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Assessing the Regioselectivity of OleD-Catalyzed Glycosylation with a Diverse Set of Acceptors

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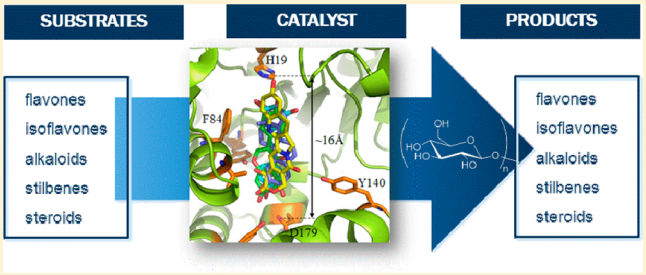
Supporting Information

ABSTRACT: To explore the acceptor regioselectivity of OleD-catalyzed glucosylation, the products of OleD-catalyzed reactions with six structurally diverse acceptors flavones—(daidzein), isoflavones (flavopiridol), stilbenes (resveratrol), indole alkaloids (10-hydroxycamptothecin), and steroids (2-methoxyestradiol)—were determined. This study highlights the first synthesis of flavopiridol and 2-methoxyestradiol glucosides and confirms the ability of OleD to glucosylate both aromatic and aliphatic nucleophiles. In all cases, molecular dynamics simulations were consistent with the determined product distribution and suggest the potential to develop a virtual screening model to identify additional OleD substrates.



The glucosyltransferase OleD from *Streptomyces antibioticus* catalyzes the glucosylation of oleandomycin using UDP-D-glucose (UDP-Glc) as the glycosyl donor (Figure 1). This enzyme, first studied by Salas and co-workers, exists as part of a prototype system for macrolide inactivation and secretion in macrolide-producing microorganisms.[1] Consistent with its role in detoxification, wild-type OleD (wtOleD) displays relatively broad substrate tolerance with a bias toward small aromatic hydroxy groups, and recent OleD-directed evolution and engineering efforts dramatically improved upon this catalyst's proficiency and range of accessible substrates.[2,3] The availability of enhanced OleD mutants and simple activated aromatic glycoside donors also enabled efforts to modulate the corresponding reaction equilibrium as a unique strategy for sugar nucleotide synthesis, glycodiversified small molecules, and even a high-throughput screen for glycosylation.[3c] Cumulatively, these studies revealed OleD variants to function as a multifunctional and iterative *O*-/*S*-/*N*-GT capable of glucosylating well over 100 diverse acceptors. However, with a few exceptions[2,3b,4] the product characterization for these studies was limited to LC-MS, and thus, the regio-/stereospecificity of OleD-catalyzed glycosyltransfer with "non-native" substrates remains poorly understood.[5] To address this limitation, herein we describe the characterization of glycosides produced via the OleD-catalyzed glucosylation of a set of six representative structural classes: flavones (daidzein), isoflavones (flavopiridol), stilbenes (resveratrol), indole alkaloids (10-hydroxycamptothecin), and steroids (2-methoxyestradiol). This study revealed OleD to glycosylate both aromatic and aliphatic nucleophiles, the regioselectivity of which was dictated by a range factors, including reaction thermodynamics, enzyme mutation, and the acceptor architecture. A parallel molecular dynamics simulation for each reaction studied was consistent with the corresponding product distribution observed and sets the stage to use virtual screening as a means to identify additional OleD substrates.

RESULTS AND DISCUSSION

Glucosylation of Daidzein. Isoflavones occur naturally in legumes and are consumed regularly in the human diet.[6] Isoflavones often exist naturally as *O*-glycosides and have attracted considerable pharmaceutical interest.[7] Daidzein is one of the most commonly occurring isoflavones, with the corresponding 4′- and 7-*O*-glucosides as well as the 7,4′-di-*O*-glucosides of daidzein isolated from numerous sources.[8] Daidzein and its corresponding *O*-glucosides are believed to be the major effective components of a traditional Chinese medicine, *Kudzu* (*Pueraria lobata*), for the treatment of a wide range of disorders since 600 A.D.[9] While daidzein has limited solubility in water, the aqueous solubility of the corresponding 7-*O*-glucoside is ~30-fold improved.[10] The unique activities of these glycosides have inspired several targeted syntheses of daidzein 4′- and 7-*O*-glucosides.[11] However, a convergent method to produce both mono- and diglucosides in parallel has not been reported.

The pilot reaction for this study utilized UDP-Glc as the donor and OleD ASP[3] as the catalyst under standard conditions (0.5 mM UDP-Glc, 0.1 mM aglycon, 16 h). On the basis of LC-MS, three products were observed (two monoglucosides and one diglucoside), with the diglucoside emerging as the major

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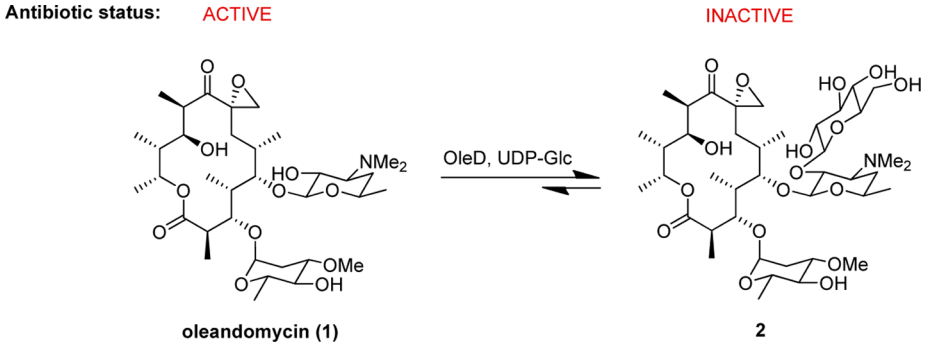

Figure 1. The native OleD-catalyzed reaction as a macrolide-producing host resistance mechanism.

