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Specific Residues Expand the Substrate Scope and Enhance the Regioselectivity of a Plant *O*-Methyltransferase

Qingyun Tang,[a] Uwe T. Bornscheuer*[a] and Ioannis V. Pavlidis*[b]

Abstract: An isoeugenol 4-*O*-methyltransferase (IeOMT) isolated from the plant *Clarkia breweri* can be engineered to a caffeic acid 3-*O*-methyltransferase (CaOMT) by replacing three concessive residues. Here we further investigated functions of these residues by constructing the triple mutant T133M/A134N/T135Q as well as single mutants of each residue. Phenolics with different chain lengths and different functional groups were investigated. The variant T133M improves the enzymatic activities against all tested substrates by introducing additional Met-π interaction with the substrates. Mutant A134N significantly enhanced the regioselectivity. It is *meta*-selective or even specific against most of the tested substrates but *para*-specific towards 3,4-dihydroxybenzoic acid. The triple mutant T133M/A134N/T135Q benefits from these two mutations, which not only expand the substrate scope but also enhance the regioselectivity of IeOMT. On the basis of our work, regiospecific methylated phenolics can be produced in high purity by different IeOMT variants.

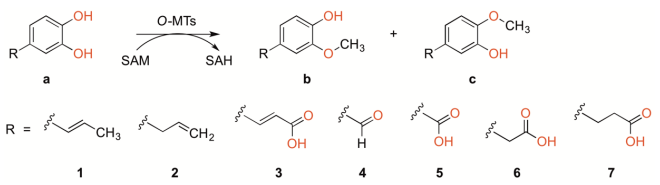
Introduction

Methylation is an important and ubiquitous process both in biological activities and chemical synthesis. Through methylation, the physicochemical properties (hydrophobicity, solubility, p*K* values), odor and taste, or the bioactivity of a compound can be altered.[1] Various biological activities and diseases are regulated through the methylation of nucleotides, proteins and hormones.[2] Methylation can be achieved by a specific enzyme class, methyltransferases (MTs, E.C. 2.1.1.x), using the biomolecule *S*-adenosyl-L-methionine (SAM) as methyl donor. Depending on the atoms to be methylated, MTs can be classified into *C*-, *O*-, *N*-, *S*-, *Se*-, *As*- and also halide ion MTs, among which *O*-MTs are the most abundant class.[3] Compared to chemical methylation methods, enzymatic methylation processes are environmentally friendly alternatives and can be superior due to their chemo-, regio- and stereoselectivity. Thus, methyltransferases are an attractive class of biocatalysts for industrial applications. So far, their potential is not utilized in industry, mainly due to two reasons. The first is relevant to the methyl donor: SAM is expensive to be provided in equimolar amounts to the substrate, while it is unstable under mild conditions. Studies on efficient *in vitro* regeneration of SAM, or the usage of other methyl donors were published recently.[4] The second reason is relevant to the reactions catalyzed: MTs were considered able to only perform methylations. However, it was shown recently that either the wild-type enzymes can have catalytic promiscuity or protein engineering could help to expand the range of residues to be accepted.[5]

Mammalian catechol *O*-MTs (COMTs) are widely studied due to their importance in the regulation of neurotransmitters and hormones (L-DOPA, dopamine, noradrenaline, adrenaline etc.), as well as their roles as drug targets.[2c, 6] COMT accepts a wide range of catechol derivatives as substrates and the majority of COMTs preferably methylate the *meta*-hydroxyl groups (*meta*-OH or 3-OH; Scheme 1).[2c, 7] They have been utilised as catalysts in the production of the flavor compound vanillin (**4b**).[8] Only a few COMTs were characterized to have *para*-preference (4-OH), which serves as a regio-complementary catalyst to the common COMTs and enables the synthesis of uncommon methylated products.[9] The regioselectivity of COMT can also be altered through protein engineering. For instance, Law and coworkers engineered several regiocomplementary mutants of the rat COMT to provide access to alkylated catechols (i.e. using 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid and 4-nitrocatechol as substrates) while they could explain the reason for the altered regioselectivity by binding models.[5d]

