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Enzymatic *N*-Glycosylation of Diverse Arylamine Aglycones by a Promiscuous Glycosyltransferase from *Carthamus tinctorius*

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Abstract: A new glycosyltransferase (UGT71E5) from *Carthamus tinctorius* exhibited a robust promiscuity towards 30 structurally diverse drug-like aromatic amine scaffolds, making it the first reported glycosyltransferase capable of catalyzing *N*-glycosylation with multiple diverse nitrogen-heterocyclic aromatic compounds. The catalytic promiscuity and reversibility of UGT71E5 was exploited to generate the rare *N*-glycoside from the abundant *O*-glycoside with high efficiency. These findings demonstrate the significant potential of UGT71E5 in the enzymatic synthesis of diverse bioactive *N*-glycosides.

Keywords: *Carthamus tinctorius*; catalytic promiscuity; enzyme catalysis; glycosylation; glycosyltransferases; *N*-glycosylation

Sugar moieties are commonly found attached to biologically active natural products and clinical drugs, which not only can contribute to the diversity of structures but also affect the physicochemical and biological properties of these glycosides.[1] The majority of the glycosides involve *O*-glycosidic bonds. By contrast, the *N*-glycosylation is relatively rare and exhibits outstanding pharmacological activity especially in some chemical drugs that have attracted considerable attention.[2] However, chemical *N*-glycosylation faces challenges in regioselectivity, stereoselectivity, and the protection and de-protection of functional groups, which limit the exploitation of these rare glycosides in drug discovery.[3] Glycosyltransferases (GTs) are generally recognized as powerful synthetic tools for bioactive glycosylated products, as they can transfer sugar moieties from donors to acceptors generating *O*-, *N*-, *S*-, and *C*-glycosidic bonds.[3a,4] Significant progress has been made in enzymatic *O*-glycosylation of natural and unnatural products by GTs.[5] However, enzymatic *N*-glycosylation still remains restricted by enzyme specificity and the availability of suitable GTs. In the past few years, several GTs with *N*-glycosylation activity have been reported, such as an engineered microbial GT OleD that exhibits *N*-glycosylation activity to some aromatic amines, hydrazines and hydrazides.[5a] In addition, some GTs from plants also possess *N*-glycosylation activity.[5f–h,6] However, the majority of the known GTs exhibit relatively narrow substrate spectra and few of them have been utilized as tools in diverse *N*-glycosylations. Thus, mining for novel GTs with catalytically promiscuous *N*-glycosylation capability is of significance, as it will allow the construction of enzymatic methods for the synthesis of bioactive *N*-glycosides. Here, we report a new GT (UGT71E5) from *Carthamus tinctorius* that can tolerate a number of structurally different drug-like aromatic amines to generate diverse *N*-glycosidic bonds. The catalytic reversibility of UGT71E5 in *O*-glycosylation allows a one-pot reaction generating rare *N*-glycosides from abundantly available *O*-glycosides.

In our previous work, we found a promiscuous GT, UGT73AE1 from *C. tinctorius*, that demonstrates a wide substrate spectrum for *O*-glycosylation.[5f] In this work, we aimed to screen for a GT with catalytic promiscuity in rare *N*-glycosylation to expand the application of GTs in diverse enzymatic glycosylations. To clone permissive GTs from *C. tinctorius*, a modified degenerate PCR primer for 5′ RACE was designed based on the conserved PSPG (*plant secondary product glycosyltransferases*) motif of six permissive plant

GTs as shown in the Supporing Information, Figure S1. Combined with 3′ RACE, 16 new different *C. tinctorius* glycosyltransferase genes (*CtGTs*) were successfully cloned by RT-PCR amplification using the total RNA from florets of *C. tinctorius* as a template and heterologously expressed in *Escherichia coli*.

