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Biocatalytic C3 β-*O*-Glycosylation of Triterpenes and Sterols to Synthesize Natural and Unnatural Saponins

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ABSTRACT

Saponins are natural products that consist of triterpene or sterol cores decorated with oxidations, glycosylations, and sometimes other modifications. Many saponins are utilized as nutraceutics (e.g., glycyrrhizin) or therapeutics (e.g., QS-21 and digitoxin/digoxin). The structure–activity relationships that govern saponin bioactivity can be identified by studying structurally related saponins; however, the production of varied sets of saponins remains challenging via either chemical (semi-)synthesis or native/heterologous biosynthesis. This report describes the discovery that the GT1 family enzyme GuUGT73F15 (*Glycyrrhiza uralensis*) can be used to biosynthesize many different saponins via triterpene/sterol C3 β-*O*-glycosylation. GuUGT73F15 utilized 22 sugar acceptors (C3 hydroxyl-containing triterpenes/sterols) and 12 sugar donors (uridine diphosphate [UDP]-sugars) as substrates to produce 130 unique monoglycosylated saponins, of which, more than 100 have not been reported as natural products to the best of our knowledge. GuUGT73F15 also accepted 13 cyclohexanol- and phenol-type molecules as minimized sugar acceptors. Based on Boltz-2x predictions, the broad substrate scope of GuUGT73F15 is hypothesized to arise from its varied sugar acceptor binding poses and consistent sugar donor binding poses. Applications of broad C3 β-*O*-glycosylation activity were exemplified via the production of antibody-saponin conjugates as well as the *in vivo* microbial biosynthesis and the *in vitro* biosynthesis of advanced QS-21 intermediates. Together, GuUGT73F15 is a versatile biocatalytic tool that can be utilized to produce libraries of high-value saponin natural products and new-to-nature saponins.

SUBJECTS

Natural Products, New-to-Nature Products, Glycoscience, Glycodiversification, Enzymology, Biocatalysis, Enzymatic Synthesis, Synthetic Biology, Saponin, Triterpene, Sterol

INTRODUCTION

Saponins are an extensive class of plant and marine natural products with triterpene or sterol cores that are decorated with oxidations, glycosylations, and sometimes other modifications like acylation or esterification.[1,2] Many saponins exhibit potent bioactivity that can be utilized for anticancer (e.g., glycyrrhizin), immunomodulatory (e.g., QS-21 and QS-7), antiarrhythmic (e.g., digitoxin and digoxin), antifungal (e.g., phytolaccoside B), antihyperlipidemic (e.g., trillin), antimicrobial (e.g., nudicaucin A), antiparasitic (e.g., avenacoside B), antiviral (e.g., α-tomatine), and insecticidal (e.g., α-solanine) applications.[3–5] Therefore, saponins and their precursors/derivatives/analogs are frequent targets for chemical (semi-)synthesis[6,7] (e.g., QS-21,[8] digitoxin,[9] and digoxin[10]), biosynthetic pathway discovery[11] (e.g., saponariosides A and B,[12] QS-21,[13] and QS-7[14]), and microbial biosynthesis[15–18] (e.g., glycyrrhizin,[19] QS-21,[20] diosgenin [dioscin precursor],[21] and verazine [cycloposine precursor][22]).

The bioactivity of structurally related saponins can be compared to identify the structure–activity relationships that govern their pharmacological properties including potency, toxicity, solubility, pharmacokinetics, and pharmacodynamics.[23] In one example, glycyrrhizin, a *Glycyrrhiza* spp. plant-produced saponin that is a natural food sweetener and is generally regarded as safe (GRAS) by the FDA, also exhibits anticancer activity *in vitro* (**Figure 1a**).[24] Changing the chemical decorations on glycyrrhizin can lead to emergent bioactivity; methylating the C30 carboxyl group and replacing the C3 glucuronic acid disaccharide with a mannose monosaccharide improved the anticancer properties of this saponin (**Figure 1b**).[25] In another example, QS-21 is a *Quillaja saponaria* plant-produced saponin that is an FDA-approved adjuvant used in vaccines for malaria (GSK's Mosquirix), shingles (GSK's Shingrix), respiratory syncytial virus (GSK's Arexvy), and COVID-19 (Novavax's COVID-19) (**Figure 1c**).[26] The chemical semi-synthesis of QS-21 analogs decorated with different oxidations, glycosylations, and acylations resulted in the discovery that the adjuvant activity of QS-21 arises from only a subset of its native decorations (**Figure 1d**),[27] forming a "core pharmacophore" that is simpler to (bio)synthesize than QS-21. In a final example, digitoxin (Digalen/Digitaline/Digitmerck) and digoxin (Digitek/Lanoxicaps/Lanoxin) are *Digitalis* spp. plant-produced saponins that are FDA-approved treatments for heart failure, although the use of these drugs is restricted by an extremely narrow