Caffeic acid/5-hydroxyferulic acid 3/5-O-MTs (CaOMTs) are key enzymes in the biosynthesis of lignin precursors found in all lignin-producing plants.[10] They catalyze methylation of the lignin precursors caffeyl alcohol, caffeic aldehyde, caffeic acid (**3a**) and 5-hydroxyconiferyl alcohol/aldehyde/acid exclusively at the *meta*-OH, leaving the *para*-OH free to form lignin polymers and to avoid oxidative radical coupling.[11] These compounds are also the building block of chalcones and flavonoids.[12]

Wang et al. have isolated a CaOMT and an isoeugenol *O*-methyltransferase (IeOMT) from *Clarkia breweri*.[13] Though the two OMTs share 83% amino acid sequence identity, they display distinct substrate preference as well as regioselectivity. While CaOMT serves to produce *meta*-methylated lignin precursors, as the other CaOMTs do, IeOMT only accepts isoeugenol (**1b**) or eugenol (**2b**) as substrates and methylates the sole free *para*-OH to produce the floral scent compounds methyl isoeugenol and methyl eugenol.



Scheme 1. *O*-MTs catalyse methylation of phenolic substrates (**a**) to give *meta*- (**b**) or *para*- (**c**) methylated products. *S*-Adenosyl-L-methionine (SAM) is provided as methyl donor and SAH (*S*-adenosyl-L-homocysteine) is generated after the methyl group donation. Structures of different substrates are shown, based on the R groups and the phenolic moieties **a**, **b** or **c**.

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Substitution of the amino acid cluster T133-A134-T135 in IeOMT with the corresponding residues in CaOMT (M135-N136-Q137) enabled IeOMT the CaOMT character, regarding the substrate specificity as well as the methylation regiospecificity.[14] However, the mechanism of the substrate discrimination has not been elucidated yet.

Herein, we investigated in detail the effects of these residues on the enzymatic function of IeOMT. Our results show that the substrate scope of IeOMT can be expanded and regioselectivity can be enhanced by specific mutations.

Results and Discussion

Investigation of the critical residues in IeOMT

As pointed out above, we aimed to study the effects of residues 133-135 in IeOMT and thus the triple mutant T133M/A134N/T135Q and the respective single mutants at each position were constructed and expressed in *E. coli*. Activities of purified enzymes against isoeugenol (**1b**), eugenol (**2b**) and caffeic acid (**3a**) were studied. As shown in Figure 1, the triple mutant T133M/A134N/T135Q of IeOMT has increased methylation activities against both substrates. Specifically, the single mutant T133M has a significant effect on the enhancement of the activities, while the T135Q largely impairs enzymatic activity. The double mutant T133M/A134N lacking T135Q was constructed to avoid its adverse effects on the enzyme activity. Nevertheless, the double mutant did not exhibit improved activity compared to the triple mutant.

IeOMT is considered an 4-*O*-MT as it displays significant activity towards the solely available *para*-OH of isoeugenol and eugenol.[14] However, it is not regioselective against caffeic acid (**3a**) which contains both free *meta*- and *para*-hydroxyl groups (Figure 1). The triple mutant T133M/A134N/T135Q has conferred *meta*-specificity to IeOMT against caffeic acid, as reported by Wang et al.[14] Interestingly, the mutants T133M and A134N at adjacent positions gave different regioselectivities against caffeic acid. T133M increases the preference for the *para*-OH, leading to the major production of isoferulic acid (**3c**), while A134N is strictly *meta*-specific. This selectivity is not substrate concentration-dependent, as the $K_{M,obs}$ of IeOMT_T133M is lower than the working concentration (Table 1). Especially for the reaction with caffeic acid (**3a**), the ratio of products **3c** to **3b** was always between 3.1 to 3.4, in substrate concentration ranging from 0.05 mM to 4 mM (data not shown). The effects of increasing enzyme activity observed for T133M and the regiospecificity of A134N are complementary in mutants T133M/A134N/T135Q and T133M/A134N, which produce pure ferulic acid (**3b**) with considerable conversions. A CaOMT from *Catharanthus roseus* (CaRo_MT) containing the same M-N-Q amino acid cluster in the corresponding positions was also investigated. It is highly active and *meta*-specific against its intrinsic substrate caffeic acid while showing minor activity against isoeugenol.

