# Probing the Regiospecificity of Enzyme-Catalyzed Steroid Glycosylation

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## Abstract
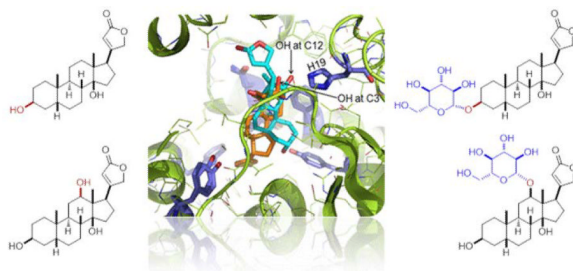
The potential of a uniquely permissive engineered glycosyltransferase (OleD ASP) as a catalyst for steroid glycosylation is highlighted. The ability of OleD ASP to glucosylate a range of cardenolides and bufadienolides was assessed using a rapid LC-UV/MS-SPE-NMR analytical platform. While a bias toward OleD-catalyzed C3 mono-glucosylation was observed, subtle alterations of the steroidal architecture in some cases, invoked diglucosylation or, in one case (digoxigenin), C12 glucosylation. This latter case represents the first, and highly efficient, synthesis of digoxigenin 12-*O*-β-D-glucoside.

Steroidal glycosides such as digitoxin, digoxin and proscillaridin have been used for centuries to treat congestive heart failure and more recently noted to display highly potent anticancer activity.[1] These ligands are known to bind the $Na^+$,$K^+$-ATPase alpha subunit with high affinity where they function to control both a range of intracellular signaling cascades critical to cell proliferation and regulate intracellular $Na^+$ and $K^+$ concentrations.[2] C3 glycosylation of the steroidal core among this natural product class has a dramatic effect upon the delicate balance between signaling (antiproliferative) versus inotropic (cardiotoxicity) activities.[3] Thus, there remains notable interest in the development of simple glycosylation platforms as a means to improve the putative therapeutic index of steroidal glycoside anticancer preclinical leads. Using a novel high throughput screen and LC-MS,[4] representative steroidal aglycons were recently identified as substrates for a set of highly permissive glycosyltransferase variants derived from the macrolideinactivating glucosyltransferase OleD.[5] Herein we extend this preliminary study through the application of a LC-UV/MS-SPE/NMR platform[6] to rapidly probe the regio-/stereospecificity of OleD-

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catalyzed glycosylation of cardenolide and bufadienolide aglycons. While this study reveals a bias toward the desired C3 regiospecificity in the context of a range of non-native substrates for this enzyme, it also highlights how subtle modifications of the steroidal aglycon can dictate and/or prohibit glycosyltransfer.

To explore the feasibility of the LC-UV/MS-SPE-NMR platform for micro-scale structural elucidation of steroidal glycosides, an initial pilot study was conducted using digitoxigenin as the model (Scheme 1). For this study, the reaction contained 600 μg of OleD ASP, 2.5 mM UDP-Glc, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 1 mM of digitoxigenin in a total volume of 1 mL. The reaction was allowed to proceed for 16 h at 25 °C and subsequently frozen, lyophilized, and the debris resuspended in 2 mL of ice cold MeOH, filtered and concentrated to 150 μl for LC-UV/MS-SPE-NMR analysis. The HPLC component of the subsequent analysis platform was accomplished using standard C18 reverse-phase chromatography with diode array detection wherein ~5 % of the flow was diverted to quadrupole time-of-flight mass (QTOF) detection. Fractions containing detected peaks were automatically diverted to pre-conditioned solid-phase extraction (SPE, C18) cartridges, which were subsequently dried via $N_2$ gas and then eluted with 30 μl $CD_3CN$ into 1.7 mm NMR tubes for direct analysis via a Bruker Avance III 600 MHz spectrometer with a 1.7 mm $^1H\{^{13}C/^{15}N\}$ cryogenic probe.

