/min. Method A: 100% H2O (2 min), 0-40% MeCN (13 min), 40-100% MeCN (5 min), 100% MeCN (2 min). Method B: 100% H2O (2 min), 0-70% MeCN (13 min), 70-100% MeCN (5 min), 100% MeCN (2 min). The compounds were detected by UV-absorption at 280 nm (254 nm for 1m) and the peaks were compared with commercially bought reference material. All references were dissolved in Hepes buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl2, addition of 20 or 50% DMSO if necessary) and used for the preparation of calibrations within concentrations from 1-50 mM. For the quantification of yatein 1k and its product desmethyl-yatein 2k, the samples (10 µL) were analyzed by normal phase HPLC-UV equipped with an IA column (Diacel Chiralpak® IA, 250 mmL x 4.6 mm, 5 µm). The substrate and the product were eluted with an isocratic gradient of 80% n-heptane and 20% isopropanol at 30 °C for 40 min (1 mL/min).

For the isolation of yatein 1k, papaverine 1j and 2,4,6-trimethoxytoluene 1i, preparative HPLC runs (Phenomenex Luna® C18 (2), 100A, 250 x 21.2 mm, 5 µm) were performed. All samples were concentrated to 1 mL and eluted with a H2O/MeCN gradient at 30 mL/min. Method C: 100% H2O (2 min), 0-30% MeCN (18 min), 30-100% MeCN (13 min), 100% MeCN (5 min). Method D: 100% H2O (2 min), 0-30% MeCN (18 min), 30-100% MeCN (8 min), 100% MeCN (7 min). Method E: 10% MeCN (2 min), 10-100% MeCN (23 min), 100% MeCN (5 min).

Table S6. Retention time and k-value of each compound analyzed by HPLC.

| Compound                        | tR [min] | k-value [mAu/mM] |
|---------------------------------|----------|------------------|
| veratrol 1a                     | 19.5     | 108.8            |
| guaiacol 2a                     | 17.7     | 119.8            |
| catechol 4a                     | 13.3     | 118.3            |
| 3,4-dimethoxytoluene 1b        | 20.9     | 113.1            |
| 2-methoxy-4-methylphenol (para) 2b | 19.7     | 113.9            |
| 2-methoxy-5-methylphenol 3b     | 19.8     | 120.9            |
| 4-methylcatechol 4b             | 15.8     | 116.8            |
| vanillin (meta) 2c              | 15.9     | 479.2            |
| isovanillin (para) 3c           | 15.7     | 449.5            |
| 3,4-dihydroxybenzaldehyde 4c    | 13.2     | 159.6*           |
| Compound                                           | Method A | Method B |
|----------------------------------------------------|----------|----------|
| 4-methoxybenzene-1,2-diol                          | 13.3     | 109.5    |
| 4-methoxybenzene-1,3-diol 3d                       | 12.2     | 133.9    |
| Vanillyl alcohol (meta) 2e                         | 11.8     | 152.3    |
| Isovanillyl alcohol (para) 3e                       | 12.4     | 138.4    |
| 3,4-dihydroxybenzyl alcohol 4e                      | 9.6      | 133.7    |
| 3-methoxy-2-naphthol 2f                             | 20.7     | 159.9    |
| 2,3-dihydroxynaphthalene 4f                         | 19.3     | 169.4    |
| 1,2,3-trimethoxybenzene 1g                          | 21.6     | 39.1     |
| 2,3-dimethoxyphenol 2g                              | 17.6     | 16.0     |
| 2,6-dimethoxyphenol 3g                              | 17.5     | 27.6     |
| 3-methoxycatechol 4g                                | 14.4     | 21.0     |
| 2-methoxyresorcinol                                 | 12.7     | 16.8     |
| Pyrogallol                                          | 9.8      | 12.2     |
| 3,4-dimethoxyphenol 2h                              | 18.1     | 6.5      |
| 3,5-dimethoxy-4-methylphenol (para) 2i             | 20.0<sup>a</sup> | 15.9<sup>b</sup> |
| 2-hydroxy-4,6-dimethoxynaphthene (ortho) 3i        | 19.7<sup>b</sup> | 17.4<sup>b</sup> |
| 6-desmethyl papaverine 2j                           | 16.6     | 80.5     |
| 4',6-didesmethyl papaverine 4j                     | 15.5     | 65.6     |
| 3.5-dimethoxytoluene 1n                             | 21.8     | 44.1     |
| 3-methoxy-5-methylphenol 2n                         | 19.5     | 69.3     |
| Oracinol 4n                                         | 14       | 70.2     |

