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Glucosyltransferase Capable of Catalyzing the Last Step in Neoandrographolide Biosynthesis

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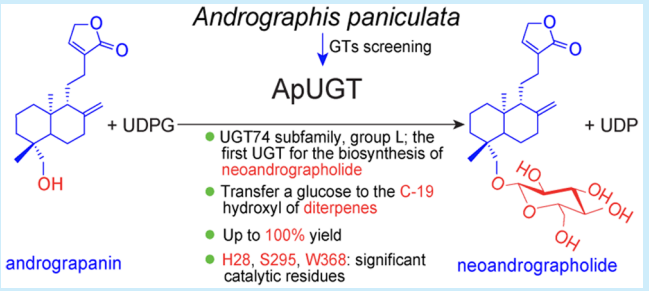
Supporting Information

ABSTRACT: ApUGT, a diterpene glycosyltransferase from *Andrographis paniculata*, could transfer a glucose to the C-19 hydroxyl moiety of andrograpanin to form neoandrographolide. This glycosyltransferase has a broad substrate scope, and it can glycosylate 26 natural and unnatural compounds of different structural types. This study provides a basis for exploring the glycosylation mechanism of *ent*-labdane-type diterpenes and plays an important role in diversifying the structures used in drug discovery.



Andrographis paniculata (Burm. f.) Wall. ex Nees, an important medicinal plant, is distributed in Southeast and South Asia and exhibits various pharmacological actions.[1] It has been included in the Dietary Supplements of the United States Pharmacopoeia. Sales of its injection, which is used for the treatment of acute lung injury,[2] reached over CNY ¥3 billion year^{-1} in China in 2015. *ent*-Labdane-type diterpenes (*ent*-LRDs) with different glycosides substituents are commonly found in *A. paniculata*.[3,4] The *ent*-LRDs in *A. paniculata* have similar structural backbones. This includes an α,β-unsaturated-γ-lactone moiety and a *trans*-fused 6/6 bicyclic framework connected by a fatty chain or a carbon−carbon double bond.[4] Their diverse chemical structures and ubiquity in nature determine the bioactivities of these diterpenoids.[5] Neoandrographolide is an *ent*-labdane-type diterpene glycoside, and its precursor aglycone is andrograpanin. Neoandrographolide and its lactone ring-containing analogues were easily modified using dehydration, double-bond migration, and isomerization. Modifying the structure of neoandrographolide, which mainly includes the conversion of the C-19 glucoside into a CH_2OH or COOH moiety, $\Delta^{8,(17)}$-double bond addition reactions, and 2-, 3-, or 7-hydroxylation of the diterpenoid ring, can produce additional diterpenoid derivatives.[6] Neoandrographolide possesses a weak cytotoxic effect and various pharmacological activities such as antiherpes simplex virus, anti-inflammatory, antioxidant, antiparasitic, and hepatoprotective activities.[7a−g] The compound is also used as a potent chemosensitizer with low cytotoxic activity against S-Jurkat and XIAP-overexpressing Jurkat cells.[7e] However, chemical glycosylation reactions have some limitations, including poor stereoselectivities and yields and tedious protection and deprotection.[8]

Glycosyltransferase assembly lines, which are exciting developments in synthetic glycobiology, were designed to better produce glycosides than chemical approaches.[9] They are considerably important because the glycan moiety forms an integral component of plant secondary metabolites, conferring properties to the compound that can help elucidate the glycoside biosynthetic pathways and have important theoretical and practical value.[10,11] The last step in the synthesis of many metabolites is a very important and beneficial step in which glycosylation frequently occurs.[12] However, a glucosyltransferase capable of catalyzing the glucosylation of the biosynthesis of neoandrographolide has not been reported. Herein, we report for the first time, the cloning and functional characterization of a glucosyltransferase from *Andrographis paniculata* (ApUGT), which exhibits high catalytic efficiency and can transfer a glucose to the C-19 hydroxyl moiety of andrograpanin to form neoandrographolide. Notably, this glucosyltransferase can catalyze the 19-*O*-glycosylation compounds with different structural types, and it is also a plant glycosyltransferase that can generate *O*-, *S*-, and *N*-glycosides.

