

Article

Rational reprogramming O-methylation regioselectivity for combinatorial biosynthetic tailoring of benzenediol lactone scaffolds

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Abstract

O-Methylation modulates the pharmacokinetic and pharmacodynamic (PK/PD) properties of small molecule natural products affecting their bioavailability, stability, and binding to targets. Diversity-oriented combinatorial biosynthesis of new chemical entities for drug discovery, and optimization of known bioactive scaffolds during drug development both demand efficient *O*-methyltransferase (OMT) biocatalysts with considerable substrate promiscuity and tunable regioselectivity that can be deployed in a scalable and sustainable manner. Here we demonstrate efficient total biosynthetic and biocatalytic platforms that use a pair of fungal OMTs with orthogonal regiospecificity to produce unnatural *O*-methylated benzenediol lactone (BDL) polyketides. We show that rational, structure-guided active site cavity engineering can reprogram the regioselectivity with substrate plasticity. These findings will guide combinatorial biosynthetic tailoring of unnatural products towards the generation of diverse chemical matter for drug discovery and the PK/PD optimization of bioactive scaffolds for drug development.

Introduction

Alkylations, most importantly the methylation of O-, C-, N- and S-centered nucleophiles are ubiquitous tailoring reactions during the biosynthesis of all major classes of small molecule natural products.¹ Among these reactions, O-methylation is used by nature and medicinal chemistry for the hydrophobic masking of peripheral hydroxyl or carboxylic acid mojeties to adjust the physicochemical and biological properties of bioactive compounds. The resulting "methyl effect" increases the lipophilicity and membrane permeability of small molecule scaffolds, enhancing their membrane transport, oral bioavailability, absorption and excretion.^{2,3} It also modulates the reactivity of small molecules by umpolung (reversal of polarity) of hydroxyl groups,⁴ affecting the in vivo metabolic stability of drugs and channeling the biosynthesis of natural products through a series of reactive intermediates.^{5,6} O-Methylation may also alter the conformation of natural product scaffolds through stereoelectronic and steric effects and confers weak interactions that contribute to binding to various targets including receptors.⁷ As opposed to chemical synthesis, enzymatic O-methylation offers regio- and stereospecific outcomes even for highly complex and reactive scaffolds without resorting to expensive multistep protection/deprotection strategies. It also proceeds with a high yield under mild, environmentally friendly reaction conditions, and does not have to contend with the formation of C-methylated side products in the case of phenolic substrates.^{8,9} In the biotechnology industries, *O*-methylation is conducted by utilizing in vivo biocatalytic platforms instead of in vitro reconstituted enzymatic reactions because the availability of the methyl donor co-substrate S-adenosyl-methionine (SAM) is limited and its in vitro regeneration is inefficient.^{1,10}

O-Methylation has been engineered in bacterial natural product biosynthetic pathways,⁷ mostly those of polyketides and nonribosomal peptides, by deleting the genes encoding native *O*-methyltransferase (OMT) enzymes or augmenting the pathways with genes for heterologous OMTs despite the often-constrained substrate flexibility of these enzymes. Extensive work has also been conducted on dissecting and altering the chemo- and regioselectivities of the mammalian and bacterial catechol OMTs, and those of the plant and bacterial flavonoid and phenylpropanoid OMTs.^{7,10} Such studies show that regio- and chemospecificity are intimately connected in each enzyme scaffold,^{11–13} although divorcing these properties is possible to some extent by directed evolution or active site cavity engineering informed by enzyme-substrate docking studies.^{14–17} However, little attention has been paid up to now to define the substrate promiscuity and modulate the regioselectivity of fungal secondary metabolite OMTs, despite these enzymes constituting the

most common tailoring activities during the biosynthesis of such natural products.

OMTs are also crucial to modulate the biological activities of benzenediol lactones (BDLs), a growing group of fungal polyketide natural products with an astonishing range of biological activities.^{18,19} BDLs are defined by a 1,3-benzenediol moiety fused to a macrocyclic lactone ring, with C2–C7 connectivity as in resorcylic acid lactones (RALs) or with C3–C8 connectivity as in dihydroxyphenylacetic acid lactones (DALs). Natural RALs contain 12- or 14-membered macrocycles (RAL₁₂ or RAL₁₄), while natural DALs most often include 12-membered macrolactones (DAL₁₂). Amongst RAL₁₂, lasiodiplodin (1a) with a 3-methoxy moiety displays ATP- and prostaglandin synthesis inhibitory activities, while its unmethylated congener, 3desmethyl-lasiodiplodin (DLD, 1) is devoid of such activities. Instead, DLD 1 is a nonsteroidal mineralocorticoid receptor antagonist and pancreatic lipase inhibitor.²⁰ RAL₁₄ with a 5-methoxy group are also important pharmacophores. Thus, hypothemycin is a selective inhibitor of mitogenassociated protein kinases; 7-oxozeaenol inhibits the tumor growth factor β activated kinase 1; while neocosmosin C is an agonist of δ -opioid receptors.^{18,19} Conversely, BDLs devoid of Omethylation also reveal exciting activities. Thus, RAL₁₄ in the pochonin/monocillin/radicicol family are potent Hsp90 inhibitors; zearalenone is an estrogen agonist; and the DAL₁₂ curvularins display anti-inflammatory and induced nitric oxide synthase inhibitory activities.^{18,19}

