ORGANIC LETTERS

2012
Vol. 14, No. 21
5424–5427

Probing the Regiospecificity of Enzyme-Catalyzed Steroid Glycosylation

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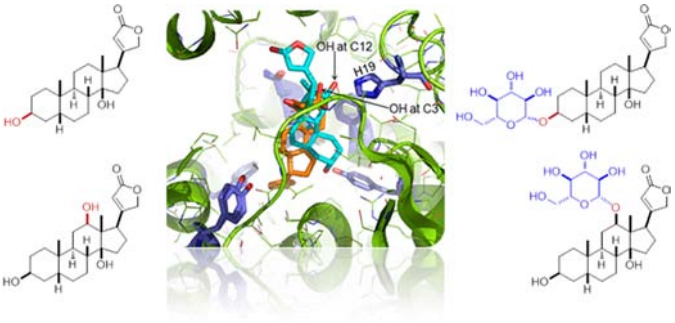
Received September 10, 2012

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 monoglucosylation 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 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 macrolide-inactivating glucosyltransferase OleD.[5]

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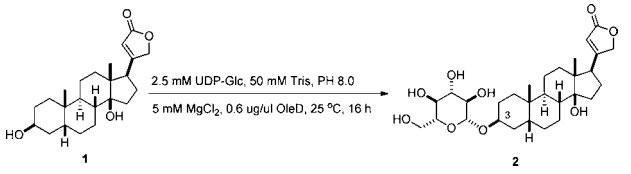
10.1021/ol3024924
Published on Web 10/18/2012

Scheme 1. OleD Catalyzed Glucosylation of Digitoxigenin



Herein we extend this preliminary study through the application of an LC-UV/MSSPE-NMR platform[6] to rapidly probe the regio-/stereospecificity of OleD-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 microscale 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 and lyophilized, and the debris was 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 reversed-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 preconditioned solid-phase extraction (SPE, C18) cartridges, which were subsequently dried via N_2 gas and then eluted with 30 μL of 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

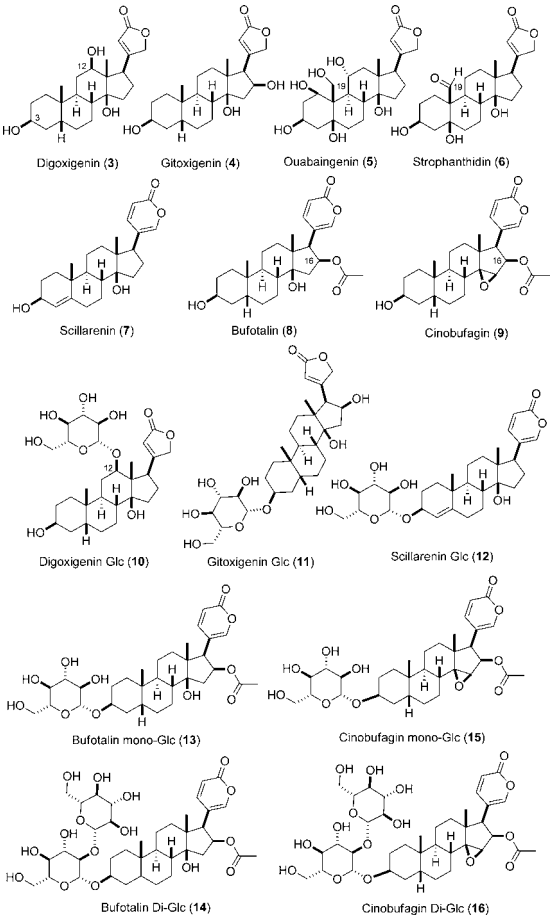


Figure 1. Potential substrates and determined products of OleD-catalyzed test reactions.

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).