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In the present study, we found that neoandrographolide can be induced by MeJA (methyl jasmonate). Following induction by MeJA, the accumulation of neoandrographolide increased for the first 48 h. To identify the enzyme responsible for the generation of neoandrographolide, time-coursed transcriptome sequencing with MeJA treatment was performed. A total of nine UGTs with accumulation patterns of neoandrographolide during MeJA induction that were consistent with the expression pattern in transcriptome (Figure S1) were designated as candidate UGTs. Based on the results of comparative transcriptomic analysis, the open reading frames of the nine candidates were extracted from the *A. paniculata* transcriptome data and assigned by the UGT Nomenclature Committee.[13] Full-length coding sequences of the selected UGTs were amplified using cDNAs, which were obtained from the leaves of *A. paniculata* that had been treated for 48 h, as templates and inserted into the HIS-MBP-pET28a vector. The predicted amino acid sequence of ApUGT specifies a polypeptide of 471 amino acids with a relative molecular weight of 52.14 kDa. At its C-terminal end, ApUGT contained the conserved plant secondary product glycosyltransferase (PSPG) domain that has been proposed to be a nucleoside−diphosphate−sugar binding site.

The corresponding proteins were heterologously expressed as His-MBP-fusion proteins in *Escherichia coli*. The nine recombinant UGTs were all purified by affinity chromatography (Figures S2) and incubated with UDP-glucose (UDPG) and andrograpanin to verify the glucosylation of andrograpanin in an in vitro catalytic reaction (50 mM Tris-HCl, pH 7.4; 320 μM andrograpanin, 1600 μM UDPG; 14 μg purified protein; 30 °C, 12 h) using an ultraperformance liquid chromatography−PDA detector (UPLC−PDA). The enzymatic analysis of these UGTs showed that only one UGT, ApUGT (UGT5), can convert one predominant peak with a shorter retention time (Figure 1). After the amplification reaction, the structure of the glycosylated product was verified by UPLC/Q-TOF-MS

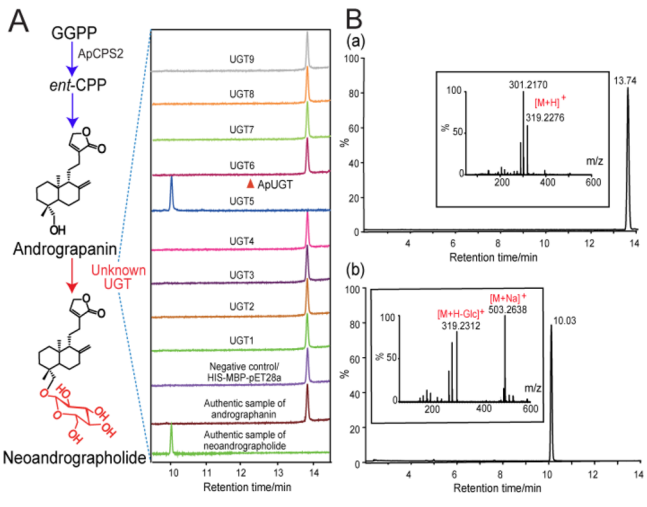

Figure 1. Cloning and identification of ApUGT catalyzing andrograpanin to neoandrographolide. (A) Enzymatic glucosylation of andrograpanin by ApUGT and UPLC analyses of the in vitro reactions of ApUGTs catalyzing andrograpanin. (B) In vitro enzyme assay for determining the activity of the candidate ApUGT by UPLC/Q-TOF-MS using andrograpanin as a substrate. (a) Reference standard of the substrate. (b) Product of the catalytic derivatization of candidate ApUGT.

(ultrahigh-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry), ^{1}H NMR and ^{13}C NMR spectroscopy supplemented in the Supporting Information (SI). MS analysis revealed the glucosylated product containing one more glucose moiety than andrograpanin (**1**), the 19-*O*-glucose moiety was further supported by NMR data, and the large anomeric proton coupling constant (J > 6.0 Hz) indicated the β-anomer and an inverting mechanism for ApUGT. Similarly, three other main *ent*-LRDs in *A. paniculata*,[14] namely 14-deoxyandrographolide (**2**), andrographolide (**4**), and 14-deoxy-11,12-didehydroandrographolide (**6**), were also glucosylated by ApUGT in vitro (Figure 2), and all of the reactions produced a predominant product, indicating the specificity of this enzyme at the 19-*O*-glycosylation.