therapeutic window (**Figure 1e**).[28] Small changes to the digoxin/digitoxin chemical structure like adding a C12 hydroxylation on digoxin led to a different *in vivo* half-life and route of metabolism/excretion.[29] This observation motivated the chemical semi-synthesis of digitoxin analogs with different C3 sugars;[30] notably, replacing the C3 digoxose trisaccharide on digitoxin with a rhamnose monosaccharide resulted in improved potency for antiproliferative and apoptosis-inducing bioactivity (**Figure 1f**).[31] These examples highlight the significant value of exploring the chemical space of saponins through diversification of their C3 saccharides.

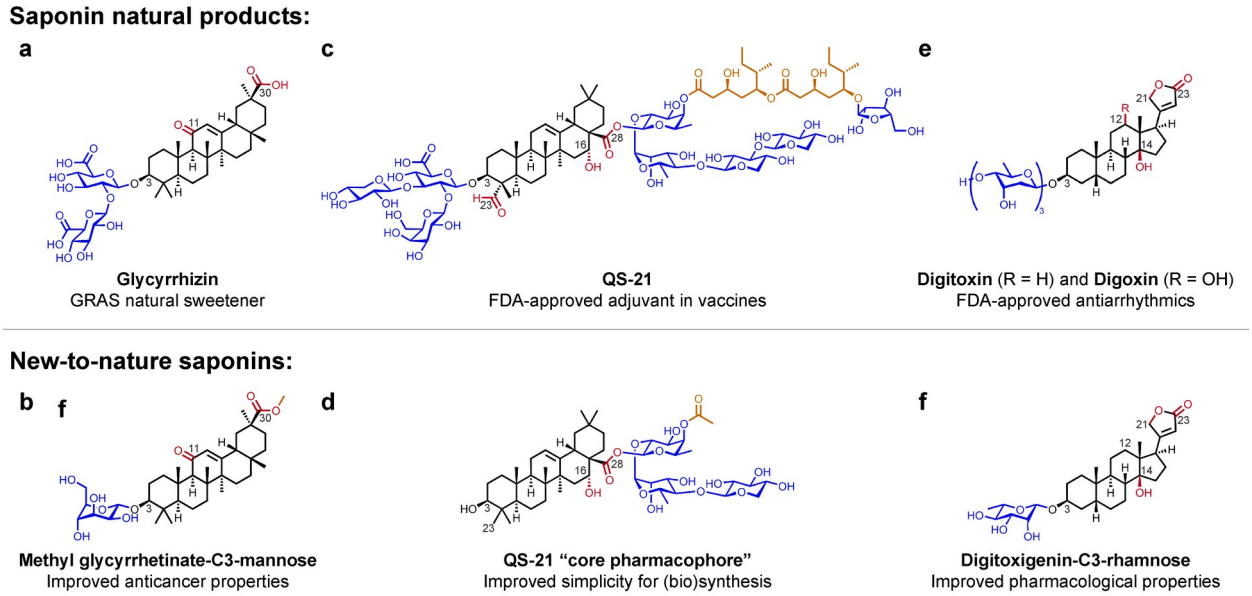

Figure 1. Chemical structures for representative saponin natural products and their new-to-nature analogs where triterpenes/sterols (black) are decorated with oxidations (red), glycosylations (blue), and other modifications (orange). Saponin natural products like (a) glycyrrhizin, (c) QS-21, and (e) digitoxin/digoxin can be modified into new-to-nature saponins with improved properties like (b) methyl glycyrrhetinate-C3-mannose, (d) the QS-21 "core pharmacophore", and (f) digitoxigenin-C3-rhamnose. Select carbons in the chemical structures are labeled with their carbon numbering (gray).

