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UGT74AN1, a Permissive Glycosyltransferase from *Asclepias curassavica* for the Regiospecific Steroid 3-*O*-Glycosylation

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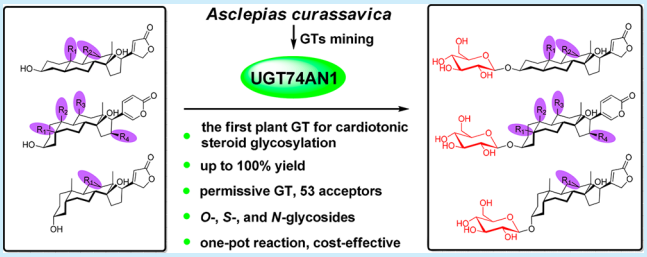
Supporting Information

ABSTRACT: A permissive steroid glycosyltransferase (UGT74AN1) from *Asclepias curassavica* exhibited robust capabilities for the regiospecific C3 glycosylation of cardiotonic steroids and C_{21} steroid precursors, and unprecedented promiscuity toward 53 structurally diverse natural and unnatural compounds to form *O*-, *N*-, and *S*-glycosides, along with the catalytic reversibility for a one-pot transglycosylation reaction. These findings highlight UGT74AN1 as the first regiospecific catalyst for cardiotonic steroid C3 glycosylation and exhibit significant potential for glycosylation of diverse bioactive molecules in drug discovery.



Cardiotonic steroid glycosides, an important class of natural products that have been used to treat congestive heart failure since the 18th century, have shown highly potent anticancer and anti-inflammatory activity.[1] C3 glycosylation of the steroidal core has been recognized as a practical way to improve their therapeutic index and enable their wider application for the treatment of other diseases.[2] Chemical synthesis of steroid 3-*O*-glycosides is faced with poor regio- and stereoselectivities, and the protection and deprotection of functional groups.[2a,3] Glycosyltransferases (GTs) are generally recognized as powerful synthetic tools which can alleviate these disadvantages.[4] Significant progress has been achieved in the application of promiscuous microbial or plant GTs for natural product glycodiversification.[5] However, the enzymatic C3 glycosylation of the cardiotonic steroids remains restricted by the availability of regiospecific GTs.[6] The plant steroid GTs which are naturally responsible for the formation of 3-*O*-cardiotonic glycosides remain undiscovered.

Cardenolides or bufadienolides attached with different types of sugar units are present in different plant groups and have been well characterized for their chemical structures.[7] However, biosynthesis of these compounds has not been studied to date.[8] A putative biosynthetic pathway has been derived from precursor feeding studies and in *vitro* crude enzyme assays,[9] indicating that steroid glycosylation may take place at various stages and should no longer be regarded as terminal biosynthetic steps.[9b] However, the powerful steroid GTs remain undetected.[10] Therefore, it is of great significance to discover novel steroid GTs from cardenolide- or bufadienolide-producing plants for cardiotonic steroid glycosylation.

Herein, we report, for the first time, the identification of a novel steroid GT (UGT74AN1) from *Asclepias curassavica*, which displays catalytic efficiency and regiospecificity toward cardenolide and bufadienolide aglycons to form 3-*O*-β-D-glucosides. Especially, UGT74AN1 catalyzes the glycosylation of C_{21} steroid precursors. Moreover, this work highlights an unexpected promiscuity of UGT74AN1 toward a diverse range of drug-like scaffolds to produce *O*-, *N*-, and *S*-glycosides, along with the catalytic reversibility for a one-pot transglycosylation reaction.