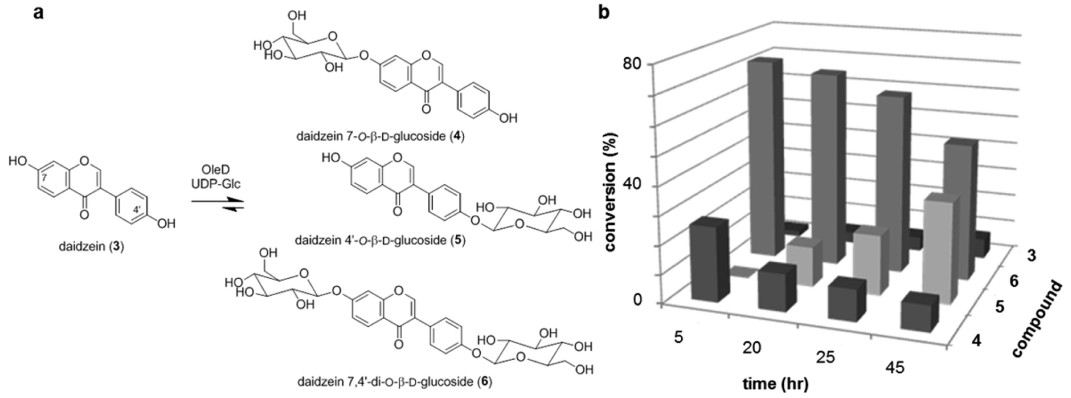

Figure 2. (a) Products deriving from OleD ASP-catalyzed glucosylation of daidzein. (b) Product distribution over time (the standard deviation of three trials was ±3%).

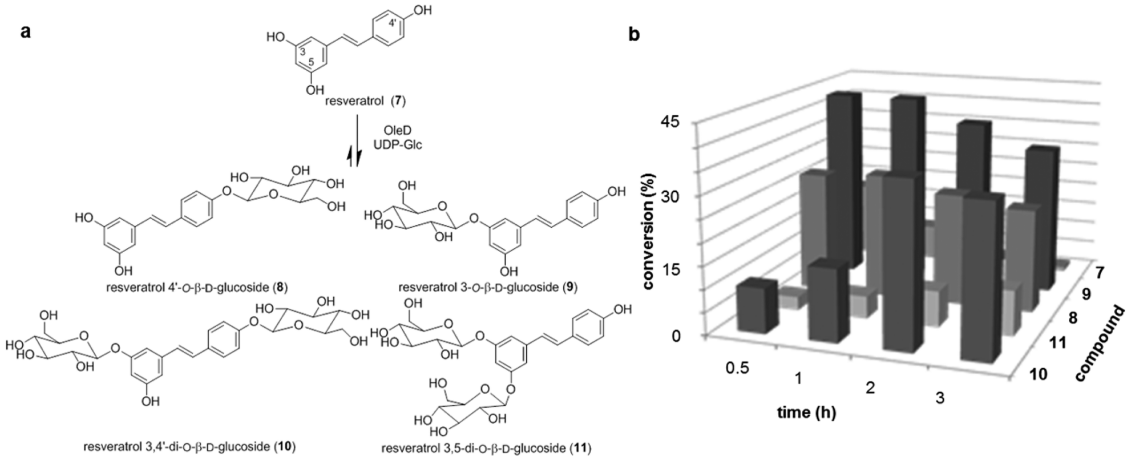

Figure 3. (a) Products deriving from OleD ASP-catalyzed glucosylation of resveratrol. (b) Product distribution over time (the standard deviation of three trials was ±2.5%).

product over time (Figure 2b). To maximize the production of all three products for subsequent characterization, a 25 h reaction was selected for the preparative-scale reaction. For this, daidzein (6.4 mg, 28.0 μmol) was dissolved in 1.25 mL of DMSO and transferred to 25 mL of assay buffer solution (50 mM Tris HCl, 5 mM $MgCl_2$, pH 8.0). The reaction was initiated via the addition of UDP-Glc (38 mg, 62.3 μmol) and 25 mg of OleD ASP. After 25 h of gentle agitation at room temperature (rt), the reaction was frozen and lyophilized to dryness. HPLC purification of the crude reaction mixture provided daidzein 7,4′-di-*O*-β-D-diglucoside **6** (5 mg, 8.6 μmol, 31%), daidzein 4′-*O*-β-D-glucoside **5** (1 mg, 2.4 μmol, 9%), and daidzein 7-*O*-β-D-glucoside **4** (2 mg, 4.8 μmol, 17%). The ^1H and ^{13}C NMR and HR-MS data of the isolated glucosides were consistent with previously reported characterization data.[11]

Glucosylation of Resveratrol. Resveratrol, a naturally occurring phytoalexin found in various plants, grape skin, peanuts, cranberries, and red wine,[12] reportedly exhibits multiple effects including life extension,[13] neuroprotection,[14] anti-inflammatory activity,[15] cardioprotection,[16] antidiabetic activity,[17] viral inhibition,[18] and cancer chemoprevention.[19] Recent work also revealed resveratrol to inhibit Wnt/β-catenin signaling, which inspired the synthesis of stilbene analogues that inhibit a unique target (methionine adenosyltransferase)