Chemical (semi-)synthesis methods can be used to form *O*-,[32] *N*-,[33] *C*-,[34] and *S*-glycosidic[35] bonds with different composition (i.e., sugar acceptor identity and sugar donor identity), connectivity (i.e., 1 → 2, 1 → 3, etc.), and configuration (i.e., α-linkage or β-linkage).[6,7] The selectivity of chemical glycosylation reactions is impacted by many factors including sugar acceptor identity/protection/activation, sugar donor identity/protection/activation, catalyst,

solvent, and temperature.[36,37] Many complex glycans and glycosides can be produced via one-pot synthesis[38] and automated solid-phase synthesis,[39] but there is no universal method for chemical glycosylation. In particular, chemical (semi-)synthesis can be used to form the glycosidic bonds in saponins (**Figure 2a**);[27,30,40,41] however, the selective synthesis of target saponins is complicated by the multiple nucleophilic functional groups on many triterpenes/sterols and sugars.[42,43] Therefore, the extensive tailoring and use of orthogonal protecting groups is required to achieve the chemical (semi-)synthesis of each glycosidic bond in each saponin, although recent progress has been made towards addressing this challenge.[44]

In Nature, some glycosyltransferases (GTs) have evolved to install *O*-, *N*-, *C*-, and *S*-glycosidic bonds between triterpenes/sterols (sugar acceptors) and activated sugars (sugar donors) to produce saponins (**Figure 2b**). The most common sugar donors in plant biosynthetic pathways for saponins are uridine diphosphate sugars (UDP-sugars).[45] Saponin-producing GTs are generally members of the carbohydrate-active glycosyltransferase 1 (GT1) enzyme family,[46] and, notably, they often exhibit stringent substrate specificity, frequently recognizing only a single UDP-sugar donor.[47] Organisms leverage this biosynthetic logic to regulate the flux and timing of glycosylation reactions and control the conversion of triterpene/sterol aglycones into either exact saponins or varied sets of saponins.[14,48] The selectivity of GT1s can be utilized in chemoenzymatic synthesis[49,50] or heterologous microbial/plant biosynthesis.[13,14,20] However, the stringency of GT1s may also provide an inherent challenge to efforts to generate new-to-nature saponins where triterpenes/sterols have non-native glycosylation patterns. The challenge of producing new-to-nature saponins is further complicated by the limited understanding of GT1 substrate recognition at the structural biology level.[51] Consequently, the enzymatic (bio)synthesis of each new-to-nature saponin often requires the discovery of novel GT1s from Nature[52] or the laboratory-based engineering[53]/evolution[54] of GT1s.