For screening the *N*-glycosylation activity of CtGTs, the small molecules, 7-amino-4-methylcoumarin (**1**) and 3,4-dichloroaniline (**4**) were used as acceptors and uridine diphosphate glucose (UDP-Glu) was used as a sugar donor. The screening reactions (50 mM Tris-HCl, pH 7.4; 0.5 mM UDP-Glu; 0.25 mM aglycone; 500 μg of crude CtGTs; 30 °C, 6 h) were analyzed by HPLC-UV/MS (high-performance liquid chromatography-UV absorption/mass spectrometry). Of the 16 recombinant CtGTs, only CtGT22 showed glycosylation activity to both **1** and **4** with high conversion rates (>90%) (Figure 1). Moreover, CtGT22 also showed high catalytic efficiency in *S*-glycosylation with small molecules (**34** and **35**) and *O*-glycosylation with flavonoids (**36**–**39**) (see Figure 1 and the Supporting Information, S35–S38). Control reactions lacking either enzyme or UDP-Glu confirmed that the reactions were dependent upon both the enzyme and UDP-Glu. The glycosylated products (**1a**, **4a**, **34a** and **35a**) were prepared from scaled-up reactions and their structures, including anomeric stereochemistry, were characterized by MS, ^{1}H NMR, and ^{13}C NMR spectroscopic analyses (Supporting Information, Figures S42–S45 and S62–S65). The large anomeric proton-coupling constants (J = 7.9, 8.4, 9.8 and 9.6 Hz for **1a**, **4a**, **34a** and **35a**, respectively) indicate the formation of the β-anomers and an inverting mechanism for CtGT22 (Supporting Information, Table S4).

The cDNA sequence of CtGT22 (1443 bp, GenBank accession number KX610759) contained an ORF encoding 480 amino acids and showed the highest identity (67%) to UGT71E1, a flavonoid GT from *Stevia rebaudiana*.[7] CtGT22 was further named UGT71E5 according to the UGT Naming Committee.[8] Recombinant His$_6$-UGT71E5 was purified using His-tag affinity chromatography and was analyzed with SDS-PAGE (Supporting Information, Figure S2).