The polyketide scaffolds of these and other BDLs are assembled in fungi by two iterative Type I polyketide synthases (iPKSs) that collaborate as BDL synthase (BDLS) subunits, and are further tailored by oxidations, reductions, halogenations, and O-methylations to afford the mature bioactive molecules.^{21–23} We have been developing combinatorial biosynthetic platforms that exploit the flexibility of secondary metabolic biosynthetic enzymes to generate structurally complex molecules that may be utilized for drug discovery and agricultural applications. We have generated novel BDL scaffolds in the heterologous host Saccharomyces cerevisiae by BDLS subunit shuffling, combinatorial domain exchanges and rational engineering of the active sites of domains,^{24–27} and elaborated these biosynthons by exploiting combinatorial tailoring reactions in total biosynthetic or biocatalytic platforms.²⁸ Here, we evaluate the combinatorial biosynthetic potential of two orthologous OMT enzymes that afford orthogonal regioisomeric outcomes when modifying the same BDL substrate. Using protein homology modeling and substrate docking, we show that subtle differences in the active site cavity architecture of these OMTs force the substrates to approach the conserved active site base in distinct poses. Rational engineering of these "plasticity residues" then allows us to morph the regioselectivity of one enzyme to that of the other, and to create nonselective or iterative OMT variants. These experiments also reveal that

regioisomer.

regioselectivity is influenced by the substrate, thus engineering artificial BDL biosynthetic

pathways demands OMTs adapted for the chosen scaffold and optimized to yield the desired

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Results and Discussion

Regiospecificity and substrate selectivity of LtOMT and HsOMT during combinatorial biosynthesis.

Previous studies identified LtOMT and HsOMT, two orthologous O-methyltransferases (OMTs) encoded in the biosynthetic gene clusters for two fungal benzenediol lactones (BDLs).^{21,29} LtOMT from Lasiodiplodia theobromae methylates the C-3 phenolic hydroxyl of the RAL₁₂ desmethyl-lasiodiplodin (DLD, 1) to produce lasiodiplodin (1a, Fig. 1).²¹ HsOMT (Hpm5) from Hypomyces subiculosus methylates the C-5 phenolic hydroxyl of the RAL₁₄ trans-14,15dehydrozearalenol (DHZ, 3) to afford 5-O-methyl DHZ 3b (Fig. 1), an intermediate of hypothemycin.²⁹ To investigate whether these apparently different regiospecificities are intrinsic to the enzymes or dictated by the substrates, we used a Saccharomyces cerevisiae host to co-express LtOMT in a heterocombination with the DHZ-synthesizing iPKS pair, and separately, HsOMT with the DLD-producing iPKSs. Gratifyingly, both OMTs accepted the BDLs offered by the noncognate iPKS pairs and efficiently processed each of these compounds to a single O-methylated product. Thus, LtOMT afforded 3-O-methyl-DHZ 3a, while HsOMT yielded 5-O-methyl-DLD 1b: these derivatives are the regioisomers of the cognate OMT products 5-O-methyl DHZ 3b and lasiodiplodin 1a, respectively (Fig. 1). This suggested that the size, shape and functionalization of the macrocyclic ring may not affect the regiospecificities of these enzymes. Co-expression of both OMTs in a single chassis with the DLD- or the DHZ-producing iPKS pairs yielded mixtures of the 3-O-methylated, the 5-O-methylated and the 3,5-O-dimethylated products 1a, 1b, and 1c, or 3a, **3b**, and **3c**, respectively. This indicated that at least one of the OMTs is able to accept the methylated product of the other OMT as an alternative substrate.



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Figure 1. Total biosynthesis of *O***-methylated BDLs using LtOMT and HsOMT.** Product profiles (reversed-phase LC-MS traces recorded at 210 nm with a photodiode array detector) are shown for cultures of *S. cerevisiae* BJ 5464-NpgA strains co-transformed with the relevant iPKS pairs,^{21,29} and with LtOMT and/or HsOMT as indicated. The identities of the products were validated by comparison with isolated and structurally characterized compounds.

To further explore the breadth of substrate tolerance and the fidelity of regioselectivity of these two OMTs, we assembled a collection of model substrates that represent the natural and "unnatural" (combinatorial biosynthetic) structural space of BDLs (Fig. 2 and *SI Appendix*, Fig. S1), including RAL₁₂ (DLD **1**, radiplodin **2** and *trans*-resorcylide), RAL₁₄ (DHZ **3**, zearalenol **4**, lasicicol **5**, radicicol, monocillin I and monocillin II), DAL₁₂ (10(11)-dehydrocurvularin and *epi*-dehydrocurvularin) and DAL₁₄ (lasilarin **6** and radilarin). To explore the requirement for a macrolactone ring, we also tested non-macrocyclic BDL congeners such as isocoumarins (IC15, IC18 and IC28), acyl resorcylic acids (ARA7 **7** and ARA8 **8**), an acyl dihydroxybenzoic acid (ADA5) and a benzaldehyde (pre-asperfuranone). Initial testing of these potential substrates was conducted in a total biosynthetic format by co-expressing the appropriate iPKS pairs with the OMTs. Successful reactions were repeated using a standardized biocatalytic platform whereby the overall rate of *O*-methylation and the product distribution was quantified after feeding purified substrates to the OMT-expressing yeast host.

The two OMTs displayed considerable substrate flexibility. Thus, six out of the 20 BDL congeners were successfully *O*-methylated by both LtOMT and HsOMT, with an additional two compounds also processed by LtOMT (Fig. 2 and *SI Appendix*, Fig. S1). Remarkably, differences in the geometry of the benzenediol lactone ring system (RAL *vs.* DAL), variations in the size (12- or 14-membered) or the functionalization of the macrocycle, or even the absence of the macrocyclic ring were all well tolerated. However, α -pyrone and benzaldehyde analogues were not accepted, nor could the substrate be simplified to a single phenolic ring as shown by the absence of *O*-methylation of orsellinic acid, 2,4-dihydroxybenzoic acid, resorcinol or phenol (*SI Appendix*, Fig. S1). For macrocyclic substrates, the presence of an enone moiety at C9-C11 was found to be incompatible with *O*-methylation by LtOMT or HsOMT, as shown by radicicol, monocillin I, *monocillin* II, *trans*-resorcylide, radilarin, 10(11)-dehydrocurvularin and *epi*-dehydrocurvularin (*SI Appendix*, Fig. S1).