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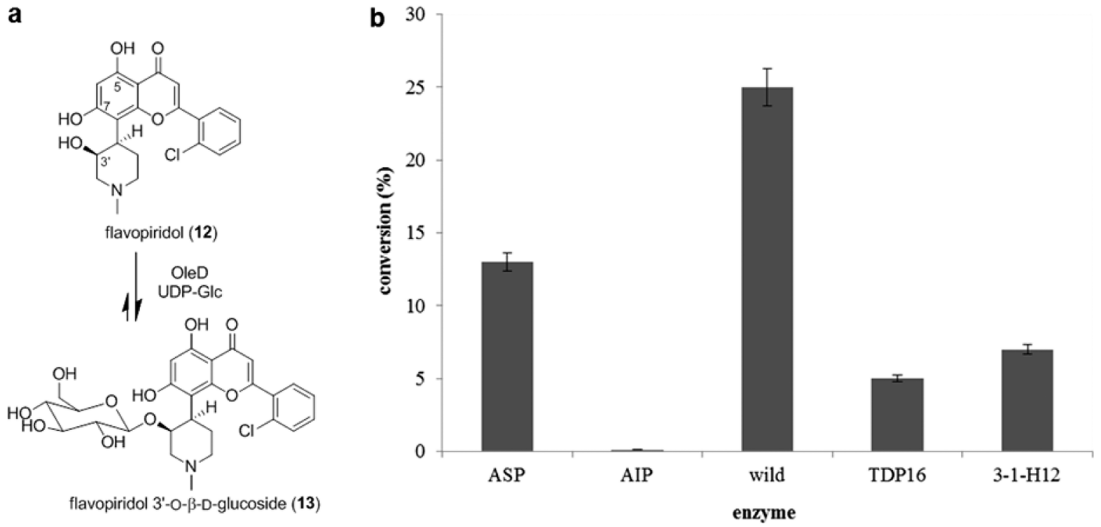

Figure 4. (a) OleD-catalyzed glucosylation of flavopiridol. (b) Flavopiridol glucosylation mediated by different OleD variants.

and display dramatic anticancer activity.[20] Despite these beneficial effects, the low bioavailability of resveratrol limits therapeutic application.[21] In humans and rats, less than 5% of an oral dose was observed as free resveratrol, with the most abundant metabolites comprising resveratrol 3-*O*-glucuronide and resveratrol 3-*O*-sulfate.[22] The *β*-D-glucosides of resveratrol, **8**,[23] **9**,[24] and **10**[25] (Figure 3), are also naturally occurring products that possess antiplatelet,[26] antioxidant,[27] and prolyl endopeptidase inhibitory activities,[28] and these activities stimulated the pursuit of various resveratrol glycosylation strategies.[29] Among these, the four glycosides **8**, **9**, **10**, and **11** were synthesized in parallel using multistep trifluoroacetimidate methodology.[29d]

The pilot reaction utilized UDP-Glc as the donor and OleD ASP as catalyst under standard conditions (2.5 mM UDP-Glc, 1 mM aglycon). The reaction was nearly complete within 3 h, leading to two diglucosides and two monoglucosides based upon LC-MS. A continuation of the reaction for longer periods of time (48 h) led to the production of two diglucosides, **10** and **11**, as the only products (data not shown). On the basis of this pilot reaction, 3 h was selected as the optimal reaction time for a preparative-scale reaction. For the preparative-scale reaction, resveratrol (5.7 mg, 25.0 *μ*mol) was dissolved in 1.25 mL of DMSO and transferred to 25 mL of assay buffer solution (50 mM Tris HCl, 5 mM $MgCl_2$, pH 8.0). The reaction was initiated via addition of UDP-Glc (38 mg, 62.3 *μ*mol) and 30 mg of OleD ASP. After 3 h of gentle agitation at rt, the reaction was frozen and lyophilized to dryness. HPLC purification of the crude reaction gave four products: resveratrol 4′-*O*-*β*-D-glucoside (**8**, 1.8 mg, 4.6 *μ*mol, 18%), resveratrol 3-*O*-*β*-D-glucoside (**9**, 2.0 mg, 5.1 *μ*mol, 20%), resveratrol 3,4′-di-*O*-*β*-D-glucoside (**10**, 2.2 mg, 4.0 *μ*mol, 16%), and resveratrol 3,5-di-*O*-*β*-D-glucoside (**11**, 1.2 mg, 2.2 *μ*mol, 9%). The ^1H and ^{13}C NMR and HR-MS data were consistent with previously reported data.[29d]

Glucosylation of Flavopiridol. Flavopiridol (also known as Alvocidib, HMR-1275, NSC 649890) is a semisynthetic analogue of the alkaloid rohitukine, a compound derived from the indigenous Indian plant *Dysoxylum binectarife*.[30] Flavopiridol is a cyclin-dependent kinase inhibitor that targets the positive transcription elongation factor P-TEFb, preventing activation of RNA polymerase II. Flavopiridol is cytotoxic to a range of cancer cell lines and initiates cell cycle arrest and p53-independent apoptosis through down-regulation of Mcl-1 and X-linked inactivator of apoptosis (XIAP).[31] Preclinical studies demonstrated the capacity of flavopiridol to induce programmed cell death, promote differentiation, inhibit angiogenic processes, and modulate transcriptional events.[32] These unique characteristics inspired extensive clinical investigation of flavopiridol.[33] Flavopiridol is eliminated via excretion in the form of both the parent drug and the C-5- or C-7-glucuronide.[34]

Using UDP-Glc as the donor and OleD ASP as catalyst (1.25 mM UDP-Glc, 0.25 mM aglycon, 16 h), the formation of a single monoglucoside (10% conversion) was observed by HPLC and LC-MS analysis (Figure 4). In an effort to boost production of this desired product, reactions catalyzed by a panel of OleD mutants were examined (Figure 4b), which surprisingly revealed wtOleD to enable the best conversion (35%). Thus, flavopiridol (11.1 mg, 25.0 *μ*mol) was dissolved in 1.25 mL of DMSO and transferred to 50 mL of assay buffer solution (50 mM Tris HCl, 5 mM $MgCl_2$, pH 8.0). The reaction was initiated via addition of UDP-Glc (76 mg, 0.125 mmol) and 15 mg of wtOleD. After 24 h of gentle agitation at rt, the reaction was frozen and lyophilized to dryness. The residue was dissolved in MeOH and subjected to HPLC purification to give flavopiridol monoglucoside product (4.9 mg, 8.7 *μ*mol) in 35% yield.