A. curassavica (L.) is a well-known cardenolide-containing species in the milkweed family Asclepiadaceae.[11] In our previous work, a wide variety of bioactive cardenolides and C_{21} steroidal glycosides had been isolated from its seeds, leaves, and stems,[12] which inspired us to search for specific GTs. We confirmed uzarigenin (**1**) was enzymatically glycosylated when incubated with UDP-glucose (UDPG) and the crude enzymes from *A. curassavica* leaves (Figure S1), which implied the corresponding steroid GT was probably a soluble enzyme that possessed the conserved PSPG (plant secondary product glycosyltransferases) motif.[13] As cardenolides evolved as a means of the plant defense system,[8a,11] the plant used in this study was treated with 100 μM methyl jasmonate (MeJA) for 24 h in order to improve the expression level of the defense-related genes.[14] To clone the specific steroid GTs, total RNA was isolated from the MeJA treated *A. curassavica* leaves and a degenerate PCR primer for 3′ RACE was designed based on the PSPG motif (Figure S2). Combined with 3′ and 5′ RACE PCR, the full length cDNAs of 11 new GT candidates (AcGTs) were finally obtained and subcloned into pET28a for expression in *E. coli*. To screen for the specific steroid GTs capable of glycosylating steroids, the detecting reactions (50 mM Tris-HCl, pH 7.5; 500 μM UDP-Glu; 200 μM uzarigenin; 5 mM $MgCl_2$; 100 μL crude AcGTs in 200 μL final volume; 37 °C, 12 h) were performed and analyzed

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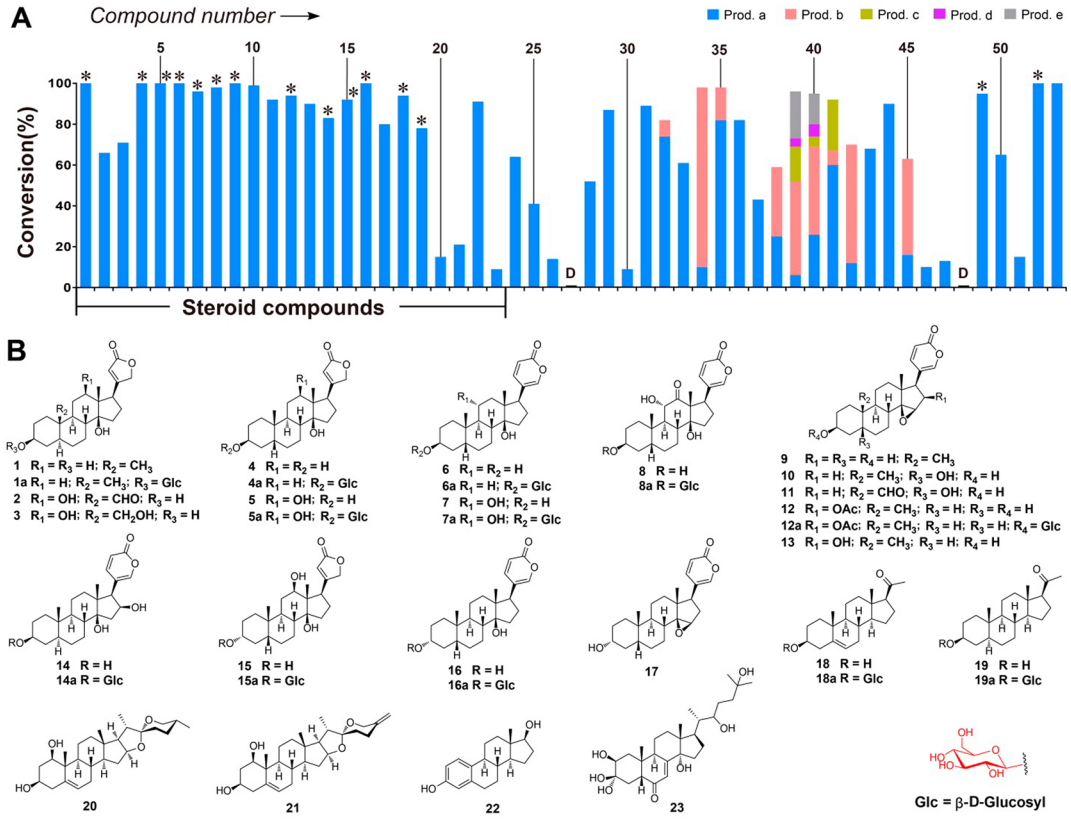