LC-MS analysis of the pilot reaction described above revealed the formation of a single glycoside in 20% yield. Structure elucidation of this glycoside, based upon $^1H$ NMR, $^1H$-$^1H$ COSY, $^1H$-$^{13}C$ HSQC, and $^1H$-$^{13}C$ HMBC, was consistent with the 3-*O*-β-D-glucoside **2** (Scheme 1).[7] Key support for the regiospecificity assignment derived from an observed HMBC correlation between the sugar anomeric C1' proton and the C3 of digitoxigenin. An observed large anomeric proton coupling constant ($δ_H$ 4.32, doublet, 8.0 Hz) supported the formation of the β-anomer, consistent with an established inverting mechanism for OleD variants studied to date[8] C3 glucosylation also led to a notably consistent large downfield $^{13}C$ shift (~7 ppm) of the C3 carbon (`glycosylation shift'),[9] which served as a convenient indicator in probing OleD-catalyzed glycosylation of alternative steroidal aglycons (Table 1).

Given the success of the digitoxigenin pilot study, steroidal aglycons **3**–**9** (Figure 1) were subsequently treated in an identical manner. Five (aglycon **3**, **4**, **7**, **8**, and **9**) out of the seven putative aglycons led to glucosylated products, three (**3**, **4**, and **7**) of which led to mono-glucosides (**10**,[13] (**11**,[14] and **12**[15]) and two (**8** and **9**) of which provided both mono- (**13**, and **15**[16]) and di-glucosides (**14**, and **16**).[17] A bias toward C3 glycosylation was observed with one surprising exception – digoxigenin, which led to C12 glucosylation exclusively in high efficiency.

The docking and molecular dynamics (MD) simulation of OleD complexed with **1** or **3** are consistent with our experimental data (Figure 2). In comparison to the binding mode of **1**, the binding model of **3** invokes an `inverse binding mode' due to electrostatic and steric repulsions between the steroid C12-OH and the Tyr140 side chain-OH (Figure S3). Similar docking and MD simulations with ligands **4**–**9**, **13** and **15** revealed the following key observations. The predicted binding of ouabaingenin (**5**) and strophanthidin (**6**) are misaligned and distant (~4.7 Å, Fig. S4, panels b and c) with the active-site base, the His19 side chain imidazole, due in part to the electrostatic attraction between the Asn80 side-chain carboxamide and functional groups at C19 in **5** or **6**. In contrast, the predicted binding modes of **8** and **9** highlight favorable dipole-quadrupole stabilization between the substrate C16 methyl ester and the Tyr140 (Figure S4), also prevalent in the binding models for the corresponding monoglucosides forms **13** and **15**, which may be important to the observed iterative (steroid C3 and subsequent glucoside C2') glucosylation of **8** and **9**.

While the impact of C3 glycosylation upon the anticancer activity of steroidal glycosides has been previously reported,[3a,18] the influence of C12 glycosylation upon bioactivity of this natural product class had not. Thus, the enzymatic synthesis of **10** was subsequently scaled to enable bioactivity assessment. Specifically, digoxigenin **3** (10 mg, 25.6 μmol) was dissolved in 0.65 mL DMSO, transferred to 25 mL assay buffer solution (50 mM Tris HCl, 5 mM $MgCl_2$, pH 8.0) and the reaction initiated via addition of UDP-Glc (62 mg, 0.124 mmol) and 16 mg of OleD ASP. After 24 hr of agitation at room temperature, the reaction was quenched and flushed though a 12 mL HLB column, which was subsequently dried with $N_2$ gas and then eluted with methanol. The methanolic eluent was concentrated and the collected residue subjected to flash chromatography using $CH_2Cl_2$/MeOH to afford digoxigenin 12-*O*-β-D-Glc **10** (13 mg, 23.5 μmol, 92%)-highlighting the first synthesis of digoxigenin 12-*O*-β-D-Glc. Consistent with the belief that steroidal glycosides bind the $Na^+$, $K^+$-ATPase alpha subunit with the lactone (and presumably C12) buried deep within the ligand binding pocket,[19] the C12 glucoside was found to be ~5–10-fold less active than the corresponding aglycon based upon in vitro cytotoxicity against two lung cancer cell lines (Table 2). The rather surprising fact that the activity of the C12 glycoside was not further diminished suggests notable flexibility in the $Na^+$, $K^+$-ATPase alpha subunit ligand-binding pocket.