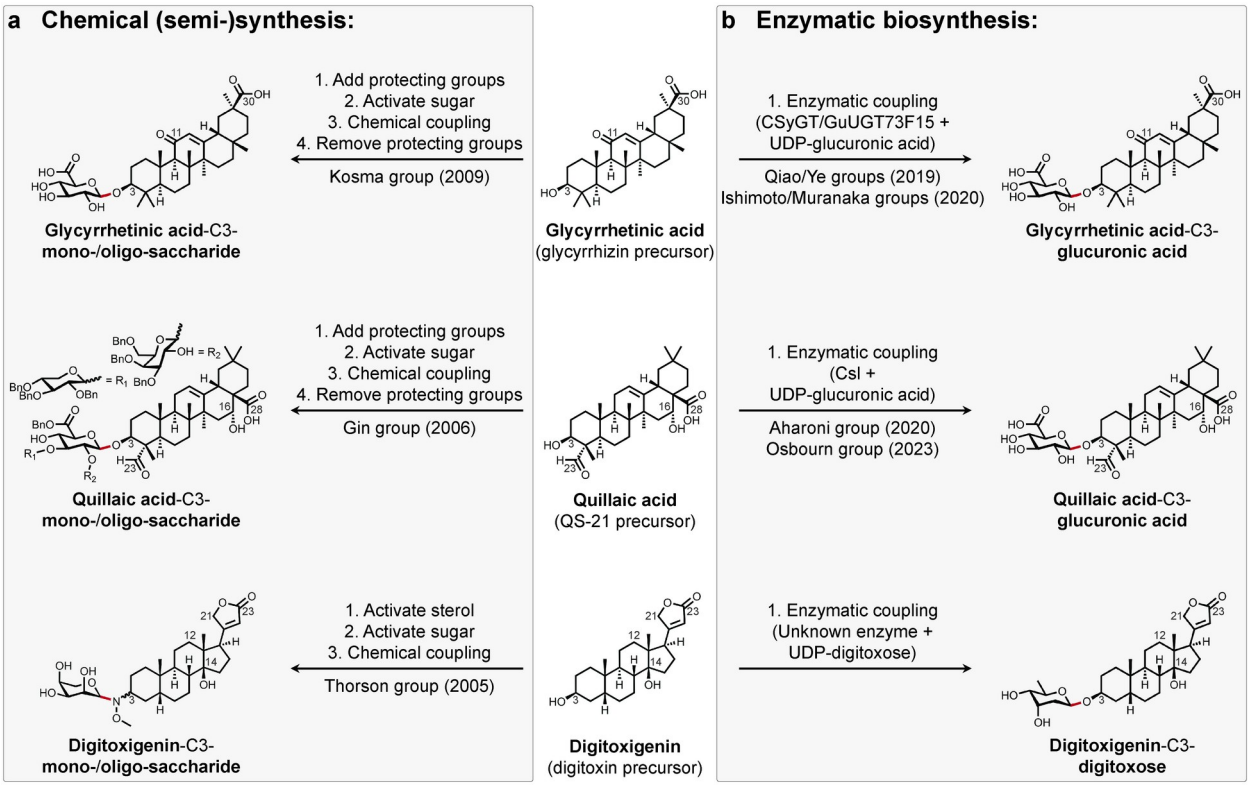

Figure 2. Different saponins can be produced by functionalizing triterpenes/sterols with C3 (oligo)saccharides via (a) chemical (semi-)synthesis[55–57] and (b) enzymatic biosynthesis.[14,19,58,59] Representative examples of saponin (bio)synthesis are included for glycyrrhetinic acid (top), quillaic acid (middle), and digitoxigenin (bottom). In the saponin chemical structures, C3 β-*O*-glycosidic bonds (and analogous C3 N-glycosidic bonds) are highlighted in red and select carbons are numbered in gray. Accessing each new saponin or set of saponins often requires significant method development such as the tailoring of protecting/activating groups or discovery/engineering/evolution of glycosyltransferases. Abbreviation: Bn = benzyl group.

Herein, this report describes the discovery that the GT1 family enzyme GuUGT73F15 (*Glycyrrhiza uralensis*) can be utilized to produce libraries of saponins via triterpene/sterol C3 β-*O*-glycosylation. Beyond its previously reported activity as a glycyrrhetinic acid C3 β-*O*-glucuronosyltransferase, GuUGT73F15 utilized 21 additional sugar acceptors (i.e., C3 hydroxyl-containing triterpenes/sterols) and 11 additional sugar donors (i.e., UDP-sugars) in saponin biosynthesis. In total, 130 unique monoglycosylated saponins were produced (**Supplementary Text 1**; **Supplementary Data 1**) and, to the best of our knowledge, more than 100 of these saponins are new-to-nature. The flexible substrate stringency of GuUGT73F15 was studied by analyzing Boltz-2x predicted protein structure and substrate/product dockings. GuUGT73F15 was then applied to

produce high-value saponins. Glycosylating a sterol with an azido sugar using GuUGT73F15 enabled the synthesis of a novel antibody-drug conjugate. This enzyme was also utilized in the microbial *in vivo* biosynthesis and the *in vitro* biosynthesis of the advanced QS-21 intermediates 3-*O*-{β-D-glucopyranosiduronic acid}-quillaic acid and 3-*O*-{β-D-xylopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-glucopyranosiduronic acid}-quillaic acid, respectively. As a whole, this report describes how GuUGT73F15 is a versatile biocatalytic tool that enables the rapid exploration of saponin chemical space as well as the future discovery of the structure–activity relationships that govern their pharmacological properties.