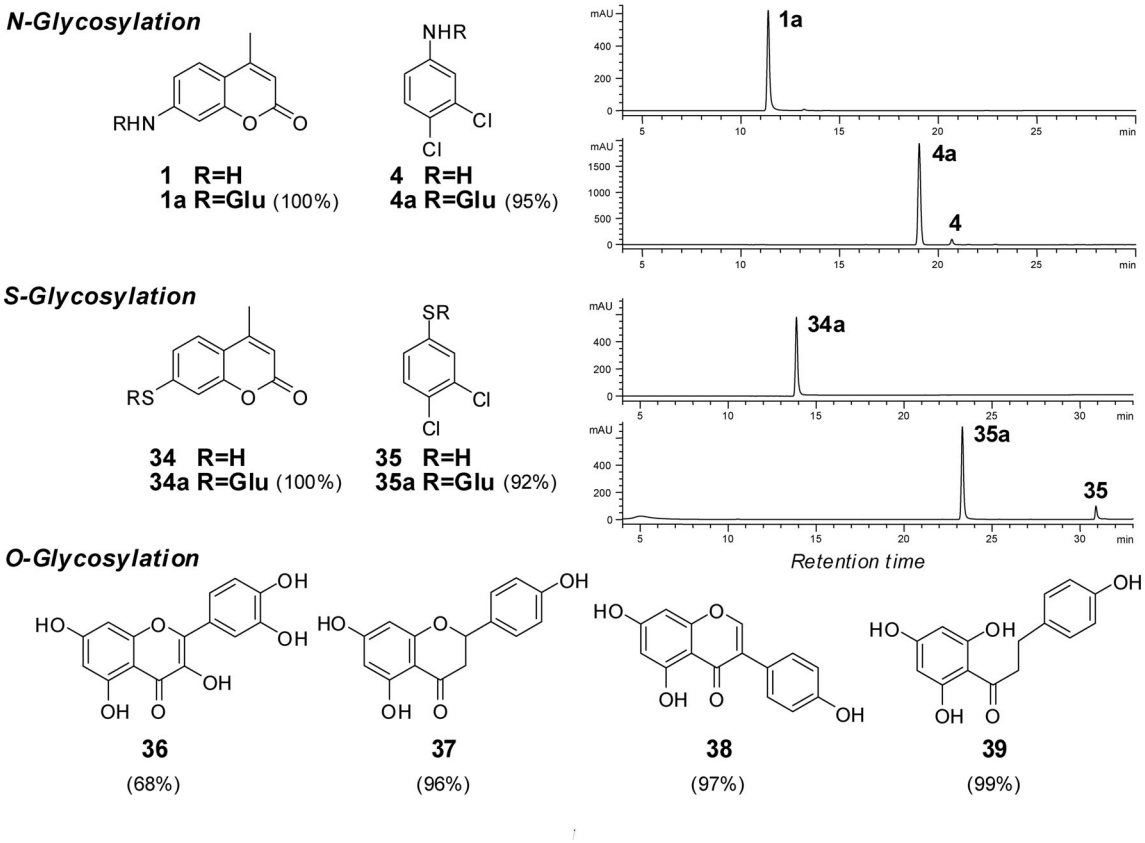

Figure 1. High catalytic efficiency of UGT71E5 in *N*-, *S*- and *O*-glycosylation. ***N*-Glycosylation:** Structures of *N*-glycosylated products and HPLC analysis of *N*-glycosylation catalyzed by UGT71E5. ***S*-Glycosylation**: Structures of *S*-glycosylated products and HPLC analysis of *S*-glycosylation catalyzed by UGT71E5. ***O*-Glycosylation**: Structures of flavonoids recognized by UGT71E5. Percent conversions of acceptors are shown underneath the structures. *N*- and *S*-glycosylated products (**1a**, **4a**, **34a** and **35a**) were isolated and confirmed by MS and ^{1}H, ^{13}C NMR.

UGT71E5 displayed the maximum catalytic activity at pH 9.0 and 30 °C and was divalent cation independent (Supporting Information, Figure S3). UGT71E5 exhibited K_m values of 274.8 μM, 76.6 μM and 181.4 μM for **1** (*N*-glycosylation), **34** (*S*-glycosylation) and **40** (*O*-glycosylation), respectively (Supporting Information, Figure S4). Given the cDNA sequence homology, the catalytic activity to flavonoids and the existence of various flavonoid glycosides in *C. tinctorius*, UGT71E5 was tentatively hypothesized as a flavonoid GT.

To systematically explore the catalytic promiscuity and probe the synthetic utility of UGT71E5 in *N*-glycosylation, a series of structurally diverse representative drugs and drug-like compounds containing *N-based* nucleophiles (amino/imino groups) was collected. Therefore, a compound library including derivatives of aniline (**1**–**16**), aminopyridine (**18**–**21**), aminopyridazine (**22**–**24**), aminopyrimidine (**25**–**29**), aminopyrazine (**30**), aminotriazine (**31**–**33**) and an aliphatic amine (**17**) was constructed (Figure 2B and C). Notably, most of the library members are clinical anticarcinogen (Lenakidomide, **2**), antileprotic (diaminodiphenyl sulfone, **5**), anesthetic (Benzocaine, **9**), cholinesterase inhibitor (Tacrine, **20**), hypotensor (Prazosin, **28**), antibacterial synergist (Trimethoprim, **29**) and bioactive natural or un-natural products with significant pharmacological activity but poor solubility and negative side effects. For example, 9-amino-camptothecin (**7**) is an inhibitor of topoisomerase I with potent antitumor activity against a wide spectrum of human tumors. However, its poor water solubility and hematologic toxicity have limited its clinical application and development.[9] Above all, 33 members with diverse structures were employed in the *N*-glycosylated modification.