HRESIMS analysis of purified glucoside yielded an $[M + H]^+$ ion at *m*/*z* 564.1644, confirming a monoglucoside of flavopiridol with a formula of $C_{27}H_{30}ClNO_{10}$. 1D and 2D NMR data support the 3′-*O*-*β*-D-glucosidic structure presented in Figure 4a. The key evidence for C-3′ glucosylation derives from the HMBC correlation between the anomeric proton and the C-3′ carbon, with the large coupling constant (8.0 Hz) of the anomeric proton (δ_H 4.28, doublet) as a key signature for the *β*-anomer (Figure S1). That OleD catalysis led to the glucosylation of the C-3′ aliphatic hydroxy was surprising given the typical bias of OleD for aromatic nucleophiles and the previously reported accessibility of flavopiridol C-5- and C-7-OH for glucuronidation.[34]

Glucosylation of 10-Hydroxycamptothecin. The indole alkaloid 10-hydroxycamptothecin from the Chinese tree *Camptotheca acuminata* inhibits the activity of DNA topo-

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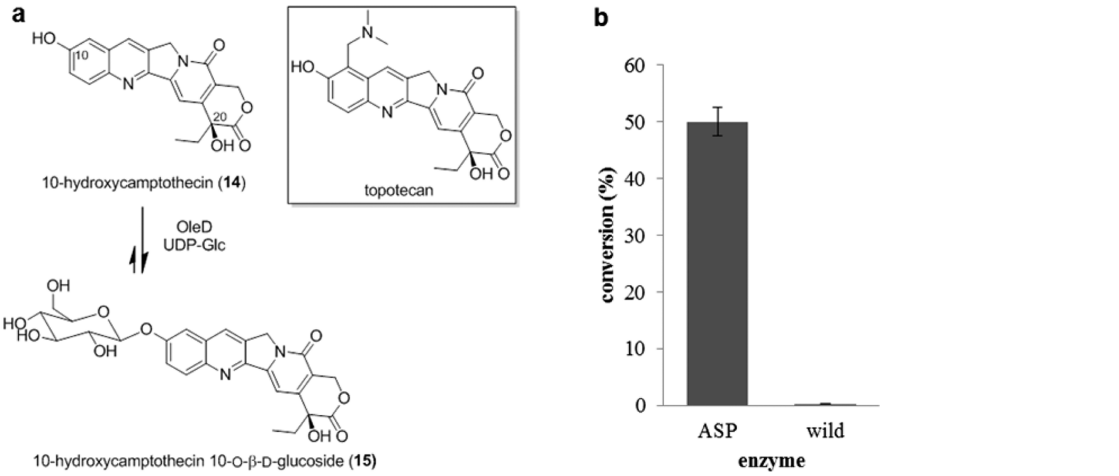

Figure 5. (a) OleD ASP-catalyzed glucosylation of 10-hydroxycamptothecin. (b) Glucosylation of 10-hydroxycamptothecin mediated by different OleD variants.

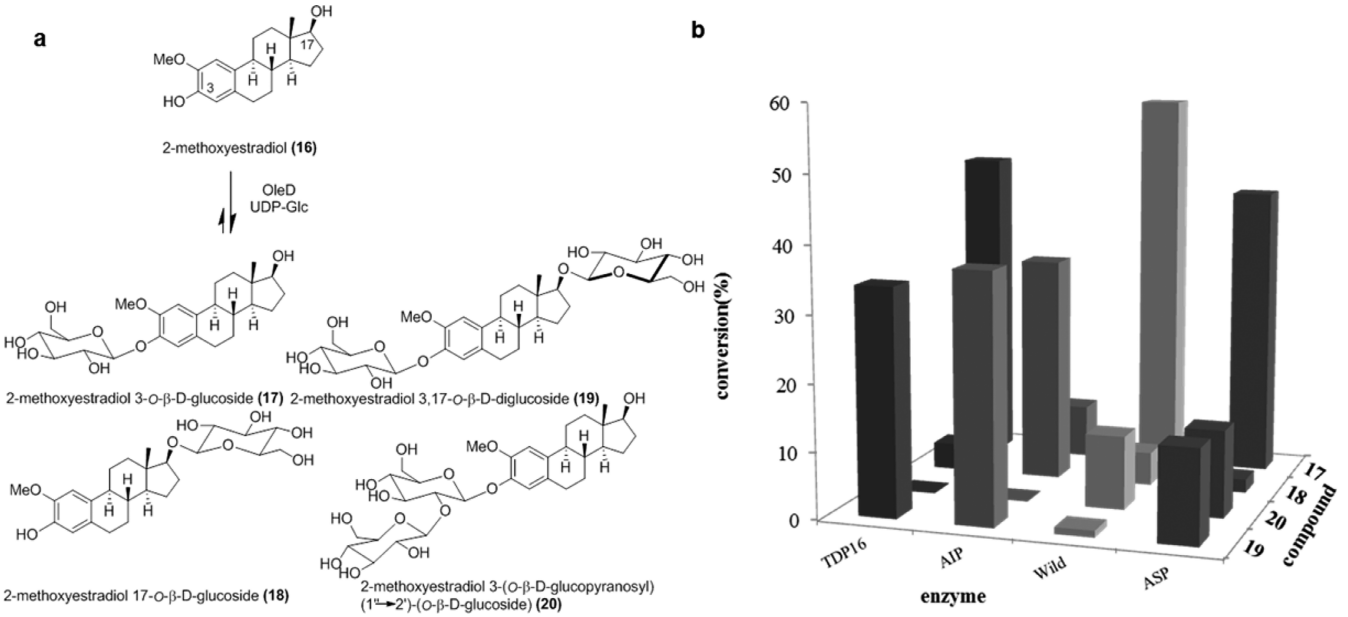

Figure 6. (a) OleD-catalyzed glucosylation of 2-methoxyestradiol. (b) Glucosylation of 2-methoxyestradiol catalyzed by a panel of OleD variants (the standard deviation of 3 trials was ±1.5%).