RESULTS AND DISCUSSION

Enzymatic Biosynthesis of Saponin Natural and New-to-Nature Products Using GuUGT73F15

The preliminary goal of this work was to increase the production of QS-21 in engineered *Saccharomyces cerevisiae* by resolving a metabolic bottleneck at the C3 β-*O*-glucuronosylation of quillaic acid.[20] This biosynthetic step is currently performed in yeast by cellulose synthase-like (Csl) enzymes from *Quillaja saponaria*.[13,14] It remains challenging to increase the activity of Csl enzymes through engineering or evolution campaigns because they are integral transmembrane proteins. Therefore, this work aimed to substitute Csl enzymes with a cytosolic C3 β-*O*-glucuronosyltransferase from the biosynthetic pathway for another saponin. GuUGT73F15 (also known as GuGT14) was selected as a promising candidate because it contains no predicted transmembrane domain and can catalyze the C3 β-*O*-glucuronosylation of glycyrrhetinic acid, a triterpene that bears considerable structural similarity to quillaic acid, during glycyrrhizin biosynthesis in *Glycyrrhiza uralensis*.[59] Furthermore, GuUGT73F15 accepts a wide scope of triterpenoids, flavones, and chalcones as sugar acceptors,[59] and the GuUGT73F15 homolog GuUGT73F13 has been utilized in heterologous saponin biosynthesis in *S. cerevisiae*.[19]

To start, GuUGT73F15 functionalized with N-terminal maltose-binding protein (MBP) tag was expressed in *Escherichia coli* and isolated using amylose affinity chromatography (**Supplementary Figures S1** and **S145**). The activity of GuUGT73F15 for its previously reported sugar acceptor glycyrrhetinic acid (**1**) and sugar donor UDP-D-glucuronic acid (UDP-**A**)[59] was

studied via *in vitro* glycosylation reactions. Reactions were monitored using reverse phase-high-performance liquid chromatography-ultraviolet absorbance spectroscopy/mass spectrometry (LC-UV/MS), where substrates/products were identified using MS and percent conversions were quantified using UV. The addition of GuUGT73F15 (10% mol) to **1** (1 equiv.) and excess UDP-**A** (12.5 equiv.) resulted in the monoglycosylation of **1** with **A** (**1**-**A**) (**Figure 3a**; **Supplementary Figures S2*ii*** and **S146*i***). The reaction conversion from **1** and UDP-**A** to **1**-**A** was estimated to be 100% by dividing the UV absorbance area for **1**-**A** by the sum of the UV absorbance areas for **1** and **1**-**A**. To facilitate this reaction on a preparatory scale and enable structural characterization to verify the site of glycosylation, the effect of both enzyme and UDP-**A** concentration on conversion efficiency were independently assayed while the other was held constant at 10% mol GuUGT73F15 or 2.5 mM UDP-**A** (**Supplementary Figure S2d**,**e**). Full conversion was only observed in conditions with ≥ 5% mol GuUGT73F15 or ≥ 1.25 mM UDP-**A**. Optimized reaction conditions utilizing 5% mol GuUGT73F15 and 1 mM UDP-**A** resulted in 95% conversion (**Supplementary Figure S2f**). Utilizing these optimized reaction conditions, **1**-**A** was produced at a ~10 mg preparatory scale and isolated using high-performance liquid chromatography (HPLC). and **1**-**A** were then characterized by nuclear magnetic resonance (NMR) spectroscopy (**Supplementary Figures S44**–**S50** and **S51**–**S57**, respectively; **Supplementary Table S7**), and a β-*O*-glycosidic bond was observed in **1**-**A** between the C3 hydroxyl group on **1** and the C1' anomeric carbon on **A** (**1**-C3-**A**). Therefore, soluble GuUGT73F15 can catalyze the C3 β-O-glucuronosylation of triterpenes.