Figure 2. Combinatorial tailoring with LtOMT and HsOMT. Stacked polar bar charts show the product distributions among o-methoxy (blue), p-methoxy (green) and o,p-dimethoxy (red) BDL congeners during biocatalytic transformation of the indicated substrates (bold numbers above the bars) with S. cerevisiase BJ5464-NpgA expressing LtOMT (substrates 1-8, 1b-4b and 6b-8b); HsOMT (substrates 1-8 and 1a-8a); and both LtOMT and HsOMT (substrates 1-8). Percent values below the compound numbers on these charts show the overall conversion of the indicated substrate into all corresponding methylated products. See SI Appendix, Table S3 for tabulated percent conversion values as the mean \pm SD from three independent experiments of three replicates each (n=9). The structures of substrates 1-8 are shown with the carbonyl "handle" (see text for details) highlighted in *yellow*, and the sites of O-methylation identified by *blue* (LtOMTtype ortho-OMT activity) and green ovals (HsOMT-type para-OMT activity). Scheme: Unmethylated substrates (X) may be converted to the o-methoxy congener (Xa) by the LtOMT, and to the *p*-methoxy congener (**Xb**) by the HsOMT enzyme. **Xa** and **Xb** may also be converted to the *o*,*p*-dimethoxy congener (\mathbf{Xc}) by the appropriate enzyme ($\mathbf{X=1-8}$). See SI Appendix Fig. S2 for examples of the resulting biocatalytic grids.

HsOMT is apparently more fastidious than LtOMT in accepting noncognate substrates. First, neither radiplodin (RDN, **2**) nor lasicicol (LCL, **5**) is *O*-methylated by this enzyme (Fig. 2). For

RDN **2**, the highly constrained macrocycle may be sterically incompatible with the active site pocket of the enzyme. For LCL **5**, enolization of the C-9 carbonyl allows the resulting hydroxyl group to serve as a proton donor, and the resulting spurious H-bond(s) may restrict the substrate from assuming a productive orientation within the active site cavity of the enzyme. Although a carbonyl is also present equidistant to the target hydroxyl group in the acceptable substrate lasilarin (LLN **6**, the skeletal isomer of LCL **5**), that carbonyl at C-1 is part of the lactone and thus would not be subject to enolization. Even for those noncognate substrates that were accepted by HsOMT, the conversion rates were frequently inferior to those of LtOMT with the same scaffold, as seen with DLD **1**, zearalenone (ZEA, **4**), and to a lesser extent with ARA7 **7** and LLN **6** (Fig. 2, *SI Appendix*, Table S3).

The 14 resulting *O*-methylated products, including 11 that are new to nature were isolated, and their structures were elucidated by comparison of their ¹H and ¹³C NMR data with those reported, and by careful analysis of their 1D NOESY, ¹H-¹H COSY, HSQC, and HMBC NMR spectra to ascertain the position of the methoxy moieties (*SI Appendix*, Fig. S6 and Table S2). For all accepted substrates, *O*-methylation regioselectivity was found to be idiosyncratic for the two OMTs, despite the large structural differences of the BDL congeners (Fig. 2). Thus, LtOMT is specific for the phenolic hydroxyl residing at the *ortho* position to the aromatic carbon bearing the carbonyl (hereafter, *ortho*-OMT activity). Conversely, HsOMT *O*-methylates the phenolic hydroxyl that is at the *para* position to the same (henceforth, *para*-OMT activity). The carbonyl "handle" for this recognition can be part of a lactone, an ester or a ketone. No "off-target" *O*-methylation was observed with either enzyme. This strict regiospecificity is in contrast to the chemical (non-enzymatic) *O*-methylation of phenolic hydroxyl groups which either affords a mixture of methoxy regioisomers and di-substituted products, or requires laborious protection-deprotection reactions to achieve regioselectivity.³⁰

Mono-O-methylated congeners as substrates for subsequent combinatorial Omethylation.

We also investigated whether preexisting *O*-methylation at one phenolic hydroxyl group would influence the suitability of a scaffold towards subsequent *O*-methylation at the other hydroxyl (Fig. 2, *SI Appendix*, Table S3). To do this, we compared results from three biocatalytic systems. First, we offered *o*-methoxy BDL congeners to the *S. cerevisiae* strain expressing HsOMT. Second, we provided *p*-methoxy compounds to the yeast host producing LtOMT. Third, we

interrogated *O*-methylation of unmethylated substrates by yeast strains co-expressing LtOMT and HsOMT in the same chassis. These experiments revealed that seemingly very similar final product distributions can result from very different processes in the coupled biocatalytic production system where two enzymes are co-expressed in the same host chassis and compete for the same unmethylated substrate, and where the mono-*O*-methylated product of either enzyme may or may not serve as a second-stage substrate for the other enzyme (Fig. 2). The resulting six *o*,*p*-dimethoxy BDL congeners, all of which are new to nature were isolated and their structures were elucidated by 1D and 2D NMR spectroscopy (*SI Appendix*, Table S2 and Fig. S6).

In some cases, preexisting *O*-methylation severely reduced the ability of both OMTs to accept a BDL scaffold as a substrate, as was seen with *O*-methylated LLN **6** congeners (Fig. 2, *SI Appendix*, Fig. S2A and Table S3). Consequently, only a small amount of the dimethoxy LLN analog **6c** was made in the LtOMT-HsOMT co-expression system. In other cases, preexisting *O*-methylation had no effect on the ability of either LtOMT or HsOMT to carry out subsequent methylation of the other phenolic hydroxyl, as was observed with methoxy congeners of the DLD **1** scaffold (Fig. 2, *SI Appendix*, Fig. S2B and Table S3). Thus, the dimethoxy DLD congener **1c** could be made either through the $1\rightarrow 1a\rightarrow 1c$ or the $1\rightarrow 1b\rightarrow 1c$ route. However, since *O*-methylation of 1/1a by HsOMT is far less complete than that of 1/1b by LtOMT, lasiodiplodin **1a** continued to accumulate in the LtOMT-HsOMT co-expression system, while 5-*O*-methyl DLD **1b** was mostly converted to the dimethoxy DLD congener **1c**.