Table 1. Observed kinetic constants of IeOMT_T133M against isoeugenol (**1b**), caffeic acid (**3a**) and 3,4-dihydroxybenzaldehyde (**4a**).

Substrate	$K_{M,obs}$ (mM)	$k_{cat,obs}$ (s^{-1})	$k_{cat,obs}$ / $K_{M,obs}$ (s^{-1}•mM^{-1})
1b	0.0058±0.0003	479 ± 7	82586
3a	0.719±0.035	64 ± 1	89
4a	0.119±0.014	226 ± 12	1899

Investigation of the substrate scope

Since some of the IeOMT mutations led to increased enzyme activity and regioselectivity, we further investigated their functions against other phenolics. 3,4-Dihydroxyphenolic compounds having different chain lengths and different functional groups were chosen (**4a**–**7a**, Scheme 1, Figure 1). Similar to the reactions with isoeugenol and caffeic acid, both T133M/A134N/T135Q, T133M/A134N and the T133M variant show improved activities against all substrates compared to the wild-type IeOMT. This is more obvious for 3,4-dihydroxybenzaldehyde (**4a**) and 3,4-dihydroxybenzoic acid (**5a**) which have one-carbon aldehyde and carboxylic group attached to the phenyl ring, respectively. Activities against 3,4-dihydroxyphenylacetic acid (**6a**) and 3,4-dihydroxyphenylpropanoic acid (**7a**) having longer carboxylic side chains did not get much improved. While CaRoMT is highly active to its intrinsic substrate caffeic acid (**3a**), it also shows high activity against 3,4-dihydroxyphenylpropanoic acid (**7a**) with the three-carbon saturated carboxylic side chain.

Interestingly, while both T133M/A134N/T135Q, T133M/A134N and A134N variants are *meta*-selective against 3,4-dihydroxybenzaldehyde (**4a**) and produce mainly vanillin (**4b**), they show *para*-specificity towards 3,4-dihydroxybenzoic acid (**5a**). CaRoMT also shows totally different regiospecificity against these two substrates.

Structural analysis

The T133M variant exhibits increased methylation activity by providing beneficial interactions to the first sphere of interaction residues.

The crystal structure of a *Clarkia breweri* IeOMT variant (T133L, E165I and F175I) with isoeugenol and SAH bound in the active site has been determined by Bhuiya and coworkers.[15] The structure is in the closed conformation, referring to the active catalytic states.[16] To perform bioinformatic analysis on the wild-type IeOMT, as well as our mutants, we performed a structure refinement (25ºC, pH 7.5) and back mutations to the wild-type sequence *in silico* and finally a subsequent energy minimization. As shown in Figure 2a, isoeugenol is surrounded and stabilized by a series of hydrophobic residues. Among them, two Met residues (M183 and M323) are highly conserved in plant aromatic OMTs and play a major role in encapsulating and orienting the hydrophobic and aromatic substrates.[17] It is reported that the -SH/π interaction between these methionines and the aromatic ring plays an important role in stabilizing the structure,[19] among with aromatic residues such as F179.