Figure 1. Exploring the substrate promiscuity of UGT74AN1 with a substrate library. (A) Percent conversion of each library number catalyzed by UGT74AN1. The library numbers are listed based on the structural scaffolds shown in the Supporting Information (SI) Figure S7. The colors in the bar graphs (Prod. a, Prod. b, Prod. c, Prod. d, and Prod. e) represent different ratios of various glycosylated products in the total product yield of each compound. The asterisks (*) represent the glycosylated products that are isolated and confirmed by LC-MS and ^{1}H and ^{13}C NMR spectroscopy. D means trace amount of products that are detected in LC-MS. (B) The structures of steroid compounds in the substrate library and corresponding glycosylated products. Compounds leading to no conversion are listed in Figure S8.

by HPLC-DAD and LC-MS. Of the 11 recombinant AcGTs, AcGT5 was the unique GT which exhibited glycosylation activity toward uzarigenin (**1**) and showed unanticipated high conversion rates (>99%) (Figure 1). Three more representative cardenolide and bufadienolide aglycons (**4**, **6**, and **16**) were tested, and all these substrates were almost entirely transformed by AcGT5 (Figure 1). After scaled-up preparative reactions, the structures of the glycosylated products were elucidated by LC-MS, ^{1}H NMR, and ^{13}C NMR spectroscopic analysis. The large anomeric proton coupling constants (J > 7.7 Hz) support the formation of the β-anomers and an inverting mechanism for AcGT5 (Table S3). The above results unambiguously established AcGT5 as the first isolated plant GTs capable of glycosylating cardiotonic steroids. The cDNA sequence of AcGT5 contained an ORF of 1416 nucleotides (GenBank accession No. MF942416) encoding a protein of 464 amino acids. AcGT5 was further named UGT74AN1 by the UGT Naming Committee[15] and showed the highest identity (60%) to CrUGT9, a predicted GT from *Catharanthus roseus*.[16] A phylogenetic tree was constructed and revealed UGT74AN1 was clustered into a new clade of UGT74 subfamily (Figure S3). To study the biochemical properties and dynamic parameters, the recombinant His$_6$-UGT74AN1 was expressed in a large-scale culture and purified to near-homogeneity by His-tag affinity chromatography (Figure S4). By using uzarigenin (**1**) as an acceptor and UDPG as a donor, the purified UGT74AN1 was found to perform the maximum conversion rates at 45 °C, pH 8.0, and was divalent cation-independent (Figure S5). K_m values of UGT74AN1 toward uzarigenin (**1**), digitoxigenin (**4**), bufalin (**6**), and 3α-hydroxybufalin (**16**) were found to be 10.3, 8.2, 20.7, and 7.3 μM, respectively (Figure S6), and the corresponding k_{cat}/K_m values for **1**, **4**, **6**, and **16** were 885.4, 706.1, 439.6, and 442.5 M^{-1} s^{-1}, respectively, which indicated the high affinity of UGT74AN1 toward the cardiotonic steroid.

To study the catalytic promiscuity, regio- and stereospecificity of UGT74AN1 for cardiotonic steroid glycosylation, 22 structurally diverse steroid compounds (**1**−**17**, **54**−**58**) were selected as substrates (Figures S7 and S8) with UDPG as the donor. Glycoside production was determined by HPLC-DAD and LC-MS analysis. The results revealed that UGT74AN1 performed unprecedented catalytic promiscuity and proficiency to glycosylate structurally different cardiotonic steroids (**1**−**17**) (Figure 1). Although the A/B ring of cardenolides from the Asclepiadaceae family was generally trans-fused,[12a] UGT74AN1 exhibited unexpected identical activity toward cardenolide and bufadienolide aglycons with a *cis* A/B ring juncture (**1**, **4**, **13**, **14**). It is important to know that UGT74AN1 was flexible enough to catalyze cardiotonic steroids with various functional groups at C5, C11, C12, C16, C19 (**2**, **3**, **5**, **7**, **8**, **10**−**15**) and capable of catalyzing stereoisomers with opposite configuration of the C3 hydroxy group (**15**−**17**). Based on ^{1}H and ^{13}C NMR analysis, UGT74AN1 was verified to be a permissive but regiospecific steroid 3-*O*-GT and established to be the first identified GT to glycosylate 3α-hydroxysteroids. It had been proven that the regiospecific C3 glycosylation of cardiotonic steroid could enhance water solubility with increased bioactivity.[2,6b] In