Preexisting *O*-methylation was often inhibitory to HsOMT but not to LtOMT (Fig. 2, *SI* Appendix, Fig. S2C and Table S3). Thus, *ortho-O*-methylation (as in 3-*O*-methyl DHZ **3a**, 3-*O*-methyl ZEA **4a**, 3-*O*-methyl ARA7 **7a**, and 3-*O*-methyl ARA8 **8a**) prevented further *O*-methylation by HsOMT, while the regioisomeric congeners (such as 5-*O*-methyl DHZ **3b**, 5-*O*-methyl ZEA **4b**, 5-*O*-methyl ARA7 **7b**, and 5-*O*-methyl ARA8 **8b**) remained excellent substrates for LtOMT. As a result, the corresponding *o*,*p*-dimethoxy derivatives could only be made via the $X \rightarrow Xb \rightarrow Xc$ route in the LtOMT-HsOMT co-expression system (X=3, 4, 7, or 8). At the same time, most of the available substrates remained trapped as the *ortho-O*-methyl congeners (as with 3-*O*-methyl ZEA **4a**, 3-*O*-methyl ARA7 **7a**, and 3-*O*-methyl ARA8 **8a**) since these could not be further converted by HsOMT. A contrary situation was observed for RDN **2** that is not a substrate for HsOMT (Fig. 2, *SI Appendix*, Fig. S2D and Table S3). Here, pre-existing *ortho-O*-methylation (as in 3-*O*-methyl RDN **2a**) made this scaffold acceptable, albeit only marginally, to serve as a substrate for HsOMT. Thus, a small amount of the *o*,*p*-dimethoxy RDN derivative **2c** was obtained through the **2**→**2a**→**2c** route in the co-expression system. Since 5-*O*-methyl RDN **2b**, an otherwise

excellent substrate for LtOMT, was not produced by HsOMT, the dimethoxy RDN congener 2c could not be made by the $2 \rightarrow 2b \rightarrow 2c$ route.

Docking identifies distinct substrate binding poses.

Despite the mutually orthogonal regiospecificities of LtOMT and HsOMT against multiple and structurally varied substrates, these enzymes are orthologous with a relatively high sequence similarity (58% identity). This suggests that the target site for O-methylation may be determined by a limited number of key amino acid residues. To identify the structural basis for the programming of these distinct regiospecificities, we constructed homology protein structure models for LtOMT and HsOMT (Fig. 3) based on the known crystal structure of the mitomycin 7-O-methyltransferase of Streptomyces lavendulae (MmcR, PDB 3GXO;³¹ Z-score 43.3, rmsd 0.9, and identity of 26% with LtOMT; and Z-score 41.6, rmsd 2.1, and identity of 24% with HsOMT). Both OMT models feature an N-terminal dimerization domain each (amino acids 1-130, SI Appendix, Fig. S3), and a C-terminal OMT Class I, α/β Rossmann-fold catalytic domain with the conserved SAM-binding signature motif DxGGGxG (amino acids 231-237)³¹⁻³³. Modeling of Sadenosyl-L-homocysteine (SAH) into LtOMT and HsOMT indicated that the key enzymecoproduct interactions are well conserved amongst LtOMT, HsOMT, MmcR³¹ and similar MTs such as the calicheamicin orsellinic acid methyltransferase CalO1 of Micromonospora echinospora³² (SI Appendix, Fig. S4). Thus, the adenine moiety of SAH is predicted to be stabilized by π -stacking with F284, while the sulfur atom of SAH could engage in hydrophobic interactions with F197 and M201. The SAH adenine N-6 amino group forms a hydrogen bond with D283, while the ribose O-2 and O-3 may engage in a hydrogen bonding network with R262 and E261, with the latter residue also interacting with the SAH adenine N-3. Finally, the terminal carboxylic acid group of SAH may form hydrogen bonds with E203 while the terminal amino group could interact with the main chain carbonyl oxygen of G233.

Docking of DLD **1** and DHZ **3** indicated that, similar to other phenolic OMT enzymes^{31,32}, substrate binding is dominated by hydrophobic and van der Waals interactions with residues such as F52* and W55* from the other subunit of the dimer (for both enzymes); residues F197, M201, A200 and M344 (for LtOMT); and amino acids V136, F197, M201 and I349 (for HsOMT). Importantly, docking also revealed characteristically different binding poses for these substrates in LtOMT *vs.* HsOMT (Fig. 3, *SI Appendix* Fig. S4). For LtOMT, both DLD and DHZ are predicted to present their OH-3 to the Nε of the deduced active site base H303³³, while OH-5 may be

contacted by the main-chain carboxyl group of D202. Conversely, rotation of both substrates within the active site cavity may position OH-5 for attack by H303 of HsOMT, while OH-3 and the C-1 carbonyl could interact with the side-chain hydroxyl group of S200. Abstraction of the proton from the substrates by H303 of either enzyme would then lead to a nucleophilic attack of the phenolate anion of the substrate on the reactive methyl group of the co-substrate *S*-adenosyl-L-methionine (SAM). Meanwhile, the catalytic base may be oriented and stabilized by a hydrogen bonding network between Nδ of H303 with D331 and E360 (LtOMT), or D331 and D360 (HsOMT), respectively³³ (Fig. 3 and *SI Appendix*, Fig. S4).