isomerase I and has a broad spectrum of anticancer activity in vitro and in vivo.[35] The unique mode of action of 10-hydroxycamptothecin has inspired many structure activity relationship studies, which ultimately led to the discovery of two water-soluble drugs used for the treatment of ovarian and lung cancer (topotecan)[36] and colon cancer (irinotecan).[37] Although these drugs are notably effective, their use suffers from dose-limiting toxicities, prompting continuing efforts to improve upon the properties of this drug class via structural modification, including glyconjugation.[38]

Using both wtOleD and OleD ASP, the pilot glucosylation of 10-hydroxycamptothecin under standard conditions (2.5 mM UDP-Glc, 1 mM aglycon) revealed the OleD ASP-catalyzed production of a single monoglucoside product (Figure 5, 50% conversion). In contrast, OleD ASP glucosylation of topotecan (Figure 5a) was low (<3%, data not shown), suggesting steric infringement imposed by substitution at C-9. Thus, OleD ASP was selected to catalyze the preparative-scale reaction. For subsequent product characterization, the preparative-scale reaction was conducted with 10-hydroxycamptothecin (6 mg, 16.25 μmol), UDP-Glc (50 mg, 81.9 μmol), and OleD ASP (16 mg) in the assay buffer solution (50 mM Tris HCl, 5 mM $MgCl_2$, pH 8.0, 50 mL total volume). After 20 h of gentle agitation at rt, the reaction was frozen and lyophilized to dryness. The residue was dissolved in MeOH and subjected to HPLC purification to give monoglucoside **15** (1.1 mg, 2.1 μmol) in 13% yield.

HRESIMS analysis of purified glucoside yielded an $[M + Na]^+$ ion at m/z 549.1484, confirming a monoglucoside of 10-hydroxycamptothecin with a formula of $C_{26}H_{26}N_2O_{10}$. 1D and 2D NMR data support the 10-*O*-β-D-glucoside structure presented in Figure 5a. The key evidence for C-10 glucosylation derives from the HMBC correlation between the anomeric proton and the C-10 carbon (Figure S1). The anomeric β-configuration is supported by the large coupling constant of the anomeric proton (δ_H 5.12, d, J = 6.0 Hz) (Figure S1). The ^{1}H NMR spectrum was consistent with previously reported data.[38e,f]

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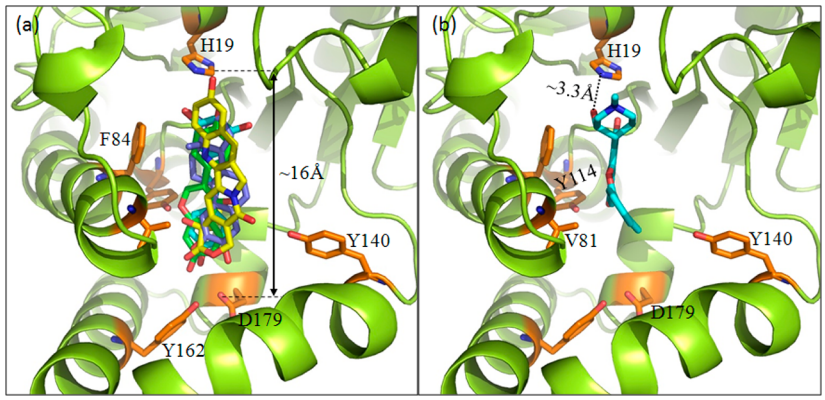

Figure 7. Binding modes based upon molecular dynamics simulations. A common extended binding mode (a) was observed for all compounds highlighted within this study with the exception of flavopiridol, which adapted a truncated binding mode (b).

Glucosylation of 2-Methoxyestradiol. The naturally occurring estrogen metabolite 2-methoxyestradiol exists at low levels in human blood serum.[39] This metabolite is notable, as it displays antiproliferative, apoptotic, and antiangiogenesis activities mediated via pathways independent of estrogen receptors.[40] Mechanistically, 2-methoxyestradiol was found to invoke microtubule stabilization via the colchicine binding site.[41] The in vitro GI_{50} of 2-methoxyestradiol against the NCI 60 cancer cell ranges from 0.08 to 5.0 μM, and this molecule has led to promising outcomes in phase I and II clinical trials as a new cancer chemotherapy.[40a,42] In addition, 2-methoxyestradiol inhibits vascular smooth muscle cell growth in arteries and induces the expression of endothelial nitric oxide synthase and production of nitric oxide.[43] However, the low aqueous solubility (5 nM in H_2O) and rapid clearance of 2-methoxyestradiol as the C-3- or C-17-glucuronide[44] has compelled continuing efforts to develop analogues with improved properties.[41,45]

Using UDP-Glc as the donor, 2-methoxyestradiol was assessed as a substrate for a panel of OleD variants under standard pilot conditions (0.5 mM UDP-Glc, 0.1 mM aglycon, 4 h). Four products, two monoglucosides and two diglucosides, were observed by HPLC and LC-MS analysis (Figure 6a), wherein product distribution was dependent on the catalyst employed. To maximize the yield of each product for subsequent characterization, OleD ASP was selected for the preparative-scale reaction.

The preparative-scale reaction utilized 2-methoxyestradiol (7.6 mg, 25.0 μmol) and UDP-Glc (76 mg, 0.125 mmol) in 50 mM Tris HCl and 5 mM $MgCl_2$, pH 8.0 (50 mL of total volume), and was initiated by the addition of 20 mg of OleD ASP. After gentle agitation at rt for 16 h, the reaction was frozen and lyophilized to dryness. The residue was dissolved in MeOH and subjected to HPLC purification to give **17** (4 mg, 8.6 μmol, 34.4%), **18** (0.1 mg, 0.2 μmol, 0.8%), **19** (1 mg, 1.6 μmol, 6.4%), and **20** (1 mg, 1.6 μmol, 6.4%). The identification of compounds **17**−**20** was confirmed by 1D and 2D NMR and HRESIMS analysis. The key evidence for C-3, C-17, or C-2′ glucosylation derives from the HMBC correlation between the anomeric proton and the carbon that was glycosylated (Figure S1). The anomeric β-configuration is supported by the large coupling constant (7.0−8.0 Hz) of the anomeric proton (Figure S1). Importantly, this study highlights the first synthesis of 2-methoxyestradiol glucosides. Also particularly intriguing from this study is the apparent C-3 glucosylation (**17**) en route to disaccharide **19** versus a switch to C-17 glucosylation (**18**) en route to the same product with the AIP variant.