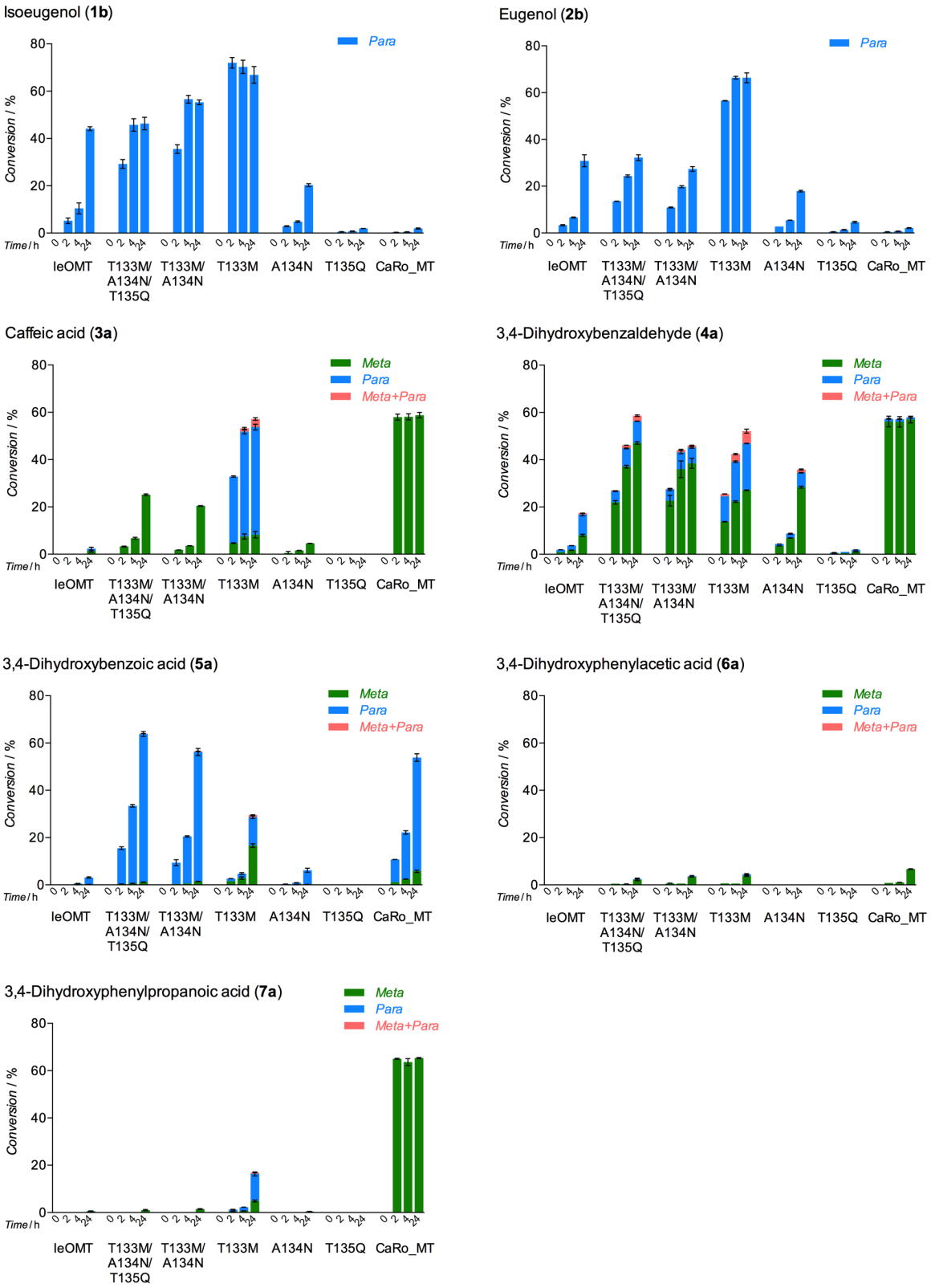

Figure 1. Methylation efficiency and regioselectivity of IeOMT and its mutants against different substrates. Values obtained for negative controls (without enzyme) were subtracted.

However, the methionine at position 133 (T133M) is far from this system, above the propenyl chain of isoeugenol. It seems that its hydrophobicity among with the higher flexibility of the side chain of methionine compared to threonine, provides a beneficial environment for this hydrophobic chain of isoeugenol (Figure 2a). Moreover, through a residue interaction network (RIN) analysis we observed the interactions with the three residues of interest (133-135) and the substrate. In this analysis we observed that the residue at position 133 (T or M) does not directly interact with the substrate, but it acts supportive to the residues that directly interact with the substrate. The most notable interactions established are the ones with the F179 residue, which as we mentioned stabilizes the aromatic residues at the active site. Thus, it seems that the T133M mutation provides the proper orientation of the phenylalanine to bring the substrate to the right position. The distance of the methyl group of SAM to the oxygen of the hydroxyl group of isoeugenol decreased from 6.1 Å in the wild-type to 5.0 Å in the T133M mutant. This is also supported from literature, as it has been also reported that T133M of IeOMT increases catalytic efficiency against coniferyl alcohol by improving substrate affinity.[19]