Figure 3. Structure modeling of LtOMT and HsOMT. Cartoon representations of homology models and predicted active site cavities of LtOMT (**A**, **C**) and HsOMT (**B**, **D**) with docked substrates DLD (carbon atoms in *orange*), DHZ (carbons in *magenta*) and co-product SAH (carbons in *green*), all represented as *sticks*. The predicted active site H303 and the residues stabilizing the active site (D331 and E360 for LtOMT, E331 and E360 for HsOMT) are also shown as *sticks* (carbons in *yellow*). Residues in the active site cavity that were found to influence substrate orientation are also shown as *sticks* (carbons in *salmon* for LtOMT, and *grey* for HsOMT), and housed in the protein segments (*orange* on the cartoon diagrams of **A** and **B**) that were

exchanged in hybrid enzymes H1 and H2 (Fig. 4). Key hydrogen bonding networks are displayed as *yellow dashes*.



Figure 4. Engineering the O-methylation regioselectivity of LtOMT. Left, schematic

representation of chimeric and mutant enzymes created from LtOMT (in *blue*) and HsOMT (in *green*), with the positions of amino acid replacements or fragment boundaries indicated. *Middle*, product profiles (reversed-phase LC-MS traces recorded at 210 nm with a photodiode array detector) of representative in vivo bioconversion reactions (DLD **1** as the substrate fed to cultures of *S. cerevisiae* BJ5464-NpgA expressing the gene encoding the indicated enzyme). The identities of the products were validated by comparison with isolated and structurally characterized compounds. *Right*, total conversion (in percent) of DLD **1** into all corresponding methylated product congeners are shown as *bar graphs*. Percentage distribution of the *O*-methylated products are represented as *pie charts* (yields of the individual *O*-methylated products divided by the total yield of all products, multiplied by 100). Color code: lasiodiplon **1a** (*blue*), 5-*O*-methyl DLD **1b** (*green*), and dimethoxy DLD congener **1c** (*red*). See *SI Appendices* Table S4 for percent conversion values (mean \pm SD from three independent experiments of three replicates each, n=9).

Remodeling of the active site cavity alters regioselectivity.

To identify the residues that may influence the choice of the *O*-methylation target site in the substrates, we built hybrid enzymes from LtOMT and HsOMT by fusion PCR, expressed these in the *S. cerevisiae* chassis, and quantified the regioselectivity and the extent of *O*-methylation after feeding DLD **1** as a model substrate. First, we created chimeras from LtOMT and HsOMT. In chimera H1, amino acids 1-329 of LtOMT were replaced with those of HsOMT. Despite the extensive replacement, the regioselectivity did not change, but the efficiency of H1 declined dramatically (Fig. 4). The complementary hybrid H2 (amino acids 330-398 from HsOMT) retained reasonable activity (26.1 \pm 3.0% total conversion), but the regioselectivity completely switched to that of HsOMT (i.e. *para*-OMT). Chimera H3 with a shorter HsOMT-derived segment (amino acids 366-398) produced **1a**:1**b** in a 3:1 ratio at 34.5 \pm 0.8% conversion. These chimeras indicated that regioselectivity is determined by the *C*-terminal end of the enzyme, and suggested that extensive replacements are detrimental to enzymatic activity.

Next, we turned to structure-guided site-directed amino acid replacements to examine the influence of residues located within 6Å of the substrates in the active site pockets of LtOMT and HsOMT. Replacement of a cluster of four of these amino acids at the *C*-terminal end of LtOMT with those of HsOMT (Q384K, G386R, W387H, and Q388H) by site-directed mutagenesis provided mutant enzyme M1 that retained the overall high conversion rate of LtOMT (90.0 \pm 2.9% of substrate converted to methylated products). M1 preserved most of the *ortho*-OMT activity of

LtOMT (lasiodiplodin **1a**, 82.9 \pm 0.8% of methylated products), but also gained HsOMT-type *para*-OMT activity as shown by the accumulation of products 5-*O*-methyl DLD **1b** (3.7 \pm 0.8%) and dimethoxy DLD congener **1c** (13.4 \pm 1.5%). Thus, replacement of these four amino acids is sufficient to accommodate the model substrate within the active site cavity in orientations that expose not only OH-3, but also OH-5 to the active site. Dissecting the influences of these amino acids by creating all possible 3-point, 2-point and 1-point replacements (Fig. 4 and *SI Appendix*, Table S4) indicated that W387H has the highest influence towards enabling a *para*-OMT activity. Q388H (and to a lesser extent Q384K) play a supplementary role when combined with W387H, while the effect of G386R is context-dependent. Thus, enzyme M4 with only the W387H mutation shows *para*-OMT activity in addition to a robust *ortho*-OMT activity as evidenced by the production of 7.7 \pm 0.3% of dimethoxy DLD congener **1c** at 76.6 \pm 20.2% overall substrate conversion. Addition of Q388H (as in M3) and further introduction of Q384K (as in M2) gradually improves the *para*-OMT activity while preserving a strong *ortho*-OMT activity (**1a**:**1b**:**1c** = 4:1:1) and continues to support high overall conversion (Fig. 4).