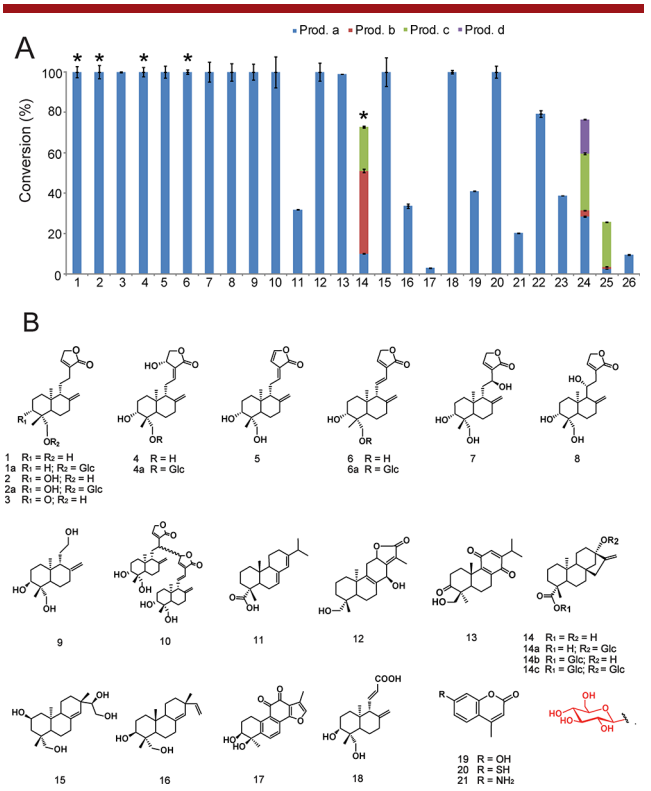

Figure 2. Exploring the catalytic promiscuity of ApUGT. (A) Percent yields of glucosylated products formed via ApUGT catalysis. Compound numbers correspond to the structures listed in Figure S4. The color of the columns (Prod. a, Prod. b, Prod. c, and Prod. d) represent different ratios of diverse glycosylated products in the total product yield of each compound. Error bars used in the figure indicate ± SDs. The asterisks (*) represent the glycosylated products that were prepared and structurally characterized by UPLC/Q-TOF-MS and ^{1}H and ^{13}C NMR spectroscopy. (B) Structures of the library members and corresponding glucosylated products. Compounds with no yields are listed in Figure S5.

To explore the scope of recognizable substrates ApUGT in vitro, a variety of representative compounds with different structures, including diterpenes and derivatives (**1**−**18**, **27**, **31**, **34**), triterpenes (**22** and **23**), flavonoids (**24**−**26**, **28**, and **29**), alkaloids (**32** and **33**), phenylpropanoid (**35**), coumarin (**30**), and simple aromatics (**19**−**21**) with various nucleophilic groups (−OH, −SH, and −NH_2), were employed in enzymatic assays with UDPG as the donor (Figure 2). Glycoside productions were determined by UPLC-PDA and UPLC/Q-

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TOF-MS analysis (Figures S11−S35). The results indicate that ApUGT not only catalyzes *ent*-LRDs (**1**−**10**) with a high conversion rate but also produces only one predominant glycosylated product. In addition, ApUGT was flexible enough to glycosylate the C-19 hydroxyl of *ent*-LRDs with substituents at C3 and C14, $\Delta^{11,(12)}$ or $\Delta^{12,(13)}$ double bond with unsaturated lactone (**1**, **2**, **4**, and **6**). Other tested diterpenes like abietane diterpenes (**11**, **13**, and **17**), *ent*-abietane diterpenes (**12**), pimarane diterpenes (**15**), isopimarane diterpenes (**16**), diterpenoid derivatives (**9** and **18**), and triterpenes (**22** and **23**) produced only one predominant glycosylation product, respectively. Interestingly, ApUGT could also transfer a glucose moiety to the C19-hydroxyl groups on the carboxyl of diterpenes (**11** and **14**). However, when steviol (**14**), an *ent*-kaurene diterpene, and some flavonoids (**24** and **25**) were used as substrates, more than one glycosylation products were obtained in the reaction, indicating a relaxed substrate specificity for ApUGT. ApUGT also exhibited robust *S*- and *N*-glycosylation activity for representative compounds (**20** and **21**).

Andrograpanin is upstream of the diterpenoid biosynthetic pathway in *A. paniculata*. Terpenoids produced by *A. paniculata* are proposed to be transformed into each other via double-bond transfer, dehydration, or hydroxyl addition (Figure S6).[15] The k_{cat}/K_m values of ApUGT for andrograpanin (**1**), 14-deoxyandrographolide (**2**), andrographolide (**4**), and 14-deoxy-11,12-didehydroandrographolide (**6**) were 3617.7, 2146.9, 3410.6, and 2561.6 M^{-1} s^{-1}, respectively. The catalytic efficiencies with the four compounds are all very similar, likely indicating that ApUGT accepts all four as substrates in vivo. It also suggest a preference for andrograpanin as its sugar acceptor because of the high k_{cat}/K_m ratio (Figure 3A). The k_{cat}/K_m value of ApUGT toward the unnatural substrate phlogacantholide B (**12**) was 3727.5 M^{-1} s^{-1}, indicating that ApUGT has a broad substrate spectrum, and can be used for glycosylation of compounds with various structures.