The unprecedented catalytic promiscuity of UGT71E5 in *N*-glycosylation was observed from a first-pass analysis with HPLC-UV/MS, which revealed that UGT71E5 was flexible enough to *N*-glycosylate sixteen (**1**–**16**) structurally different library members (Figure 2A and B). Of particular note, most of the acceptors have large structural differences, including varied skeletons. As *N*-heteroarylamine compounds are common drug scaffolds, exploring and expanding the utility of UGT71E5 in the glycosylation of heteroarylamines is significant. Thus, by focusing on heteroarylamines, another 16 library members (**18**–**33**), including derivatives of N_1-, N_2-, and N_3-heterocycles, were employed. UGT71E5 was sufficiently flexible to *N*-glycosylate all the derivatives of aminopyridine (**18**–**21**) (Figure 2A and C). To our surprise, derivatives of aminopyridazine (**22**, **23**), aminopyrimidine (**25**–**29**), aminopyrazine (**30**), and aminotriazine (**31**, **33**) were also recognized by UGT71E5. The importance of aromatic properties is evidenced by the fact that when more carbon atoms in the aromatic rings were substituted by nitrogen, the conversion rates were lower. Introduction of nitrogen atoms into the aromatic ring may affect, but not completely destroy, the aromatic properties of the acceptors.[10] Additionally, the heterocyclic ring might form a π–π interaction with an enzyme amino acid residue around the active site and help to allocate the substrate in an appropriate orientation.[6a] Hence, UGT71E5 glycosylated these *N*-heteroarylamine compounds, utilizing its robust catalytic promiscuity. In total, 30 structurally diverse unnatural compounds, including 14 (**18**–**23**, **25**–**31** and **33**) derivatives of nitrogen heterocycles, were shown as acceptors of UGT71E5 to generate *N*-glycosides. This remarkable catalytic promiscuity for nitrogen heterocycles is uncommon for a plant GT. To the best of our knowledge, this is the first reported GT capable of catalyzing *N*-glycosidic bond formation with such diverse nitrogen heterocyclic aromatic compounds. This finding will expand the applications of GTs in the field of enzymatic glycosylation.

In addition to *N*-glycosylation, UGT71E5 also exhibited *S*-glycosylation activity toward the SH function of an nitrogen heterocyclic derivative (2-mercapto-4-methylpyridine, **41**), which has not been reported for other GTs (Supporting Information, Figures S29 and S33). Moreover, a different pattern of *S*-glycosylation was observed when 2,4-dichlorobenzyl thiol (**42**), which only possesses an aliphatic thiol, was used as an acceptor (Supporting Information, Figures S29 and S34).

To further confirm the catalytic properties of UGT71E5 and the structures of the glycosylated products, eight *N*-glucosylated (**1a**, **4a**-**8a**, **10a** and **20a**) and three *S*-glucosylated (**34a**, **35a** and **42a**) products were isolated from the preparative-scale reactions, four (**6a**, **7a**, **10a** and **20a**) of which were novel compounds (Figures 2 and the Supporting Information, S29). The structures were identified by mass and NMR spectroscopic analyses (Suppoorting Information, Figures S42–S67). For example, ^{1}H and ^{13}C NMR, HSQC and HMBC were employed to confirm the structure of **7a**. In the ^{1}H NMR spectrum, the presence of the glucosyl moiety was suggested by the characteristic signals for H-Glc$_{1'}$ (δ_H = 4.55, 1H) and H-Glc$_{2'\text{-}6'}$ (δ_H = 3.16–3.72, 6H). The location of the glucosyl moiety was indicated by the correlation between H-Glc$_{1'}$ and C-9 (δ_C = 143.5) in the HMBC spectrum and the unchanged hydroxy proton signal of OH-20 (δ_H = 6.52, 1H) in the ^{1}H NMR spectrum. The large coupling constant (J = 8.0 Hz) of H-Glc$_{1'}$ indicated the β configuration of the glucosidic linkage. Thus, the glycosylated product of 9-amino-camptothecin (**7**) was identified as 9-amino-camptothecin 9-*N*-β-D-glucoside (**7a**). The water solubility of the glycosylated products was greatly increased while the pharmacological activity needs to be further investigated. For *N*-glycoside **7a**, the water solubility increased nearly