Introduction of segment 330-366 of HsOMT into the four-point mutant M1 yielded enzyme HM1 that acted exclusively as a *para*-OMT, indicating that this short region is decisive in setting regioselectivity. Surprisingly, the chimeric mutant HM1 converted more of the DLD substrate to 5-O-methyl DLD **1b** than HsOMT, the native para-OMT parent (58.3 \pm 11.4% vs. 41.6 \pm 1.2%). We then investigated two residues of this segment that are situated within 6Å of the substrates. Adding the F330V mutation to those already present in enzyme M1 (as in M5) improved the para-OMT activity as evidenced by an increase of the **1b** yield to $13.3 \pm 5.5\%$ (cf. $3.7 \pm 0.8\%$ for M1). while the production of the 3,5-di-O-methoxy product 1c and the total conversion rate remained unchanged. Introduction of the T356M mutation into M1 (as in M6) led to a dramatic reversal of the regioselectivity with 5-O-methyl DLD 1b representing $74.3 \pm 4.2\%$ of the products and lasiodiplodin 1a reduced to $10.0 \pm 1.9\%$, albeit at the expense of a slight penalty in product yield $(70.1 \pm 5.8\%$ total conversion in M6 vs. $90.0 \pm 2.9\%$ in M1). Introduction of both changes into M1 afforded enzyme M7 (a six-point mutant) with an almost perfect para-OMT specificity ($94.1 \pm 1.0\%$ **1b**) and high overall conversion $(93.7 \pm 1.1\%)$. Thus, mutant enzyme M7 far surpassed the parent HsOMT as a DLD para-OMT. Surprisingly, mutant M8 with only a single amino acid replacement (T356M) had an apparently balanced ortho-OMT/para-OMT activity with the three product congeners present in roughly 1:1:1 at $80.1 \pm 10.2\%$ total conversion. Thus, this residue seems to relax regioselectivity, and allows the substrate to assume either of the two binding poses with equal likelihood. Introduction of a second mutation into enzyme M8 (W387H, cf. mutant M4) yielded

M9 that afforded the 3,5-di-O-methyl-DLD 1c almost exclusively, and with a very high productivity (96.4 \pm 0.7% conversion of the substrate).

To further characterize the native and the engineered enzymes, LtOMT, HsOMT and selected variants of LtOMT (M1, M6-M9) were heterologously expressed in *E. coli* as His-tagged proteins and purified to substantial homogeneity (*SI Appendix*, Fig. S5). The purified enzymes afforded optimal conversion of the model substrate DLD **1** in the presence of 10 mM MgCl₂. While NaCl (0-100 mM), CaCl₂ (10 mM) or MnCl₂ (10 mM) did not influence the activity, EDTA, CuCl₂ or ZnSO₄ (each at 10 mM) were strongly inhibitory. The enzymes showed a broad optimum at pH 7.5-9.5 in Tris or phosphate buffers, with the activity disappearing at pH 5.0 and above 10.0. The temperature optimum was 30 °C, with residual activity still measurable at 4 °C and at 65 °C.

The kinetic parameters for LtOMT and its variants with DLD **1** as the substrate are shown in Table 1. HsOMT was found to be kinetically slow and the reaction did not reach saturation under the conditions tested, thus K_m and V_{max} were not determined for this enzyme. Interestingly, the K_m of LtOMT and its five variants were similar. We speculate that the substrate binding cavity of LtOMT is suitably spacious to accommodate the altered amino acid residues without reducing the affinity of the mutant enzymes towards binding the model substrate. In contrast, the turnover rate (k_{cat}) and thus the catalytic efficiency (k_{cat}/K_m) of the LtOMT parent enzyme were more than two orders of magnitude higher than those of the mutants. This may indicate that the altered substrate with the mutant enzymes. Nevertheless, the turnover rates of the formation of the transition state with these of many wild type secondary metabolite methyltransferases, such as the GenL gentamicin C1/C2 methyltransferase $(k_{cat} 0.043 \text{ sec}^{-1})^{34}$ or many flavone OMTs from plants $(k_{cat}$ often in the range of 0.06 to 0.09 sec^{-1})^{15,16}.

Taken together, structure-based site-specific replacements of a few key "plasticity residues" in the active site of LtOMT remodeled this regiospecific *ortho*-OMT enzyme to afford an almost exclusive DLD *para*-OMT (M7, 6 amino acids replaced); an apparently unselective DLD *ortho*-OMT/*para*-OMT (M8, single amino acid replacement); and a DLD *o,p*-di-OMT (M9, two amino acids replaced). While homology modeling-based "keyhole surgery" was successful in modulating the regioselectivity of LtOMT, protein crystallographic studies would be necessary to disentangle the exact contributions of the individual amino acid replacements in forcing the substrates to adopt altered binding poses, leading to modulated regioselectivities and catalytic efficiencies. Increasing kinetic efficiency would also require further optimization. This is analogous to natural evolution where functional innovation in the product and substrate spectrum is often the consequence of a

few key amino acid changes, followed by the accumulation of compensatory mutations that optimize the novel activity.³⁵

 Table 1. Kinetic parameters of LtOMT and its engineered variants with DLD (1) as the substrate

	K _m [mM]	V _{max} [nmol sec ⁻¹ mg ⁻¹]	k_{cat} [sec ⁻¹]	$k_{\text{cat}}/K_{\text{m}}$ [sec ⁻¹ mM ⁻¹]
LtOMT	1.58 ± 0.01	329.8 ± 102.8	14.585 ± 4.547	9.209
M1	2.03 ± 0.07	1.5 ± 0.5	0.068 ± 0.023	0.033
M6	1.15 ± 0.02	0.6 ± 0.1	0.028 ± 0.002	0.024
M7	2.49 ± 0.22	1.0 ± 0.2	0.044 ± 0.008	0.018
M8	1.40 ± 0.03	1.2 ± 0.1	0.053 ± 0.005	0.038
M9	1.52 ± 0.30	1.4 ± 0.5	0.064 ± 0.022	0.042

 $k_{\text{cat}}/K_{\text{m}}$ was calculated using the averages of k_{cat} and K_{m} . Values represent the mean \pm SD from three independent experiments with three replicates each (n=9).

Regioselectivity of the engineered enzymes is influenced by the substrate.