Homology modeling and optimization of the 3D structure of ApUGT were performed. The crystal structure of UGT74F2 was selected as a suitable template to identify the crucial amino acids involved in the glucosylation reaction. Based on the protein sequence similarity (40%) and closely related biological functions of these two enzymes, we predicted the key catalytic sites using molecular docking (Figure S9). Enzyme assays following single site-directed mutagenesis revealed that mutations to five key sites (H28A, S295A, E347A, W368A, and E373A) decreased the andrograpanin glucosylation activity. In particular the H28A and W368A mutants, showed 86% and 85% reduced activity, respectively, compared with the wild-type enzyme. The mutations S295A, E347A, and E373A resulted in 53%, 3%, and 33% decreases in ApUGT activity toward andrograpanin, respectively (Figure 3B). Our results suggest that the mutations in ApUGT have modest effects, which is seen in some glycosyltransferase mutants but not typically.[16] H28A, S295A, and W368A may be significant catalytic residues in the biosynthetic glucosylation of neoandrographolide.

A phylogenetic tree of ApUGT was constructed with plant UGTs (Figure S10), and the tree revealed that ApUGT5 was clustered into a subclade of the UGT74 subfamily and belongs to group L. Members of this group can catalyze the formation of glycosidic bonds that recognize hydroxyl groups on a variety of different substrates, including diterpenes, triterpenes, and

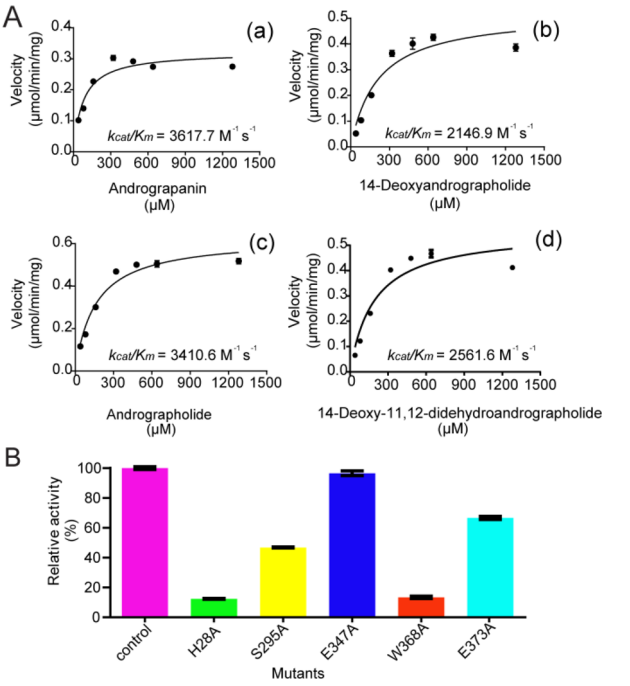

Figure 3. Biochemical analysis of ApUGT. (A) Determination of kinetic parameters for the recombinant ApUGT. Kinetic parameters detected with (a) andrograpanin, (b) 14-dehydroandrographolide, (c) andrographolide, and (d) 14-deoxy-11,12-didehydroandrographolide as acceptors and UDPG as the donor. (B) Relative catalytic activities of ApUGT wild-type and mutants when andrograpanin was used as substrate. Error bars used in the figure indicate ± SDs.

flavonoids, etc. In addition to SrUGT74G1, which is a diterpene glycosyltransferase, ApUGT5 is another diterpene glycosyltransferase belonging to UGT74 subfamily group L and is the first UGT reported to glycosylate the C-19 hydroxyl moiety of *ent*-LRDs precursors, suggesting that ApUGT is a new diterpene glycosyltransferase of this subcluster.

In summary, we have identified a glucosyltransferase (ApUGT) capable of catalyzing the 19-*O*-glucosylation in the biosynthesis of neoandrographolide with broad substrate spectrum and high catalytic efficiency. As a plant UGT, ApUGT showed robust glucosylation activity over a series of structurally diverse compounds. It can catalyze *O*-, *S*-, and *N*-glycosidic bond formation. Phylogenetic analysis also indicated that ApUGT is the first *ent*-LRDs glucosyltransferase in the plant UGT74 family group L (Figure S10), further demonstrating the novelty of ApUGT in the plant UGT74 family. The enzyme reported here provides a potential molecular basis for studying glycosylations in the biosyntheses of diterpenoid glycosides and paves the way for the utilization of UGTs for high-yield productions of neoandrographolide, which will promote the research and development of new neoandrographolide-based drugs, further ensuring the safety and effectiveness of clinical medications. Further studies on enzyme engineering and the modification of these compounds using enzymatic methods or synthetic biology will be conducted.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b02146.

Experimental procedures, UPLC-Q-TOF-MS characterization data, and NMR spectra of glycosylated products (PDF)

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Notes

The authors declare no competing financial interest.

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