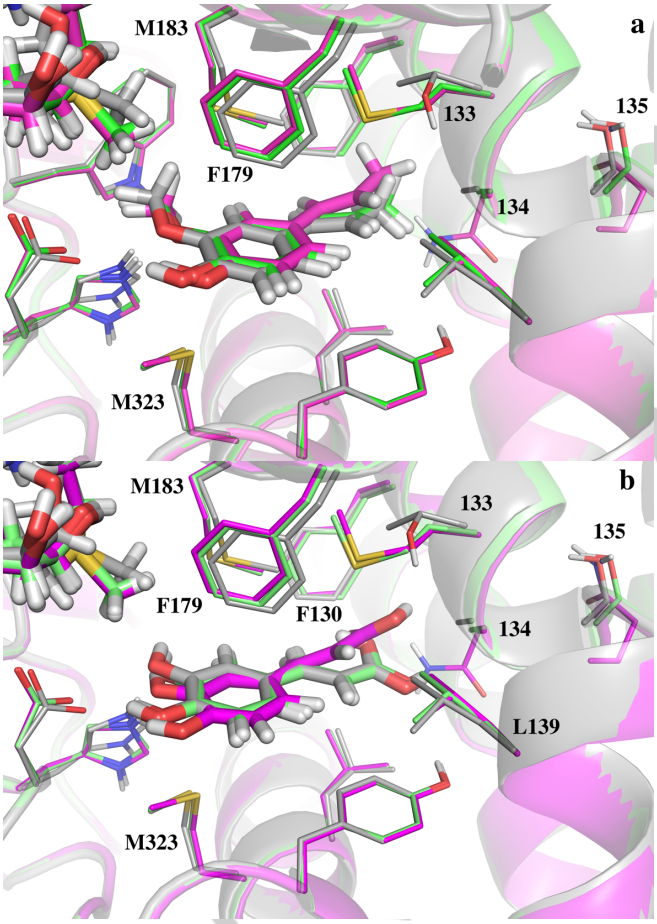

Figure 2. Models of *Clarkia breweri* IeOMT wild-type (grey), T133M (green) and T133M/A134N/T135Q (magenta) with SAM and isoeugenol (a) and caffeic acid (b) in the active site. The residues interacting with the substrate are shown as lines, the most important are labeled.

In our case we observed the increased enzymatic activities of T133M mutant also against caffeic acid (**3a**), 3,4-dihydroxybenzaldehyde (**4a**) and 3,4-dihydroxybenzoic acid (**5a**). It is interesting to note that CaRoMT, which also has the "MNQ" motif, is highly active against caffeic acid (**3a**), 3,4-dihydroxybenzaldehyde (**4a**) and 3,4-dihydroxyphenyl-propanoic acid (**7a**). This supportive role of the position 133 can also explain the reason why the beneficial effect was also observed with substrates with more polar R-groups, such as caffeic acid (**3a**) that has a carboxylic function. As seen in Figure 2b, M133 interacts better with F130, L139, F179, and even establishes interactions with M183, while in the wild-type T133 interacts weakly only with the first three residues.
Interesting is the effect of the position 135, which is further away from the substrate and its side chain does not even point towards the binding site. It seems that the negative effect that the T135Q mutation has on the activity is primarily related to secondary interactions that potentially break the interactions established with the substrate.

Mechanism of regioselectivity.
Both IeOMT and CaOMT belong to the type I plant *O*-MT family and catalyze methylation via a general acid/base-mediated S_N2-type nucleophilic substitution.[15-16, 20] The substrate hydroxyl groups will be deprotonated by the catalytic residues and carry out nucleophilic attack on the reactive methyl group of SAM afterwards. The mechanism of regioselectivity can be better explained by the overimposed active sites of the *Clarkia breweri* IeOMT (4-*O*-MT) and *Perennial Ryegrass* CaOMT (3-*O*-MT; Figure 3). Since the substrate is stabilized by a series of hydrophobic residues in the enzyme active site, the interaction between the substrate side chain and the adjacent residues affects the orientation of the substrate.