The regiospecificities of the wild type LtOMT and HsOMT enzymes were found to be independent of the substrates used (Fig. 2): for any given substrate accepted by the enzyme, only a single *O*-methylated derivative was obtained, and the targeted phenolic OH was always *ortho* (LtOMT) or *para* (HsOMT) to the aromatic carbon bearing the carbonyl "handle". In contrast, the mutant LtOMT enzymes possess both *ortho*-OMT and *para*-OMT activities to various degrees. To discover whether the ratio of these activities is an intrinsic characteristic of these enzymes or if regioselectivity is also influenced by the substrates, we selected five mutants (M1, M6-M9) with characteristically different regioselective outcomes when acting on the DLD model substrate. We conducted biocatalytic reactions with these enzymes using our panel of eight structurally varied BDL substrates and their *o*-methoxy or *p*-methoxy congeners (Fig. 5, *SI Appendix*, Table S5).

The LtOMT variant enzymes processed DLD **1** and RDN **2**, the two RAL_{12} scaffolds of the panel in a similar manner. The T356M mutation in LtOMT variant M8 created a previously nonexistent *para*-OMT activity against both the unmethylated compounds DLD **1** and RDN **2** and their *o*-methoxy congeners lasiodiplodin **1a** and 3-*O*-methyl RDN **2a**. The *ortho*-OMT activity

against the unmethylated and the p-methoxy congeners remained operational, thus the o,pdimethoxy congeners were produced through both the $X \rightarrow Xa \rightarrow Xc$ and the $X \rightarrow Xb \rightarrow Xc$ routes (X=1 or 2). Adding the W387H mutation (as in M9) caused these *o,p*-dimethoxy congeners to become the main products with substrates DLD 1 and RDN 2. This was the result of improvements in both the *para*-OMT activity towards lasiodiplodin 1a and 3-O-methyl RDN 2a, and an increase in the ortho-OMT activity towards 5-O-methyl DLD 1b and 5-O-methyl RDN 2b. Supplementing the T356M and the W387H mutations with the Q384K, G386R, and Q388H changes (as in M6) reduced activity towards the mono-methoxy congeners, but improved the *para*-OMT activity towards the unmethylated substrates DLD 1 and RDN 2. As a result, the amounts of the dimethoxy congeners were reduced, but the *p*-methoxy congeners **1b** and **2b** accumulated. Removing the T356M mutation (as in M1) did not affect the *ortho*-OMT activity, but eliminated the *para*-OMT activity against lasiodiplodin 1a and 3-O-methyl RDN 2a and limited it against DLD 1 and RDN 2. Introducing the F330V mutation into M6 (as in M7) did not seem to affect the *ortho*-OMT activity against 1/1b and 2/2b. However, it improved the para-OMT activity against DLD 1 and RDN 2 but not against the *o*-methoxy congeners 1a and 2a. As a result, 5-O-methyl DLD 1b became the main product for the DLD scaffold, while 5-O-methyl RDN 2b production reached its highest level for the RDN scaffold with this enzyme variant.

The unmethylated RAL₁₄ congeners were found to be less amenable than the RAL₁₂ compounds towards *para-O*-methylation, perhaps as a consequence of the increased steric bulk of the larger macrocycles. In particular, LCL **5** (and its *o*-methoxy congener **5a**) remained completely impervious to *para-O*-methylation just as with the other parent enzyme HsOMT, indicating that the suggested inhibitory role of the enol tautomer of the C-9 carbonyl remains a problem even in the context of an LtOMT scaffold. Thus, molding the active site cavity modulated only the *ortho*-OMT activity against LCL **5**. For the DHZ **3** and ZEA **4** scaffolds, no mutants were able to accommodate the *o*-methoxy congeners **3a** and **4a**, perhaps because the increased bulk of the 3-methoxy group obstructs access to the active site in the conformation that is conducive to the *para*-OMT activity. Consequently, the $X \rightarrow Xa \rightarrow Xc$ route (X=3 or 4) towards the dimethoxy analogs remained unavailable for all enzyme variants. In contrast, the *p*-methoxy congeners **3b** and **4b** remained excellent substrates for all LtOMT mutants, indicating that the methoxy group in the *ortho*-OMT binding pose does not encounter similar steric restrictions. Consequently, the first-stage products 5-*O*-methyl DHZ **3b** and 5-*O*-methyl ZEA **4b** did not accumulate but were converted to the dimethoxy congeners through the $X \rightarrow Xa \rightarrow Xc$ route (X=3 or 4).

The only DAL scaffold recognized as a substrate by the two parental enzymes was the 14-

membered BDL, LLN **6**, although the conversion rate was only moderate for both enzymes (37.7 \pm 1.4% for LtOMT and 30.7 \pm 3.1% for HsOMT, Fig. 2). The introduced mutations allowed the production of 7-*O*-methyl LLN **6a**, 5-*O*-methyl LLN **6b**, and the dimethoxy LLN congener **6c** by all engineered enzymes, with the mono-methoxy congeners **6a** and **6b** accepted as substrates for a second round of *O*-methylation by all mutants except M1. However, overall conversion declined precipitously to 2-5% with M1 and M6-M8, recovering to 15.8 \pm 1.4% only with M9.

Just as with the RAL₁₄ scaffolds, the *p*-methoxy congeners of the two ARA (5-*O*-methyl ARA7, **7b** and 5-*O*-methyl ARA8, **8b**) could be methylated by LtOMT and all its mutants, while the *o*-methoxy congeners (3-*O*-methyl ARA7, **7a** and 3-*O*-methyl ARA8, **8a**) remained recalcitrant towards subsequent methylation. Presumably, the bulky *o*-methoxy group prohibits the substrate from binding in the active site cavity in a pose that could offer the *para* phenolic hydroxyl to the active site base H303, but can be accommodated in the pose that leads to *ortho-O*-methylation.