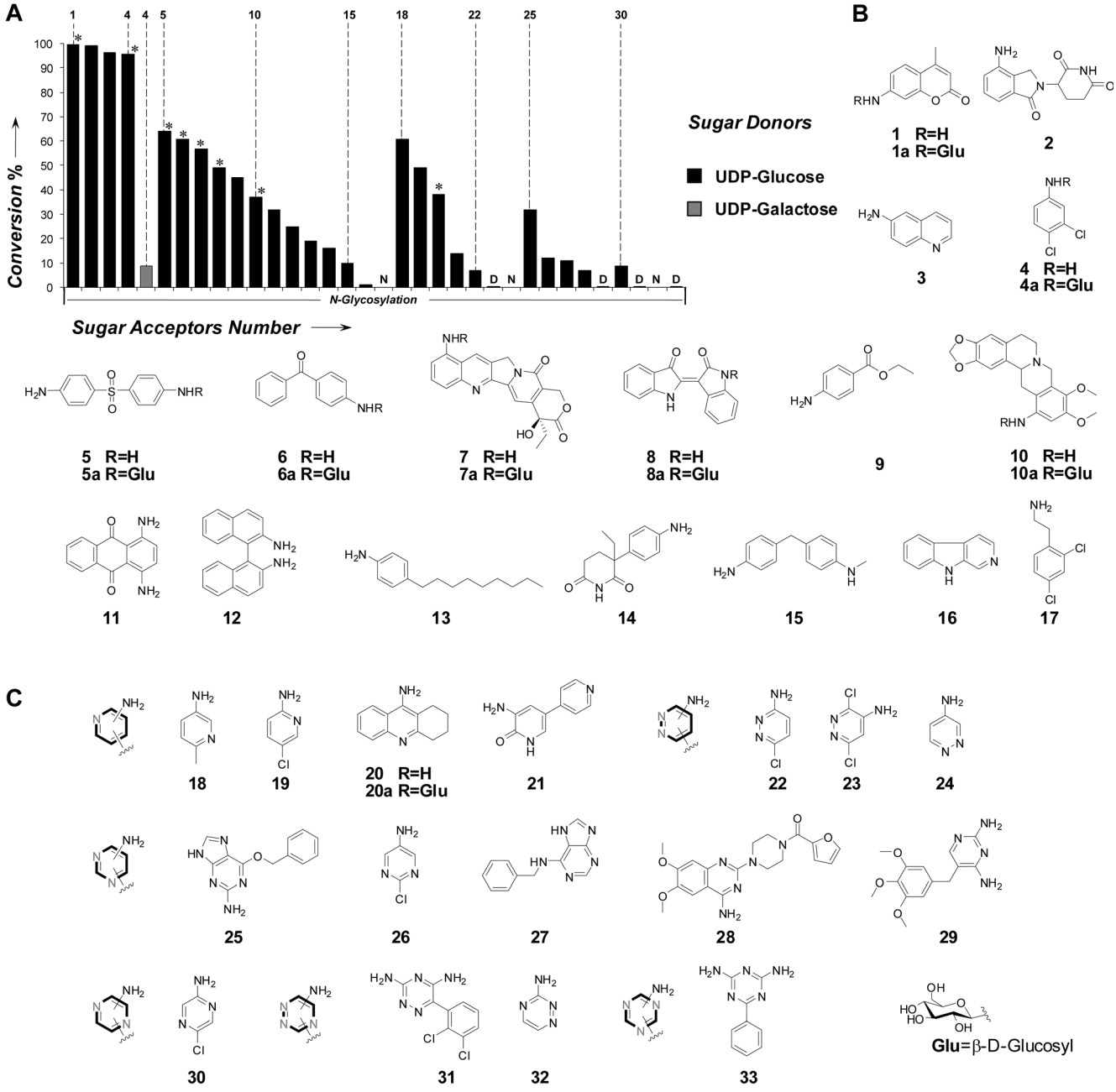

Figure 2. Catalytic promiscuity of UGT71E5 in *N*-glycosylation. (**A**) Percent conversions of *N*-glycosylated products catalyzed by UGT71E5. The library numbers are listed based on the structural scaffolds shown in parts **B** and **C**. The black and grey columns represent conversions of glucosides and galactosides, respectively. * = glycosylated products were isolated and confirmed by MS and NMR; D = detected in MS; N = not detected. (**B**, **C**) The structures of the library members and corresponding *N*-glycosylated products.

39-fold compared to the aglycone (Supporting Information, Table S3). The anomers of all the isolated products were in the β configuration, as evidenced by anomeric protons with large coupling constants ($J >$ 7.0 Hz) (Supporting Information, Table S4).

Given the current low diversity of *N*- and *S*-glycosides, exploitation of universally applicable green chemical approaches to generate rare *N*- and *S*-glycosides from abundant *O*-glycosides is highly desired, but, however, has not been achieved due to the lack of suitable GTs. Thus, based on the tri-functional features of UGT71E5 in *N*-, *S*-, and *O*- glycosylation including the catalytic reversibility in *O*-glycosylation,[11,5g] model reactions were performed with 4-nitrophenyl β-D-glucopyranoside (**43**) as a sugar donor and 7-amino-4-methylcoumarin (**1**) or 7-mercapto-4-methylcoumarin (**34**) as an acceptor under the catalysis of UGT71E5 (Figure 3). The desired *N*- and *S*-glu-

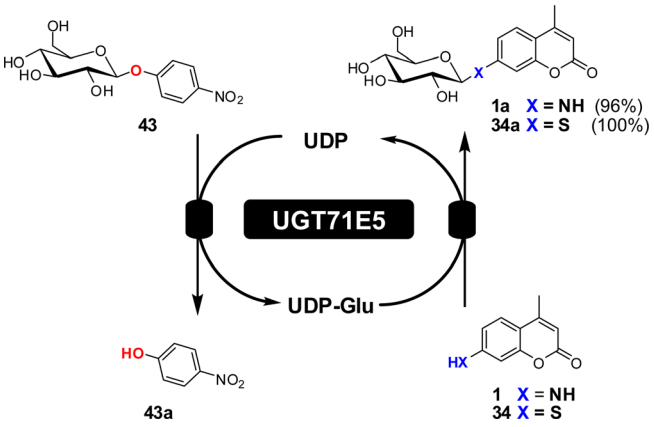