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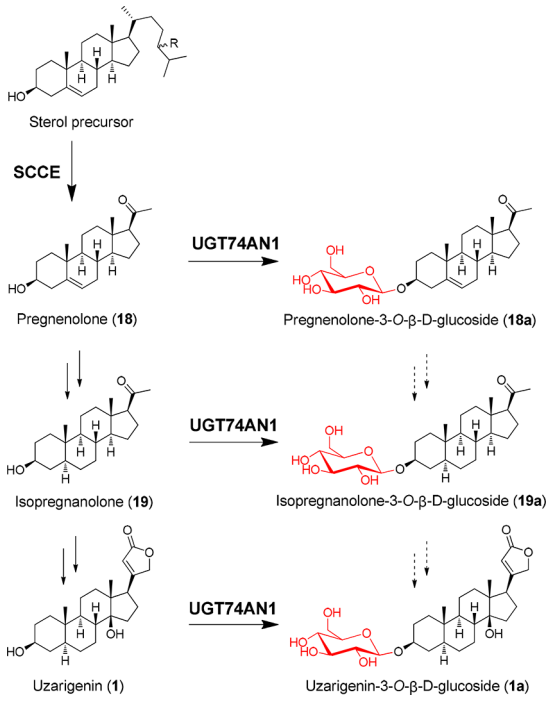
comparison, the promiscuous microbial GT YjiC1 exhibited limited regiospecificity to form varied regioisomeric species with reduced bioactivity,[6b] and OleD showed low tolerance to the functional groups at the C19 position,[6a] which was also recognized to be vital for anticancer activity.[17]

Based on the above results, we further accessed the catalytic ability of UGT74AN1 toward other steroid compounds (**18**−**23**, **59**−**62**) and observed that C_{21} steroids (**18**, **19**), steroid sapogenins (**20**, **21**), and other steroid compounds (**22**, **23**) were also glycosylated by UGT74AN1 (Figure 1, Figures S15−S20). Of particular note, UGT74AN1 effectly transformed pregnolone (**18**) and isopregnanolone (**19**) into pregnolone 3-*O*-glucoside (**18a**) and isopregnanolone 3-*O*-glucoside (**19a**) (Figures S76−S81), respectively, which demonstrated UGT74AN1 to be the first identified GT to glycosylate C_{21} steroid precursors. Given the robust catalytic activity toward cardenolide aglycons and C_{21} steroid precursors, UGT74AN1 was tentatively hypothesized as a steroid GT which probably plays a role in the 3-*O*-glycosylation of cardenolides in *vivo* (Scheme 1).[9d,11] Unlike previously

Scheme 1. Proposed Biosynthetic Pathway of Cardenolides in *A. curassavica*[a]



[a]SCCE: cholesterol side chain cleaving enzyme.

reported sterol GTs which are mostly insoluble membrane bound enzymes with a narrow substrate range,[18] UGT74AN1 is a soluble enzyme with a broad substrate range, which makes it an unusual kind of steroid GT. UGT74AN1 could glycosylate C_{21} steroid precursors in *vitro*, which indicates that the substrate promiscuity of steroid GT could be one of the reasons why steroid glycosylation takes place at various stages.