Taken these together, it is striking that the apparent *ortho*-OMT/*para*-OMT preference of a given enzyme variant is not an intrinsic and immutable property of the enzyme but emerges from an intimate interplay with the various substrate scaffolds. Similar phenomena were also noted for bacterial and mammalian catechol OMTs^{11–13}. Product distribution among *o*-methoxy, *p*-methoxy and *o*,*p*-dimethoxy derivatives is determined by two major factors: 1, how well the active site accommodates the unmethylated substrate in the two poses that expose one or the other phenolic hydroxyl to the active site; and 2, whether the *o*-methoxy and the *p*-methoxy first-stage reaction products can be re-accommodated in the complementary pose that offers the free OH to the active site. It is thus perhaps not surprising that mutant enzymes selected by using a single model substrate (in this case, DLD 1) would reveal very different product profiles when offered a different substrate. Analogous phenomena are often encountered in directed enzyme evolution projects, prompting the formulation of the "first law of directed evolution": you get what you screen for.³⁶



Figure 5. Different substrate scaffolds modulate the apparent regiospecificities of selected engineered OMTs. Stacked polar bar charts show the product distributions among *o*-methoxy (*blue*), *p*-methoxy (*green*) and *o*,*p*-dimethoxy (*red*) BDL congeners during biocatalytic conversion of the indicated substrates (*bold numbers above the bars*) with *S. cerevisiase* BJ5464-NpgA expressing LtOMT and its variants M1 and M6-M9. *Percent values* below the compound numbers on these charts show the overall conversion percentage of the indicated substrate into all corresponding methylated products. See *SI Appendix*, Table S5 for tabulated percent conversion values as the mean \pm SD from three independent experiments of three replicates each (n=9). *Scheme*: Unmethylated substrates (**X**) may be converted to the *o*-methoxy congener (**Xa**) by the *ortho*-OMT activity, and to the *p*-methoxy congener (**Xb**) by the *para*-OMT activity of the enzymes. Either or both of these first-stage products may also be converted to the *o*,*p*-dimethoxy congener (**Xc**) by the enzyme (**X=1-8**).

Conclusions

O-Methylation of reactive phenolic hydroxyl groups by regiospecific OMT enzymes is used by BDL-producing fungi in nature to modulate the biological activities of these ecologically

important small molecule natural products. For combinatorial biosynthesis of drug-like BDL scaffolds in the laboratory, appropriate tailoring enzymes need to be employed to process unnatural congeners produced by engineered polyketide biosynthetic pathways. Amongst these tailoring enzymes, OMTs need to display significant promiscuity to accept structurally varied BDL scaffolds as substrates, while these enzymes must also offer appropriate regioselectivity to deliver the desired methoxy regioisomer. Our work shows that two orthologous OMT enzymes from different BDL biosynthetic pathways possess considerable substrate flexibility but display strict orthogonal regiospecificity. Protein homology modeling and substrate docking reveals that nature used subtle alterations of the amino acids lining the active site cavity to force the substrates to adopt different binding poses. In turn, this exposes distinct phenolic hydroxyls to the conserved catalytic base, accounting for the observed orthogonal regiospecificity. This apparent plasticity of the active site cavity architecture then allows us to re-program the regioselectivity of BDL OMT enzymes by adjusting just a few amino acids in a keyhole surgery-like manner. Doing so, we can transpose the strict regiospecificity of a native OMT to generate a mutant enzyme that produces the orthogonal *p*-methoxy derivative of the model substrate almost exclusively. We can also engineer a mutant enzyme that apparently eschews regioselectivity towards the model substrate to afford o-methoxy, *p*-methoxy, and *o*,*p*-dimethoxy congeners in equal proportions; or another one that specializes in recursive methylation to yield mostly the *o*,*p*-dimethoxy derivative. We also show that apparently similar product distributions may result from different scenarios involving dissimilar recognition and processing of the unmethylated, the o-methoxy, and the p-methoxy substrates by the ortho-OMT and the *para*-OMT activities engineered into the same enzyme chassis. As their active site cavity is being remodeled, the enzymes may acquire or lose one or the other activity, and come to accept or reject certain substrates. In accord with these dynamic changes, the regiospecificities and the resulting product ratios of the mutant OMT enzymes turn out to be context dependent, as they are modulated by the complex interactions of the structurally varied substrates with the enzymes. Thus, engineering an OMT to generate a desired methoxy regioisomer of a given "unnatural product" during combinatorial biosynthesis is analogous to enzyme evolution in nature where catalytic innovations emerge in the context of a particular molecule that provides fitness advantage to the producer, and is accomplished by a few key mutations in "plasticity residues" followed by the acquisition of supplementary mutations to optimize the novel activity. Recapturing this process in synthetic biology by directed enzyme evolution towards improved catalytic efficiency, coupled with chassis metabolic engineering and fermentation process optimization would doubtless be also necessary to deploy engineered OMTs for practical, industrial scale applications, as was seen with the recent development of a commercial process for vanillin production that also employs an

OMT.^{40–42} Even so, extension of the scalable, sustainable and tunable enzymatic approach described here towards the regioselective *O*-methylation of a wider variety of phenolic polyketide natural products, such as the isocoumarins, isochromanones anthraquinoids, and the flavonoids^{7,10,43,44} offers advantages over the well-known chemical methods that require high temperature/pressure and longer reaction times often leading to undesired *C*-methylated byproducts.^{8,9}

Data availability

Experimental and bioinformatic procedures, descriptions on the isolation and structure elucidation of compounds, spectroscopic data, tabulated biocatalytic conversion rates, supplementary figures including additional structures, dissection of demethylation routes, and protein topology and docking models are all available in the Supplementary Information. All other data are available from the authors upon reasonable request.

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Figure 1. Total biosynthesis of O-methylated BDLs using LtOMT and HsOMT.

84x44mm (300 x 300 DPI)





Figure 2. Combinatorial tailoring with LtOMT and HsOMT.



Figure 3. Structure modeling of LtOMT and HsOMT.



Figure 4. Engineering the O-methylation regioselectivity of LtOMT.





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Figure 5. Different substrate scaffolds modulate the apparent regiospecificities of selected engineered OMTs.





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