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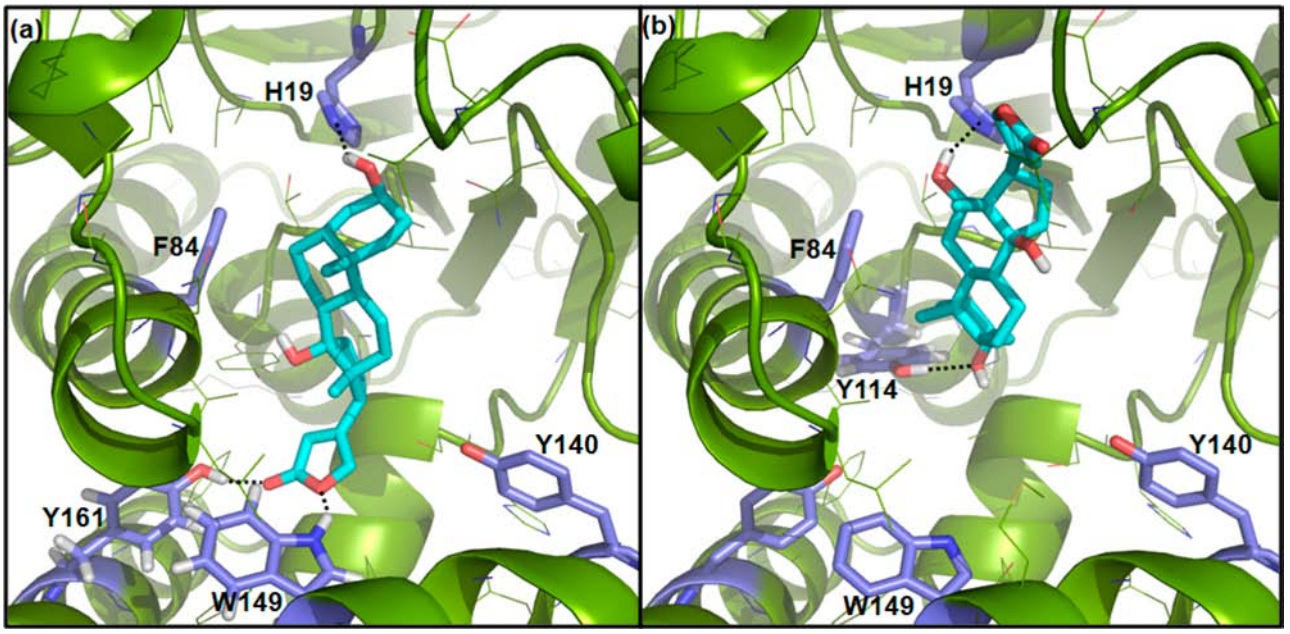

Figure 2. Simulated ligand-bound models for OleD-**1** (a) and OleD-**3** (b). Ligands are represented in cyan, OleD in green, key active-site residues in purple, and H-bonds as dashed lines. The calculated binding energy of the compounds **1** and **3** with OleD are −12.1 and −11.9 kcal/mol.

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[3c]	**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.

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 monoglucosides (**10**,[13] **11**,[14] and **12**[15]) and two (**8** and **9**) of which provided both mono- (**13** and **15**[16]) and diglucosides (**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.

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.

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, Figure 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**.

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(17) The diglucosylation was evidenced by the HMBC coupling of the anomeric proton of the second sugar with the C2 carbon of the first sugar.

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 the 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 of DMSO and transferred to 25 mL of assay buffer solution (50 mM Tris HCl, 5 mM $MgCl_2$, pH 8.0), and the reaction was initiated via addition of UDP-Glc (62 mg, 0.124 mmol) and 16 mg of OleD ASP. After 24 h 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 was 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 the regioselectivity of the reaction. As such, this study serves to provide additional information for understanding the 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]

Acknowledgment. This work was supported by NIH AI52218 (J.S.T.) and also made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH Grants P41RR02301 (BRTP/NCRR) and P41GM66326 (NIGMS). Additional equipment was purchased with funds from the University of Wisconsin, the NIH (RR02781, RR08438), the NSF (DMB-8415048, OIA-9977486, BIR-9214394), and the USDA.

Supporting Information Available. Experimental and computational procedures, characterization, and biological testing. This material is available free of charge via the Internet at http://pubs.acs.org.

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