In summary, this study highlights the capabilities of OleD ASP in the context of steroidal glycoside construction and reveals how subtle alterations of the steroidal architecture can dramatically influence regioselectivity of the reaction. As such, this study serves to provide additional information for understanding substrate specificity of the uniquely permissive OleD ASP variant and also sets the stage for rapid enzymatic glycorandomization of this class of natural product through the use of alternative sugar donors.[20]

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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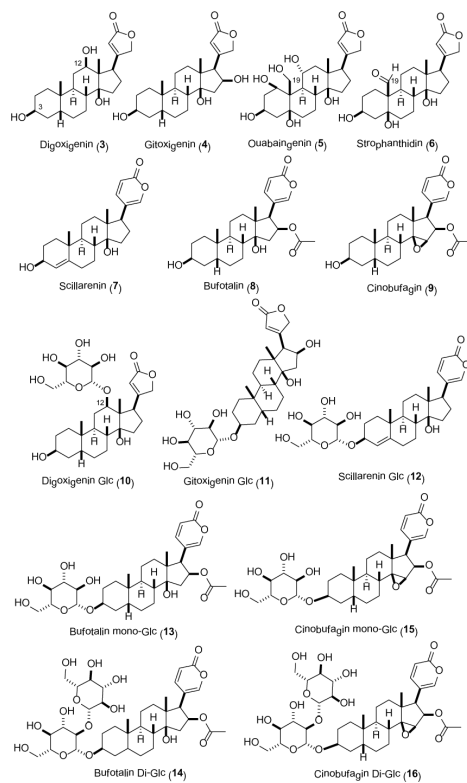
**Figure 1.**
Potential substrates and determined products of OleD-catalyzed test reactions.
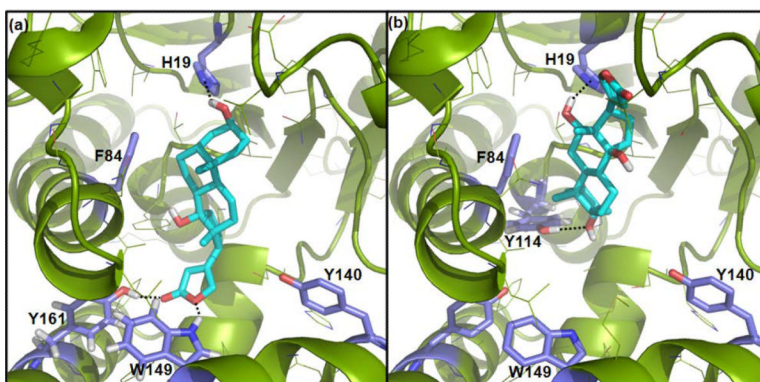
**Figure 2.**
Simulated ligand-bound models for OleD-**1 (a)** and OleD-**3 (b)**. Ligands are represented in cyan, OleD in green, key active-site residues in purple and H-bonds as dashed lines. The calculated binding energy of the compounds **1** and **3** with OleD are −12.1 and −11.9 kcal/mol.
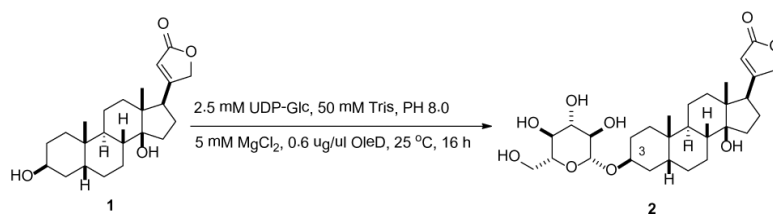
**Scheme 1.**
OleD catalyzed glucosylation of digitoxigenin.

**Table 1**

C3 $^{13}$C chemical shifts of substrates and products

| compound | C3 shift (ppm) | compound | C3 shift (ppm) |
|---|---|---|---|
| **1** | 66.7 | **2** | 73.5 |
| **3** | 66.3[a] | **10** | 66.0[b] |
| **4** | 66.8[10] | **11** | 73.6 |
| **7** | 67.7[3(c)] | **12** | 75.6 |
| **8** | 66.7[11] | **13** | 73.7 |
| | | **14** | 74.8 |
| **9** | 66.6[12] | **15** | 73.2 |
| | | **16** | 74.1 |

[a]C12 $^{13}$C chemical shift 73.7 ppm;

[b]C12 $^{13}$C chemical shift 82.1 ppm

**Table 2**

In vitro cytotoxicity (μM)

| compound | H460[a] | A549[b] |
|----------|---------|---------|
| **3** | 0.38 ± 0.05 | 0.30 ± 0.02 |
| **10** | 3.60 ± 0.24 | 1.49 ± 0.07 |

[a]H460, large cell lung carcinoma;

[b]A549, small cell lung carcinoma