Molecular Dynamics Simulations. The binding modes of all 18 structures by OleD were studied by molecular docking and included all five native aglycons (**3**, **7**, **12**, **14**, and **16**), all monoglucoside products/intermediates (**4**, **5**, **8**, **9**, **13**, **15**, **17**, and **18**), and all diglucosides (**6**, **10**, **11**, **19**, and **20**) (Figure 7). This cumulative analysis revealed all compounds studied, with the exception of flavopiridol (**12**), to bind in a manner recently described for steroidal glycosides.[4b] Specifically, ligand binding within this model is mediated via distal hydrogen-bonding interactions with key residues (Y114, Y162, and D179) deep within a polar pocket located ~16 Å from the proximal catalytic H19. This analysis indicates an average length of substrates examined to be ~12.5 Å, and in cases where diglucosides were formed, modeling revealed the distal pocket to readily accommodate the larger glucosides with only minor shifts in the H19-aglycon nucleophile distance/alignment. Also consistent with the product distribution observed, docking simulations with **3**, **7**, and **16** each revealed two modes of binding (head first versus tail first) with similar binding affinity. While flavopiridol was found to occupy the same substrate cavity, the model revealed unique proximal interactions, specifically, side chain hydrogen bonding with Y116 and van der Waals contacts with V81 and F84, to accommodate the substrate's smaller size. In addition, this docking model revealed the C-3′ aliphatic hydroxy of flavopiridol as the only aglycon nucleophile in proximity to the critical H19. Importantly, this collective analysis highlights common binding constraints of a range of non-native substrates for this unique glycosyltransferase and, as such, serves as a basis for the application of virtual screening in an attempt to rapidly identify new potential substrates for enzyme-catalyzed glycoconjugation.[46]

In summary, this work is an important key follow-up to studies that revealed the uniquely permissive nature of a range of engineered/evolved OleD variants. Specifically, the full characterization of product distributions deriving from OleD-catalyzed reactions with the diverse set of non-native substrates highlighted within this study provides important insights into the regioselectivity of each target reaction and a critical basis for the development of future predictive models for the substrate specificity scope of this fascinating catalyst.

EXPERIMENTAL SECTION

General Experimental Procedures. All chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise stated. NMR

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spectra, including ^{1}H, ^{13}C, gCOSY, TOCSY, gHSQC, and gHMBC, were recorded on a Varian UNITY INOVA 400 or 500 MHz spectrometer. Mass spectra were acquired on a Bruker MaXis high-resolution quadrupole time-of-flight mass spectrometer.

Protein Expression and Purification. A single colony of *E. coli* BL21(DE3)pLysS (Stratagene) transformed with the pET28a-based OleD expression vector (wt, ASP, AIP, TDP16, 3-1-H12)[3] was used to inoculate 3 mL of LB medium supplemented with 50 μg/mL kanamycin and cultured overnight at 37 °C with shaking (250 rpm). The starter culture was then transferred to 1 L of LB medium supplemented with 50 μg/mL kanamycin and grown at 37 °C with shaking (250 rpm) until the OD_{600} reached ∼0.7. Isopropyl β-D-thiogalactoside was subsequently added to a final concentration of 0.4 mM, and the culture was incubated at 28 °C for approximately 18 h with shaking at 250 rpm. Cell pellets were collected by centrifugation at 10000*g* and 4 °C for 20 min, and the supernatant was discarded. Cell pellets were resuspended in 10 mL of chilled lysis buffer (20 mM phosphate buffer, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and were lysed by sonication (8 pulses of 40 s each) in an ice bath. The cell debris was removed by centrifugation at 10000*g* and 4 °C for 20 min. The cleared supernatant was immediately applied to 3 mL of nickel nitrilotriacetic acid resin (QIAgen) pre-equilibrated with wash buffer (20 mM phosphate buffer, pH 7.4, 0.3 M NaCl, 10 mM imidazole). Protein was allowed to bind for 30 min at 4 °C with gentle agitation, and the resin was subsequently washed with 4 × 50 mL wash buffer. Finally, the enzyme was eluted from the resin via incubation with 2 mL of wash buffer containing 250 mM imidazole for 15 min at 4 °C with gentle agitation. The purified protein was applied to a PD-10 desalting column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 8.0) and eluted as described by the manufacturer. Protein aliquots were immediately flash frozen in liquid nitrogen and stored at −80 °C. Protein purity was confirmed by SDS-PAGE to be >95%, and protein concentration for all studies was determined using the Bradford protein assay kit from Bio-Rad.

General Pilot-Scale Reaction. Reactions were conducted in a final volume of 100 μL under standard conditions (50 mM Tris HCl, 5 mM $MgCl_2$, pH 8.0, 0.5 μg/μL purified enzyme, 2.5−5 equiv of UDP-Glc, 1 equiv of aglycon, 25 °C). Two separate control reactions that withheld either enzyme or UDP-Glc were performed in parallel. Reactions were allowed to proceed at 25 °C for a specific time period, quenched with 100 μL of cold MeOH, and centrifuged at 10000*g* for 10 min, and the supernatant was removed for analysis. The clarified reaction mixtures were analyzed by analytical reverse-phase HPLC [Phenomenex 250 mm × 4.6 mm Gemini 5 μ C_{18} column; 1 mL/min; gradient of solvents A (0.1% TFA) and B (100% CH_3CN): (a) 0−20 min, 10−75% B; (b) 20−21 min, 75−100% B; (c) 21−26 min, 100% B; (d) 26−29 min, 100−10% B; and (e) 29−35 min, 10% B; A_{254} detection]. HPLC peak areas were integrated using the Star Chromatography Workstation software (Varian), and the total percent conversion was calculated as a percent of the total peak area of substrate and products.