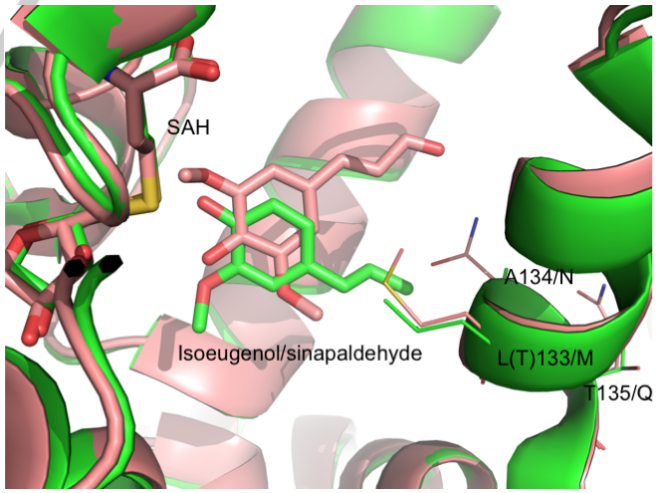

Figure 3. Overimposed active sites of the *Clarkia breweri* IeOMT (green) and *Perennial Ryegrass* CaOMT (PDB ID: 3p9i; pink; with SAH and sinapaldehyde bound). The mutated sites in IeOMT and the corresponding residues in *Perennial Ryegrass* CaOMT are shown. Interactions between the substrate side chain and residues 133 and 134 affect the orientation of the substrate. The hydroxyl group which is closer to the sulfur atom of SAH (elemental colouring) will be preferably methylated.

We hypothesize that the position 134 due to backbone interactions with the 135 can negate the negative effect and that is the reason why we did not observe this inactivation in the triple mutant.

Depending on whether the *meta*- or the *para*-hydroxyl group is closer to the methyl group of SAM (within *ca.* 3 Å to the activated methyl carbon of SAM or *ca.* 4 Å to the sulfonium moiety) they get preferably methylated and thus determine the regioselectivity.[20c] Since the added Met residue in mutant T133M is located above the isoeugenol side chain and stabilizes it through hydrophobic interaction, it attracts the substrate side chain to move downwards (from the view of Figure 3) and makes the *para*-hydroxyl group well-exposed to SAM. Similarly, T133M is also *para*-selective to caffeic acid. However, T133M does not show obvious regioselectivity against 3,4-dihydroxybenzaldehyde (**4a**) or 3,4-dihydroxybenzoic acid (**5a**), probably because these substrates have shorter side chains and therefore are more flexible in the enzyme active site.

In mutant A134N, the mutated residue has steric hindrance with the substrate side chain and expels it upwards (from the view of Figure 3) and hence the substrate positions its *meta*-hydroxyl group to the methyl donor SAM. This makes the variants T133M/A134N/T135Q, T133M/A134N and A134N strictly *meta*-specific towards caffeic acid. Interestingly, while these mutants are *meta*-selective against 3,4-dihydroxybenzaldehyde (**4a**), they are *para*-specific to 3,4-dihydroxybenzoic acid (**5a**). In our model (Figure 2b) the residue N134 of the triple mutant is the only one that directly interacts with the caffeic acid, forming a hydrogen bond and thus probably orienting it in the proper position for the observed *para*-selectivity.

The regioselectivity of the mammalian COMT is regulated through a different mechanism. Unlike IeOMT, in which the substrate can rotate in the active site pocket, the two hydroxyl groups of the catechol derivative substrate are coordinated and stabilized by the COMT-bound Mg^{2+} ion and only one hydroxyl group is exposed to SAM and being methylated.[5d, 21] The substituent on the catechol moiety can be located at either the *meta*- or *para*-position of the methylated hydroxyl group. Law et al. have reported that polar and charged substrate side chains tend to orientate towards the solvent and it results in *meta*-methylation, while neutral or hydrophobic substituents are more likely to approach the hydrophobic part of the active site and promote *para*-methylation.[5d] Therefore, the regioselectivity of COMT depends on the property of the substrate side chain and its relative position to the methylated hydroxyl group.

Conclusions

In our study, we discovered that a triple mutation T133M/A134N/T135Q in the isoeugenol *O*-MT, which is involved in substrate discrimination, exhibits expanded substrate scope and also enhanced regioselectivity. Further investigations showed that variant T133M improved the enzymatic activities against all tested compounds by introducing additional Met-π interaction with the substrates. The enhancement of regioselectivity derives from mutant A134N, which drives the enzyme *meta*-selective to most of the tested phenolics and *para*-specific towards 3,4-dihydroxybenzoic acid. As a result, ferulic acid (**3b**), isoferulic acid (**3c**), vanillin (**4b**) and isovanillic acid (**5c**) in high purity can be produced by different IeOMT variants.