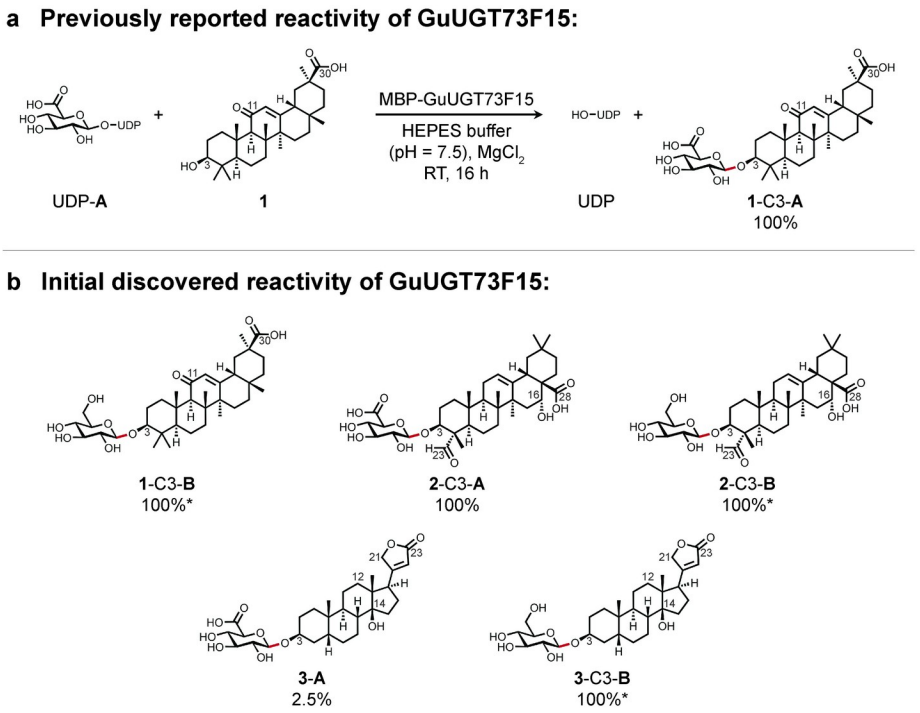

Figure 3. The activity of GuUGT73F15 was characterized via *in vitro* glycosylation reactions with (a) its previously reported substrates and (b) other substrates. In the saponin chemical structures, C3 β-*O*-glycosidic bonds are highlighted in red and select carbons are numbered in gray. The saponin name abbreviation (top) and the reaction percent conversion (bottom) are listed below the saponin chemical structures. The following reaction conditions were used: 2.5 mM UDP-sugar, 200 μM triterpene/sterol, 20 μM MBP-tagged GuUGT73F15, 50 mM HEPES (pH = 7.5), and 2 mM $MgCl_2$ for 16 h at room temperature. Reaction mixtures were characterized by LC-UV/MS and percent conversions for reactions were quantified from absorbances at λ = 215 nm. *Reactions where some products that contain multiple glycosylations were observed.

The substrate scope of GuUGT73F15 was explored next to discover the feasibility of implementing this enzyme in QS-21-producing *S. cerevisiae*. An *in vitro* glycosylation reaction of GuUGT73F15 with quillaic acid (**2**) and UDP-**A** resulted in the production of the monoglycosylated saponin **2**-**A** with 100% conversion (**Figure 3b**; **Supplementary Figure S3*ii*** and **S147*i***). Utilizing the same optimized reaction conditions as was done for **1**-C3-**A**, **2**-**A** was prepared on the ~10 mg preparatory scale and purified by HPLC. NMR characterization of **2** (**Supplementary Figures S65–S71**; **Supplementary Table S8**) and **2**-**A** (**Supplementary Figures S72–S78**; **Supplementary Table S8**) enabled the identification of a C3 β-*O*-glycosidic bond in **2**-**A** between **2** and **A** (**2**-C3-**A**). This result shows that GuUGT73F15 exhibits the target enzymatic activity; however, the utilization of this enzyme in QS-21-producing yeast requires that