General Procedure for Preparative-Scale OleD-Catalyzed Glycosylation Reaction. Aglycon was dissolved in 5% DMSO and transferred to pH 8 buffer solution (50 mM Tris HCl, 5 mM $MgCl_2$). UDP-Glc was added followed by OleD catalyst. After the designated time of agitation at rt, the reaction was subsequently frozen and lyophilized, and the debris was resuspended in 2 mL of ice-cold MeOH and filtered. Product *O*-glucosides were isolated by collecting fractions from semipreparative reverse-phase HPLC [Phenomenex 250 mm × 10 mm Gemini 5 μ C_{18} column; 4.5 mL/min; solvent A (0.1% trifluoroacetic acid) and B (100% CH_3CN) using the gradient 0−20 min, 10−75% B; 20−21 min, 75−100% B; 21−26 min, 100% B; 26−29 min, 100−10% B; and 29−35 min, 10% B; A_{254} detection]. The product-containing fractions were subsequently collected and lyophilized to provide the corresponding *O*-glucosides. The compound was then characterized using 1D and 2D NMR, including ^{1}H, ^{13}C, gCOSY, TOCSY, gHSQC, and gHMBC.

Flavopiridol 3′-*O*-β-D-glucoside (13): white powder; ^{1}H NMR (CD_3OD, 500 MHz) 7.81 (dd, *J* = 7.5, 2.0 Hz, 1H), 7.63 (dd, *J* = 7.0, 2.0 Hz, 1H), 7.60−7.50 (m, 2H), 6.48 (s, 1H), 6.37 (s, 1H), 4.33 (m, 1H), 4.28 (d, *J* = 8.0 Hz, 1H), 3.71 (m, 1H), 3.67−3.57 (m, 3H), 3.54 (dd, *J* = 11.0, 3.0 Hz, 1H), 3.30−3.24 (m, 2H), 3.19 (dd, *J* = 11.0, 6.0 Hz, 1H), 3.06 (ddd, *J* = 9.5, 6.0, 3.0 Hz, 1H), 3.00 (dd, *J* = 9.5, 8.5 Hz, 1H), 2.99 (dd, *J* = 9.0, 8.0 Hz, 1H), 2.89 (s, 3H), 2.06 (m, 2H); ^{13}C NMR (CD_3OD, 125 MHz) 184.0, 164.5, 163.0, 162.7, 162.2, 133.6, 133.4, 133.1, 132.4, 131.8, 129.0, 111.8, 106.8, 105.9, 101.7, 100.0, 77.9, 77.6, 75.3, 74.2, 72.3, 63.5, 58.8, 56.7, 44.2, 37.5, 24.5; HRESIMS *m/z* 564.16438 $[M + H]^+$ (calcd for $C_{27}H_{31}ClNO_{10}$, 564.1631).

2-Methoxyestradiol 3-*O*-β-D-glucoside (17): white powder; ^{1}H NMR (CD_3OD, 500 MHz) 6.91 (s, 1H), 6.88 (s, 1H), 4.84 (d, *J* = 7.5 Hz, 1H), 3.89 (m, 1H), 3.84 (s, 3H), 3.71 (m, 1H), 3.68 (m, 1H), 3.48 (m, 2H), 3.40 (m, 2H), 2.78 (m, 2H), 2.35 (dd, *J* = 14.0, 3.0 Hz, 1H), 2.20 (m, 1H), 2.06 (m, 1H), 2.00 (m, 1H), 1.90 (dd, *J* = 12.0, 2.5 Hz, 1H), 1.72 (m, 1H), 1.60−1.17 (m, 7H), 0.80 (s, 3H); ^{13}C NMR (CD_3OD, 100 MHz) 148.8, 146.1, 136.2, 130.9, 118.8, 111.6, 103.3, 82.6, 78.4, 78.0, 75.1, 71.6, 62.7, 57.2, 51.4, 45.9, 44.5, 40.5, 38.2, 30.9, 30.3, 28.7, 27.8, 24.2, 11.8; HRESIMS *m/z* 487.2304 $[M + Na]^+$ (calcd for $C_{25}H_{36}O_8Na$, 487.2302).

2-Methoxyestradiol 17-*O*-β-D-glucoside (18): white powder; ^{1}H NMR (CD_3OD, 500 MHz) 8.51 (s, 1H), 6.77 (s, 1H), 6.45 (s, 1H), 4.34 (d, *J* = 8.0 Hz, 1H), 3.83 (dd, *J* = 12.0, 2.0 Hz, 1H), 3.80 (d, *J* = 8.0 Hz, 1H), 3.77 (s, 3H), 3.65 (dd, *J* = 12.0, 6.0 Hz, 1H), 3.40−3.15 (m, 4H), 2.68 (m, 2H), 2.63 (s, 1H), 2.17−2.10 (m, 3H), 1.66 (m, 2H), 1.54−1.15 (m, 7H), 0.86 (s, 3H); ^{13}C NMR (CD_3OD, 125 MHz) 147.1, 145.4, 132.8, 130.4, 116.6, 110.5, 104.9, 90.0, 78.3, 78.0, 75.6, 71.9, 63.0, 56.7, 51.3, 45.4, 44.4, 40.4, 39.0, 30.2, 30.1, 28.7, 28.0, 24.2, 12.3; HRESIMS *m/z* 487.2304 $[M + Na]^+$ (calcd for $C_{25}H_{36}O_8Na$, 487.2302).