Figure 3. Exploiting *O*-GT reversibility of UGT71E5 to generate *N*- and *S*-glucosides from a simple *O*-glucoside (**43**). Conversions of **1a** and **34a** were 96% and 100%, respectively. The HPLC analysis is shown in the Supporting Information, Figures S39 and S40.

cosylated products (**1a** and **34a**) were obtained with conversions of 96% and 100%, respectively. These high conversions occurred in the presence of only a catalytic amount of UDP (1/10 of **43**), which revealed that UDP was under cyclic utilization throughout the coupled reactions. Therefore, *O*-deglycosylation and *N*- or *S*-glycosylation were successfully coupled together with UDP *via* UGT71E5. To investigate the substrate spectrum toward the NDP moieties, UGT71E5 was subsequently probed with **1** and four other commercially available NDPs in one-pot reactions. Interestingly, UGT71E5 displayed broad substrate specificity, recognizing dTDP, ADP, CDP and GDP, as well as their corresponding activated glucoses. In those reactions, glucose was transferred from dTDP-, ADP-, CDP- and GDP-Glu donors at 50% that of UDP-Glu (Supporting Information, Figure S41). This feature allows UGT71E5 to be applied in *N*-glycodiversification from *O*-glucosides *in vivo* with different NDPs of host strains.[12] Above all, this one-pot reaction mediated by UGT71E5 generating *N*- and *S*-glycosides from abundant *O*-glycosides without adding activated sugars establishes a cost-effective enzymatic method for synthesizing diverse bioactive *N*- and *S*-glycosides. To be noticed, UGT71E5, as a tri-functional *N*-, *S*- and *O*-GT with catalytic promiscuity and high efficiency, can be applied to glycodiversification.