All standards were measured with HPLC Method A, $\lambda = 280$ nm

<sup>a</sup> Standards were measured with HPLC Method A, $\lambda = 262$ nm

<sup>b</sup> Standards were measured with HPLC Method B, $\lambda = 280$ nm
HPLC chromatograms

Figure S12. HPLC chromatogram of the demethylation of veratrol 1a coupled to concomitant methylation of orcinol 1n. Reaction conditions: substrate 1a (10 mM, 1.4 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, 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl2) at 35 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. Total volume 500 µL. All reactions were quenched by the addition of MeCN (60 vol. % final conc.) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV (Method A, 280 nm). The peak at 20.5 min appears independently and is assigned as system peak.
Figure S13. HPLC chromatogram of the demethylation of 3,4-dimethoxytoluene 1b enabled by the methylation of orcinol 1n. 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, 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 μM ZnCl₂) at 35 °C, 800 rpm in Eppendorf® Thermomixer® (1.5 mL) for 24 h. Total volume 500 μL. All reactions were quenched by the addition of MeCN (60 vol. % final conc.) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV (Method A, 280 nm). The peak at 20.5 min appears independently and is assigned as system peak.

Figure S14. Preparative HPLC chromatogram of the demethylation of papaverine 1j on semi-preparative scale. Reaction conditions: substrate 1j (10 mM, 170 mg), methyl acceptor 1n (20 mM, 125 mg), vdmB (40 mg/mL cell-free extract, ≡ 0.077 mM vdmB), CP (31 mg/mL, 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 μM ZnCl₂) at 35 °C, 800 rpm in Eppendorf® Thermomixer® (1.5 mL) for 24 h. Total volume 500 μL. All reactions were quenched by the addition of MeCN (60 vol. % final conc.) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV (Method A, 280 nm). The peak at 20.5 min appears independently and is assigned as system peak.
ZnCl₂) at 35 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. All reactions were quenched by the addition of MeCN (60 vol. % final conc.) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV (Method C).

Figure S15. Preparative HPLC chromatogram of the demethylation of 2,4,6-trimethoxytoluene 1i on semi-preparative scale. Reaction conditions: substrate 1i (10 mM, 23 mg), methyl acceptor 1n (20 mM, 17.5 mg), vdmB (40 mg/mL cell-free extract, ≡ 0.077 mM vdmB), CP (31 mg/mL, 1.5 mM pure CP loaded with methyl cobalamin) in HEPES buffer (50 mM, pH 6.5, 150 mM KCl, 20 µM ZnCl₂) at 35 °C, 800 rpm in Eppendorf Thermomixer® (1.5 mL) for 24 h. All reactions were quenched by the addition of MeCN (60 vol. % final conc.) after 24 h and the conversions were analyzed via calibration curves of the corresponding reference compounds on HPLC-UV (Method D).
Figure S16: HPLC chromatogram of the substrate papaverine 1j at 280 nm. Papaverine was eluted at 18.2 min on the HPLC-UV (Method A).

Figure S17: HPLC chromatogram of the isolated, mono-demethylated product 6-desmethylpapaverine 2j at 280 nm. 6-desmethylpapaverine was eluted at 16.5 min on the HPLC-UV (Method A). The impurity peak at 14.1 min referred to orcinol 4n in small quantity which could not be fully separated from the product. The peak at 20 min appears independently and is assigned as system peak.
Figure S18: HPLC chromatogram of the isolated, di-demethylated product 4’,6-didesmethyldapaverine 4j at 280 nm. 4’,6-didesmethyldapaverine was eluted at 15.7 min on the HPLC-UV (Method A). Minor traces of impurities were found which could not be fully separated from the product. The peak at 20 min appears independently and is assigned as system peak.
NMRs:
2-hydroxy-4,6-dimethoxytoluene (2i):

\[ \text{H NMR (300 MHz, CDCl}_3\text{)} \delta 6.18 - 5.98 (m, 2H), 4.70 (s, 1H), 3.79 (s, 3H), 3.76 (s, 3H), 2.03 (s, 3H). \]

\[ \text{C\{H\} NMR (75 MHz, CDCl}_3\text{)} \delta 159.2, 159.1, 154.9, 104.1, 93.3, 91.4, 55.8, 55.5, 7.6. \]

Figure S19. \[ ^1\text{H-NMR spectrum of 4-hydroxy-2,6-dimethoxytoluene (2i).} \]
Figure S20. $^{13}$C-$^1$H-NMR spectrum of 4-hydroxy-2,6-dimethoxyltoluene (2i).
4-hydroxy-2,6-dimethoxytoluene (3i):

\( ^1H \text{ NMR (300 MHz, CDCl}_3\) } \delta 6.07 (s, 2H), 3.78 (s, 6H), 2.00 (s, 3H).