Experimental Section

Materials

*Dpn*I and *S*-adenosyl-L-methionine (32 mM solution) were purchased from New England Biolabs, Inc. All oligonucleotides were purchased from ThermoFisher Scientific. Competent cells were self prepared. All other chemicals were purchased from Sigma or TCI and were of analytical grade or higher purity.

Gene construction, mutagenesis, expression and purification

The codon optimized genes (for expression in *E. coli*) for IeOMT from *Clarkia breweri* (accession number of protein: O04385) and for CaOMT from *Catharanthus roseus* (accession number of protein: Q8W013) with C-terminal His_6-tag were synthesized and subcloned into the *E. coli* expression vector pET-21a(+) by GenScript USA Inc.. Mutagenesis to generate the triple mutant T133M/A134N/T135Q was carried out with a modified version of the FastCloning method, by amplifying the whole pET-21a(+)-IeOMT vector using primers with 18 bp overlapping at their 5' ends.[22] Other mutants were constructed following the QuikChange (Stratagene) protocol. Primers are as below:

T133M/A134N/T135Q
fw: 5'-ATGAATCAAGACAAGGTTCTGCTGGAGCCGTGGTTC-3';
T133M/A134N/T135Q
rv: 5'- AACCTTGTCTTGATTCATCAGCAGAAACGGCGCCAG-3';
T133M/A134N
fw: 5'- GCCGTTTCTGCTGATGAACACCGACAAGGTTCTG-3';
T133M/A134N
rv: 5'- CAGAACCTTGTCGGTGTTCATCAGCAGAAACGGC-3';
T133M fw: 5'-CGTTTCTGCTGATGGCGACCGACAAGG-3';
T133M rv: 5'-CCTTGTCGGTCGCCATCAGCAGAAACG-3';
A134N fw: 5'-CCTTGTCGGTCGCCATCAGCAGAAACG-3';
A134N rv: 5'-GAACCTTGTCGGTGTTGGTCAGCAG-3';
T135Q fw: 5'-GCTGACCGCGCAAGACAAGGTTCTGC-3';
T135Q rv: 5'-GCAGAACCTTGTCTTGCGCGGTCAGC-3'.

The wild-type IeOMT templates in PCR products were digested by *Dpn*I before transforming into *E. coli* Top 10 chemically competent cells for plasmid amplification. After confirming the correct constructs by DNA sequencing (Eurofins, Germany), the newly constructed mutant plasmids were transformed into *E. coli* Bl21 (DE3) chemically competent cells for protein expression. The *E. coli* containing IeOMT or mutant plasmids were grown in terrific broth (TB) containing 100 μg/mL ampicillin at 37ºC until A_{600nm} reached 0.8-1.0. After induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), the cultures were grown overnight (18-22 h) at 20ºC. Then the cells were harvested by centrifugation, suspended in lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 7.5) and lysed by French press. After centrifugation, the supernatant was loaded over a Ni-NTA gravity flow column (Carl Roth, Germany), washed with 10 bed volumes of lysis buffer and the targeted proteins were eluted with elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 300 mM imidazole, pH 7.5). Then the purified proteins were desalted by Sephadex G-25 in PD-10 desalting columns (GE healthcare, USA) and stored in 50 mM sodium phosphate buffer, pH 7.5. The protein purities were confirmed by SDS-PAGE.