it is selective for **2** and UDP-**A** in the presence of many triterpenes/sterols (i.e., ergosterol, β-amyrin, erythrodiol, oleanolic acid, hederagenin, and gypsogenin) and UDP-sugars (i.e., UDP-D-glucose, UDP-D-galactose, UDP-D-xylose, UDP-L-arabinopyranose, UDP-L-arabinofuranose, UDP-L-rhamnose, UDP-D-fucose, UDP-4-keto-6-deoxy-D-glucose, and UDP-D-apiose), respectively.[20] Replacing UDP-**A** with UDP-D-glucose (UDP-**B**) in the glycosylation reaction resulted in the biosynthesis of the monoglycosylated saponin **2**-**B** and diglycosylated saponin **2**-(**B**)$_2$ with 100% consumption of **2** (**Figure 3b**; **Supplementary Figures S3*iii*** and **S147*ii***). Previous *in vitro* studies of GuUGT73F15 have shown a large excess of UDP-**B** can elicit the installation of disaccharides on select acceptors.[59] Therefore, the effects of both enzyme loading and UDP-**B** concentration on product distribution were independently assayed. Full consumption of **2** was observed with ≥ 1% mol GuUGT73F15 and ≥ 312 μM UDP-**B** (**Supplementary Figure S3d**,**e**). In contrast to previous investigations reporting that GuUGT73F15 functions naturally as a glucuronosyltransferase, these data suggest that GuUGT73F15 likely recognizes a different cognate sugar (possibly UDP-**B**). Furthermore, the **2**-(**B**)$_2$:**2**-**B** ratio was positively correlated with increasing amounts of GuUGT73F15 or UDP-**B**; this suggests that the production of the diglycosylated product is dependent on enzyme efficiency (i.e., k_{cat}) and substrate binding (i.e., K_M). Optimizing the reaction conditions to utilize only 1% mol GuUGT73F15 and 400 μM UDP-**B** led to complete consumption of **2** and almost exclusive production (94%) of monosaccharide product **2**-**B** (**Supplementary Figure S3f**). Utilizing these optimized reaction conditions, the enzymatic synthesis of **2**-**B** was carried out on the ~10 mg preparatory scale and **2**-**B** was purified by HPLC. Similar to **2**-C3-**A**, NMR characterization of **2**-**B** revealed glucosylation occurred at the C3 hydroxyl group on **2** (**2**-C3-**B**) (**Supplementary Figures S79–S85**; **Supplementary Table S8**). Together, these results suggest that it may be challenging to utilize GuUGT73F15 for *in vivo* production of **2**-**A** without additional engineering/evolution to impart sufficient stringency to solely utilize UDP-**A** in the presence of other UDP-sugars.

While these data suggest that the native GuUGT73F15 enzyme is unsuitable for the *in vivo* production of **2**-C3-**A** as originally envisioned, the observed *in vitro* activity of GuUGT73F15 motivated further exploration of its substrate scope. An *in vitro* glycosylation reaction of GuUGT73F15 with **1** and UDP-**B** resulted in the biosynthesis of monoglycosylated saponin **1**-**B**