Inspired by the unprecedented catalytic efficiency for steroid compounds, we further investigated the capability of UGT74AN1 toward structurally diverse scaffolds by using 37 representative natural and unnatural compounds (**24**−**53**, **63**−**69**) as substrates (Figures S7 and S8). The results were summarized in Figure 1, which revealed the excellent capability of UGT74AN1 to catalyze 30 of the 37 structurally diverse scaffolds to form mono- or multiple products (Figures S21−S41). It is noteworthy that chloramphenicol (**47**) and erythromycin (**48**) led to monoglucosylated products, which implied UGT74AN1 as the first reported plant GT capable of catalyzing antibiotics. Besides, UGT74AN1 exhibited robust *N*- and *S*-glycosylation activity toward representative compounds containing *N*-based (**49**−**51**) and *S*-based (**52**, **53**) nucleophiles. Overall, UGT74AN1 had higher efficiency and more regioselectivity toward steroid compounds containing a 6/6/6/5 ring system in comparison with other scaffolds. In this study, a total of 53 drug-like acceptors were glycosylated by UGT74AN1 and the structures of 15 glucosylated products were elucidated by ^{1}H NMR and ^{13}C NMR analysis (Figures S45−S85).

To establish a green, cost-effective chemoenzymatic platform,[5d,19] we exploited the catalytic reversibility of UGT74AN1 for one-pot transglycosylation reactions. As shown in Figure 2,

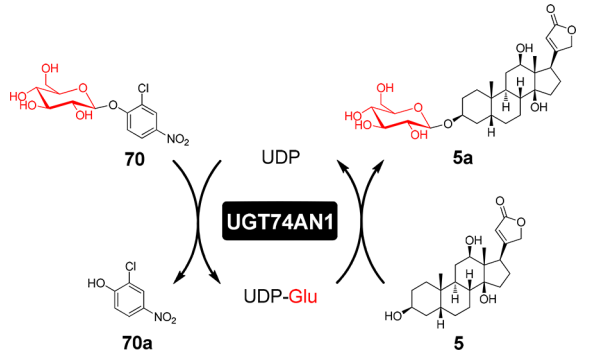


Figure 2. Exploiting the catalytic reversibility of UGT74AN1 to generate digoxigenin 3-*O*-β-D-glucoside (**5a**) from a simple donor (**70**). The HPLC analysis is shown in the SI, Figures S42−S44.

the model reactions were carried out in the presence of only a catalytic amount of UDP and led to the formation of expected products **1a**, **5a**, and **15a** with an average high yield of 90% (Figures S42−S44). Given the established substrate promiscuity of UGT74AN1, the extension of the one-pot reaction suggests UGT74AN1 can be applied to glycodiversification of bioactive molecules without adding UDPG.

In summary, we reported for the first time, the discovery of a novel steroid GT from *A. curassavica*, UGT74AN1, which displayed catalytic efficiency and regiospecificity toward cardenolide and bufadienolide aglycons, and even catalyzed C_{21} steroid precursors to form 3-*O*-β-D-glucosides. Unprecedentedly, UGT74AN1 was capable of catalyzing structurally different cardenolide and bufadienolide aglycons with *cis* or *trans* A/B ring fusion. Furthermore, UGT74AN1 showed great tolerance to various functional groups at the steroid core and served as the first reported GT catalyzing the glycosylation of 3α-hydroxy group of cardiotonic steroids. This study highlights UGT74AN1 to be the first reported promiscuous steroid GT to catalyze numerous drug-like natural and unnatural products to form *O*-, *N*-, and *S*-glycosides, along with catalytic reversibility for the one-pot transglycosylation reaction. UGT74AN1 accepted antibiotics as a substrate, which had not yet been reported in plant GTs. Cumulatively, this study demonstrated, for the first time, that a novel steroid GT, which probably played a role in the glycosylation of cardenolides in *vivo*, could serve as a powerful enzymatic tool for the regiospecific glycosylation of drug-like scaffolds. The enzyme reported here hints at more exciting and

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powerful plant steroid GTs as efficient biocatalysts in drug discovery.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b03619.

Experimental procedures, HPLC-HRESIMS characterization data, NMR, and HSQC spectra of glucosylated products (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Prof. Qi-Tai Zheng on the occasion of his 80th birthday.

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