\( ^{13}C\{^1H\} \text{ NMR (75 MHz, CDCl}_3\) } \delta 159.0, 154.7, 106.7, 91.9, 55.8, 7.8.

Figure S21. \(^1H\)-NMR spectrum of 2-hydroxy-4,6-dimethoxytoluene (3i).
Figure S22. $^{13}$C-$^1$H-NMR spectrum of 2-hydroxy-4,6-dimethoxytoluene (3i).
Papaverine (1j):

$\textsuperscript{1}H$ NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 8.35 (d, $J_1 = 5.7$ Hz, 1H), 7.42 (d, $J_1 = 5.7$ Hz, 1H), 7.33 (s, 1H), 7.03 (s, 1H), 6.77 (m, 3H), 4.53 (s, 2H), 3.98 (s, 3H), 3.89 (s, 3H), 3.80 (s, 3H), 3.75 (s, 3H).

$\textsuperscript{13}C\{^1H\}$ NMR (75 MHz, CDCl\textsubscript{3}) $\delta$ 157.0, 154.1, 152.5, 149.5, 148.3, 136.9, 129.3, 128.0, 122.5, 121.5, 120.8, 112.5, 111.3, 106.0, 104.9, 56.8, 56.5, 56.3, 55.8, 36.5.

Figure S23. $\textsuperscript{1}H$-NMR spectrum of papaverine (1j).
Figure S24. $^{13}$C-$^1$H-NMR spectrum of papaverine (1j).
6-desmethyl papaverine (2j):

\[
\begin{align*}
\text{HO} & & \text{MeO} \\
\text{MeO} & & \text{OMe}
\end{align*}
\]

\(^1\)H NMR (300 MHz, d3-MeOD) \(\delta\) 8.09 (d, \(J_1 = 5.9\) Hz, 1H), 7.53 – 7.36 (m, 2H), 7.08 (s, 1H), 6.91 (d, \(J_1 = 1.4\) Hz, 1H), 6.82 (s, 2H), 4.51 (s, 2H), 3.89 (s, 3H), 3.75 (s, 3H), 3.73 (s, 3H).

\(^{13}\)C\{\(^1\)H\}-NMR (75 MHz, d3-MeOD) \(\delta\) 159.3, 158.1, 151.8, 150.6, 149.2, 138.7, 136.3, 133.2, 123.3, 121.9, 119.9, 113.5, 113.2, 110.0, 105.4, 56.5, 56.4, 56.3, 41.4.

**Figure S25.** \(^1\)H-NMR spectrum of 6-desmethyl papaverine (2j).
Figure S26. $^{13}$C-$^1$H-NMR spectrum of 6-desmethyl papaverine (2j).
Figure S27. $^1$H-$^1$H-NOESY NMR spectrum of 6-desmethyl papaverine (2j).
Figure S28. $^1$H-$^1$H COSY NMR spectrum of 6-desmethyl papaverine (2j).
Figure S29. $^1$H-$^{13}$C-HSQC NMR spectrum of 6-desmethyl papaverine (2j).
Figure S30. $^1$H-$^{13}$C-HMBC NMR spectrum of 6-desmethylpapaverine (2j).
4',6-desmethyl papaverine (4j):

\[ \delta \text{H NMR (700 MHz, d3-MeOD)} \ \delta 8.08 (d, J_1 = 5.0 \text{ Hz}, 1H), \ 7.46 - 7.44 \text{ (m, 2H)}, \ 7.08 \text{ (s, 1H)}, \ 6.86 \text{ (s, 1H)}, \ 6.69 \text{ (s, 2H)}, \ 4.49 \text{ (s, 2H)}, \ 3.90 \text{ (s, 3H)}, \ 3.74 \text{ (s, 3H)}. \]

Figure S31. $^1$H-NMR spectrum of 4',6-desmethyl papaverine (4j).
Due to the limited amount of product obtained and to the consequent lack of $^1$H-$^{13}$C-HSQC NMR and $^1$H-$^{13}$C-HMBC NMR, the assignment of the $^1$H NMR peaks was done accordingly to the 6-desmethylpapaverine (2j) $^1$H NMR (considering the high similarity of the two molecules). Consequently the disappearance of the NOE of the H 5’ with the methoxy group 12 (demethylation in position 4’) in the NOESY NMR spectrum allowed the confirmation of the 4’,6-didesmethyl papaverine (4j) compound.
Figure S32. $^1$H-$^1$H-NOESY NMR spectrum of 4',6-didesmethyl papaverine (4j).
rac-yatein (1k):