Enzyme assays and HPLC analysis

A typical reaction mixture was composed of 0.1 mg/mL purified enzyme, 5 mM DTT, 1 mM substrate, 1 mM SAM and 25% *E. coli* cell lysate, in 50 mM phosphate buffer, pH 7.5. Negative controls were also performed without adding MTs. Assays were performed in triplicate. Reactions were incubated at 28°C with 1000 rpm agitation in an Eppendorf Thermomixer. Samples were taken after 2, 4 and 24 h reaction time and an equal volume of acetonitrile was added to quench the reactions. The samples were centrifuged at full speed for 30 min to remove protein precipitate and then 200 µL supernatant were transferred to HPLC sample vial inserts prior to HPLC analysis. Analysis were performed on VWR Hitachi Elite LaChrom system equipped with the Kinetex EVO C18 (4.6 x 250 mm column, 5 µm particle size, Phenomenex) reversed-phase column. The mobile phase for the separation of isoeugenol (**1b**) and the methylated product was 10 mM sodium 1-heptanesulfonate, 20 mM phosphate, pH 4.4 and 50 % (v/v) acetonitrile; 50% water and methanol were used for detection of eugenol (**2b**) and methyl eugenol; 0.1 % acetic acid and acetonitrile were used for the separation of caffeic acid (**3a**) (18 % acetonitrile), 3,4-dihydroxybenzoic acid (**5a**) (10 % acetonitrile), 3,4-dihydroxyphenylacetic acid (**6a**) (20 % acetonitrile) and 3,4-dihydroxyphenylpropanoic acid (**7a**) (20 % acetonitrile) and their methylated products. A modified linear gradient with 0-2 min 5%-20% methanol, 2-11 min 23% methanol, 11-13 min 95% methanol, 13-16 min 5% methanol and lasted until 20 min, mixed with 0.1 % acetic acid was applied for the separations of 3,4-dihydroxybenzaldehyde (**4a**) and products.[5d] Wavelengths for the detections of isoeugenol, eugenol, caffeic acid, 3,4-dihydroxybenzaldehyde and the phenolic acids were 260, 280, 320, 320 and 280 nm, respectively. All analyses were performed at a flow rate 1 mL/min and the column temperature was 35ºC. The identities of *meta*-, *para*- and double-methylated products were confirmed by comparison with chromatographic elution times of commercial standards. Substrate and product concentrations were determined by comparing the peak areas to the calibration curves of each compound and the conversions were calculated. The kinetic constants ($K_{M,obs}$) of IeOMT_T133M were determined at various substrate concentrations and 1 mM SAM under the same reaction condition as in the enzyme assays using appropriate amounts of purified enzyme. Initial reaction rates were measured and fit to the Michaelis-Menten model using GraphPad Prism 6.0 (GraphPad Software Inc.) to determined the $V_{max,\ obs}$ and $K_{M,obs}$ values for each substrate. Then $k_{cat,obs}$ was defined by dividing $V_{max,obs}$ by the enzyme concentration and $k_{cat,obs}$ / $K_{M,obs}$ was calculated.

Bioinformatic analysis

The bioinformatic analysis was performed with YASARA 18.11.21. First the structure of 3REO was back-mutated to its wild-type sequence and the SAH was transformed to SAM by the addition of the methyl group and this structure was refined at pH 7.5, 25ºC for 500ps, taking a snapshot every 25 ps. The structure with the lowest energy was selected for the further experiments. For the mutants, the respective amino acids were swapped with subsequent energy minimization. The same was performed for the exchange of substrate..As we knew the binding pattern of isoeugenol, we swapped the atoms of the R-group to form the caffeic acid and subsequently minimized the energy. The figures were prepared with Pymol. The RIN analysis was performed with RINalyzer according to literature, with the use of Cytoscape 3.7 and Chimera 1.13.[23]

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Keywords: biocatalysis • methyltransferases • catalytic promiscuity • regioselectivity

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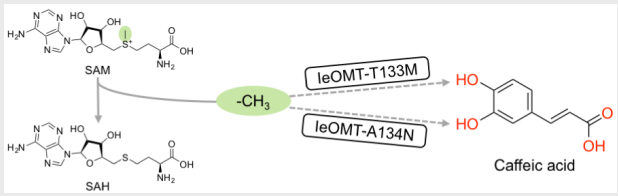

Isoeugenol *O*-methyltransferase (IeOMT) methylates the hydroxyl group of isoeugenol by utilising *S*-adenosyl-L-methionine (SAM) as methyl donor. We discovered that some mutations enable IeOMT to methylate caffeic acid. Specifically, the T133M variant methylates the *para*-OH while A134N methylates the *meta*-OH. Besides, these mutants also show expanded substrate scope and enhanced regioselectivity of IeOMT.

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Specific Residues Expand the Substrate Scope and Enhance the Regioselectivity of a Plant *O*-Methyltransferase