UGT71E5 also showed tolerance to sugar donors. The commercially available sugar donors UDP-Glu, UDP-galactose (UDP-Gal) and UDP-glucuronic acid (UDP-GA) were employed to investigate the promiscuity of sugar donors. UGT71E5 was able to accept UDP-Glu and UDP-Gal, generating *N*-glycosidic bonds when 3,4-dichloroaniline (**4**) was used as an acceptor. For 3,4-dichlorobenzenethiol (**35**), UGT71E5 can recognize UDP-Glu, UDP-Gal, and UDP-GA to form *S*-glycosidic bonds (Supporting Information, Figures S29, S31 and S32). The broad acceptor and donor spectra make UGT71E5 a powerful enzymatic tool in the synthesis of glycosides and combinatorial application for the future drug discovery.

In summary, the astonishing catalytic diversity of UGT71E5 in *N*-glycosylation was highlighted. As a plant GT, UGT71E5 exhibited robust *N*-glycosylation to a series of drug and drug-like compounds. In particular, the surprising glycosylation activity to $-NH_2$ and –NH– functions of nitrogen heterocyclic derivatives makes UGT71E5 the first reported GT capable of catalyzing *N*-glycosylation with multiple diverse nitrogen heterocyclic compounds. In addition to the acceptor diversity, UGT71E5 also showed an interesting tolerance to sugar donors in *N*- and *S*-glycosylation, which had not yet been reported in plant GTs. Moreover, the remarkable enzymatic conversion of *O*- into *N*- and *S*-glycosidic bonds may create new opportunities for the synthesis of rare bioactive *N*- and *S*-glycosides. So far, GTs with *N*-glycosylation activity have not been well studied due to the lack of suitable models.[13] UGT71E5 might be an OGT *in vivo* while possessing potent *N*-glycosylation catalytic activity *in vitro*, that is a good model for deciphering the promiscuity of plant GTs *in vitro*. Cumulatively, the present study demonstrated, for the first time, the significant potential of a plant enzyme as a powerful biocatalyst for the synthesis of rare bioactive natural and unnatural glycosides.

Experimental Section

Materials

Chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), J&K Scientific Ltd. (Beijing, China), InnoChem Science & Technology Co., Ltd. (Beijing, China) and BioBioPha (Kunming, China).

Methods

UGT71E5 was amplified by PCR, and the gene fragment was then inserted into plasmid pET-28a. The resultant plasmid was then transformed into *E. coli* (TransGen Biotech, China) for heterologous expression. Recombinant UGT71E5 was purified using His-tag affinity chromatography. The analyses of enzymatic products were performed on an Agilent 1200 series HPLC system (Agilent Technologies, Germany) coupled with an LCQ Fleet ion trap mass spectrometer (Thermo Electron Corp., USA) equipped with an electrospray ionization (ESI) source. HR-ESI-MS data were measured with an Agilent Technologies 6520 Accurate Mass Q-TOF LC/MS spectrometer. Compounds were characterized by ^{1}H NMR at 400 or 600 MHz and ^{13}C NMR at 150 MHz on Mercury-400 and Bruker AVIIIHD-600 spectrometers.

The detailed experimental procedures, including *CtGTs* cloning, expression, reaction analysis and products purification protocols, LC/MS, HRESIMS and NMR characterization data and spectra of glucosylated products can be found in the Supporting Information.

Acknowledgements

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