2-Methoxyestradiol 3,17-di-*O*-β-D-glucoside (19): white powder; ^{1}H NMR (CD_3OD, 500 MHz) 6.86 (s, 1H), 6.83 (s, 1H), 4.80 (d, *J* = 7.0 Hz, 1H), 4.34 (d, *J* = 7.5 Hz, 1H), 3.83 (m, 1H), 3.79 (s, 3H), 3.66 (d, *J* = 5.0 Hz, 1H), 3.64 (d, *J* = 5.0 Hz, 1H), 3.73−3.63 (m, 3H), 3.54 (dd, *J* = 12.0, 4.0 Hz, 1H), 3.45 (m, 2H), 3.38 (m, 2H), 3.30 (m, 1H), 3.26 (m, 1H), 3.19 (m, 1H), 2.79 (m, 2H), 2.35 (dd, *J* = 14.0, 3.0 Hz, 1H), 2.21 (m, 1H), 2.06 (m, 1H), 2.00 (m, 1H), 1.90 (dd, *J* = 12.0, 2.0 Hz, 1H), 1.72 (m, 1H), 1.60−1.17 (m, 7H), 0.81 (s, 3H); ^{13}C NMR (CD_3OD, 125 MHz) 148.7, 145.3, 136.1, 130.8, 118.7, 111.5, 104.9, 103.2, 89.9, 78.36, 78.32, 78.04, 78.00, 75.6, 75.1, 71.8, 71.6, 62.9, 62.7, 57.1, 51.3, 45.9, 44.7, 40.5, 40.2, 30.3, 30.1, 28.6, 27.9, 24.1, 12.3; HRESIMS *m/z* 649.2838 $[M + Na]^+$ (calcd for $C_{31}H_{46}O_{13}Na$, 649.2831).

2-Methoxyestradiol 3-(*O*-β-D-glucopyranosyl)-(1″→2′)-(*O*-β-D-glucoside) (20): white powder; ^{1}H NMR (CD_3OD, 500 MHz) 6.91 (s, 1H), 6.85 (s, 1H), 5.13 (d, *J* = 7.5 Hz, 1H), 4.78 (d, *J* = 8.0 Hz, 1H), 3.90 (m, 1H), 3.82 (s, 3H), 3.78 (dd, *J* = 9.0, 8.0 Hz, 1H), 3.73−3.63 (m, 3H), 3.54 (dd, *J* = 12.0, 4.0 Hz, 1H), 3.45 (m, 2H), 3.38 (m, 2H), 3.30 (m, 1H), 3.26 (m, 1H), 3.19 (m, 1H), 2.79 (m, 2H), 2.35 (dd, *J* = 14.0, 3.0 Hz, 1H), 2.21 (m, 1H), 2.06 (m, 1H), 2.00 (m, 1H), 1.90 (dd, *J* = 12.0, 2.0 Hz, 1H), 1.72 (m, 1H), 1.60−1.17 (m, 7H), 0.81 (s, 3H); ^{13}C NMR (CD_3OD, 125 MHz) 148.0, 145.8, 135.6, 130.7, 117.0, 111.4, 104.2, 100.9, 82.6, 82.5, 78.0, 77.93, 77.88, 77.78, 75.7, 71.2, 71.0, 62.7, 62.0, 57.1, 51.4, 45.9, 44.5, 40.5, 38.3, 30.9, 30.4, 28.7, 28.0, 24.2, 11.9; HRESIMS *m/z* 649.2835 $[M + Na]^+$ (cacld for $C_{31}H_{46}O_{13}Na$, 649.2831).

Molecular Dynamics Simulations. Multiple conformations of each ligand were generated using OMEGA (Open Eye Scientific Software). The conformations of each ligand were fitted in the binding cavity of OleD (PDBID 2IYF)[1f] using Sabre and Fred software packages.[47] The docking strategy exhaustively explored all possible positions of each ligand in the binding site and generally focused upon two parameters: shape and optimization. During the shape fitting, the ligand was placed into a grid box encompassing all active-site atoms (including hydrogen atoms) using smooth Gaussian potential.[48] Two optimization filters were subsequently processed: rigid-body optimization and optimization of the ligand pose in the dihedral angle space. The pose ensemble was then filtered to first reject poses that did not have sufficient shape complementarity with the active site of the protein followed by rejection of those lacking at least one heavy atom hydrogen bond with the His19 imidazole.

In separate docking runs, the binding poses of the ligand structure were refined by MD simulations followed by MM-GBSA calculations using the Sander module from the Amber11 package[49] as previously described.[46] Briefly, the OleD−ligand binding complex was neutralized by adding appropriate counterions and was solvated in a rectangular box of TIP3P H_2O molecules with a minimum solute−wall distance of 10 Å.[50] The partial atomic charges used for the ligand were the electrostatic potential-fitted atomic charges, calculated at the ab initio HF/6-31G* level using the Gaussian03 program.[51] The solvated systems were energy-minimized and carefully equilibrated. These systems were gradually heated from T = 10 K to T = 298.15 K in 100 ps before running the MD simulation. The MD simulations were performed with a periodic boundary condition in the NPT ensemble at T = 298.15 K with Berendsen temperature coupling and constant pressure (P = 1 atm) with isotropic molecule-based scaling.[52] A time step of 2.0 fs was used, with a cutoff of 12 Å for the nonbonded interactions, and the SHAKE algorithm was employed to keep all bonds involving hydrogen atoms rigid.[53] Long-range interactions were handled using the particle mesh Ewald algorithm.[54] As we did in our previous work,[4b] only the ligand and residue side chains in the binding pocket were permitted to move during the energy minimization and MD simulations. We used the constraint to prevent any changes in the OleD structure due to the presence of residues in the loops on the top of the protein active site. MD simulations were then carried out for 4.0 ns. During the simulations, the coordinates of the system were collected every 10 ps.

■ ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C spectroscopic data, molecular docking data, and individual docking illustrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest: J.S.T. is a co-founder of Centrose (Madison, WI, USA).

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