\[ \text{H NMR (300 MHz, CDCl}_3\text{)} \delta 6.73 - 6.65 (m, 1H), 6.50 - 6.42 (m, 2H), 6.35 (s, 2H), 5.93 (q, J_1 = 1.4 Hz, 2H), 4.25 - 4.09 (m, 1H), 3.87 (dd, J_1 = 9.2, J_2 = 7.3 Hz, 1H), 3.82 (m, 9H), 3.02 - 2.79 (m, 2H), 2.72 - 2.36 (m, 4H). \]

\[ \text{C}{^1}{^H} \text{ NMR (75 MHz, CDCl}_3\text{)} \delta 178.7, 153.4, 148.0, 146.5, 139.3, 133.4, 131.6, 121.6, 108.9, 108.4, 106.29, 101.2, 71.3, 61.0, 56.2, 46.6, 41.1, 38.4, 35.3. \]

Figure S33. \( ^1\text{H-NMR spectrum of rac-yatein (1k).} \)
Figure S34. $^{13}$C{^1}H-NMR spectrum of rac-yatein (1k).
rac-4-demethylyatein (2k):

$^1$H NMR (300 MHz, CDCl$_3$) δ 6.74 – 6.65 (m, 1H), 6.50 – 6.43 (m, 2H), 6.36 (s, 2H), 5.93 (q, $J_1 = 1.4$ Hz, 2H), 5.43 (s, 1H), 4.15 (dd, $J_1 = 9.2$, $J_2 = 7.0$ Hz, 1H), 3.86 (m, 7H), 2.90 (d, $J_1 = 5.7$ Hz, 2H), 2.68 – 2.34 (m, 4H).

$^{13}$C {$^1$H} NMR (75 MHz, CDCl$_3$) δ 178.8, 148.0, 147.2, 146.5, 133.7, 131.7, 128.8, 121.7, 108.9, 108.4, 106.0, 101.2, 71.3, 56.4, 46.8, 41.0, 38.5, 35.2.

Figure S35. $^1$H-NMR spectrum of rac-4-demethylyatein (2k).
Figure S36. 13C{1H}-NMR spectrum of rac-4-demethyleatein (2k).
Table S7: Comparison of observed NMR data of rac-4-demethyleatein (2k) with literature values.\(^8\)

|          | 1H-NMR |          | 13C-NMR |          |
|----------|--------|----------|---------|----------|
|          | observed | Literature\(^8\) | observed | Literature\(^8\) |
| ppm      | J  | ppm      | J  | Δppm     |
| 6.74 – 6.65 | -  | 6.70      | 7.0, 1.5 | 0.01   |
| 6.50 – 6.43 | -  | 6.47      | 7.0 | -       |
| 6.36     | -  | 6.36      | 9.0 | -       |
| 5.93     | 1.4 | 5.94      | 1.1 | 0.01   |
| 5.43     | -  | -        | -   | -       |
| 4.15     | 9.2, 7.0 | 4.15     | 9.3, 6.8 | -     |
| 3.86     | -  | 3.87      | 5.9 | 0.01   |
| 3.86     | -  | 3.86      | -   | -       |
| 2.90     | 5.7 | 2.90      | 5.9 | -       |
| 2.68 – 2.34 | -  | 2.7-2.4 | - | -       |
3-methoxy-5-methylphenol (2n):

\[
\begin{align*}
\text{\(^1\text{H} NMR (300 MHz, CDCl}_3\}) & \delta 6.33 (s, 1H), 6.26 (d, J = 0.6 \text{ Hz}, 1H), 6.23 (t, J = 2.1 \text{ Hz}, 1H), 3.76 (s, 1H), 2.27 (s, 1H). \\
\text{\(^{13}\text{C} \{^1\text{H}\} NMR (75 MHz, CDCl}_3\}) & \delta 160.87, 156.58, 140.75, 108.77, 107.48, 98.69, 55.38, 21.72.
\end{align*}
\]

Figure S37. \(^1\text{H}-\text{NMR of the 3-methoxy-5-methylphenol (2n) isolated from a biotransformation, namely the demethylation of 2,4,6-tri-methoxytoluene (1i). For reaction conditions see Figure S15. The insert displays the reference material (3-methoxy-5-methylphenol, Sigma Aldrich). The NMR corresponds to data reported in literature.\(^9\)
Figure S38. $^{13}$C-{'^1$H}-NMR of the 3-methoxy-5-methylphenol (2n) isolated from a biotransformation, namely the demethylation of 2,4,6-trimethoxytoluene (1i). For reaction conditions see Figure S15. The insert displays the reference material (3-methoxy-5-methylphenol, Sigma Aldrich). The NMR corresponds to data reported in literature.\(^9\)
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