and diglycosylated saponin **1**-(**B**)$_2$ with 100% consumption of **1** (**Figure 3b**; **Supplementary Figures S2*iii*** and **S146*ii***). Utilizing the same optimized reaction conditions for the ~10 mg scaled production of **2**-C3-**B**, the enzymatic synthesis of **1**-**B** was scaled and the product purified by HPLC. Similar to **1**-C3-**A**, **2**-C3-**A**, and **2**-C3-**B**, structural characterization by NMR confirmed that **1**-**B** contained a C3 β-*O*-glycosidic bond (**1**-C3-**B**) (**Supplementary Figures S58–S64**; **Supplementary Table S7**). Both **1** and **2** are triterpenoids biosynthetically derived from β-amyrin and feature identical pentacyclic carbon backbone structures and therefore encompass a relatively small structural perturbation with regard to acceptor binding to GuUGT73F15. Other UGT73 family C3 β-*O*-glycosyltransferases are reported to exhibit activity with sterols/steroids,[60] so the activity of GuUGT73F15 with digitoxigenin (**3**) as a sugar acceptor was studied next. The addition of GuUGT73F15 to **3** and UDP-**A** produced the monoglycosylated saponin **3**-**A** with 2.5% conversion (**Figure 3b**, **Supplementary Figures S4*ii*** and **S148*i***) and its addition to **3** and UDP-**B** resulted in the production of the monoglycosylated saponin **3**-**B** and the diglycosylated saponin **3**-(**B**)$_2$ with 100% consumption of **3** (**Figure 3b**, **Supplementary Figures S4*iii*** and **S148*ii***). The observed difference in conversion efficiency utilizing UDP-**A** with structurally distinct acceptors (i.e., **1** or **2** vs. **3**) suggests that the binding of each reactant impacts the efficiency of GuUGT73F15, possibly mediated by perturbation of its active site architecture. As done previously with acceptors **1** and **2**, the effect of GuUGT73F15 and UDP-**B** concentration on production distribution and conversion efficiency was tested. Consistent with observations for **1** and **2**, lower enzyme and UDP-**B** loading resulted in efficient conversion to **3**-**B** while decreasing production of **3**-(**B**)$_2$ (**Supplementary Figure S4d**,**e**,**f**). Utilizing identical reaction conditions for the scaled production of **1**-C3-**B** and **2**-C3-**B**, the enzymatic synthesis of **3-B** was performed at the ~10 mg preparatory scale and the product purified by HPLC. NMR characterization of **3** (**Supplementary Figures S86–S92**; **Supplementary Table S9**) and **3**-**B** (**Supplementary Figures S93–S99**; **Supplementary Table S9**) led to the identification of a C3 β-*O*-glycosidic bond between **3** and **B** (**3**-C3-**B**). Together, GuUGT73F15 catalyzed C3 β-*O*-glycosylation with three different triterpenes/sterols and two different UDP-sugars to produce two saponin natural products (i.e., **1**-C3-**A** [glycyrrhizin precursor] and **2**-C3-**A** [QS-21 precursor]) as well as four new-to-nature saponins (i.e., **1**-C3-**B**, **2**-C3-**B**, **3**-**A**, and **3**-C3-**B**).

Acceptor and UDP-sugar Donor Substrate Scope of GuUGT73F15

The substrate scope of GuUGT73F15 was further explored with a varied set of triterpene/sterol natural products from commercial vendors. These triterpenes/sterols included oleananes (i.e., echinocystic acid [**4**], gypsogenin [**5**], hederagenin [**6**], and oleanolic acid [**7**]); ursanes (i.e., ursolic acid [**8**]); sterols (i.e., alphadolone acetate [**9**], caudatin [**10**], epiandrosterone [**11**], estradiol [**12**], and qingyangshengenin [**13**]); and steroidal alkaloids (i.e., cyclopamine [**14**], ebeiedinone [**15**], solanidine [**16**], solasodine [**17**], teinemine [**18**], tomatidine [**19**], verazine [**20**], yibeissine [**21**], and zhebeirine [**22**]) (**Figure 4a**; **Supplementary Table S1**). *In vitro* glycosylation reactions with GuUGT73F15 were set up with each individual triterpene/sterol and UDP-**B**. In addition to **1**-**B**, **2**-**B**, and **3**-**B** (described above), these reactions produced the monoglycosylated saponins **4**-**B** (**Supplementary Figure S5*ii***), **5**-**B** (**Supplementary Figure S6*ii***), **6**-**B** (**Supplementary Figure S7*ii***), **7**-**B** (**Supplementary Figure S8*ii***), **8**-**B** (**Supplementary Figure S9*ii***), **9**-**B** (**Supplementary Figure S10*ii***), **10**-**B** (**Supplementary Figure S11*ii***), **11**-**B** (**Supplementary Figure S12*ii***), **12**-**B** (**Supplementary Figure S13*iii***), **13**-**B** (**Supplementary Figure S14*iii***), **14**-**B** (**Supplementary Figure S15*iii***), **15**-**B** (**Supplementary Figure S16*iii***), **16**-**B** (**Supplementary Figure S17*ii***), **17**-**B** (**Supplementary Figure S18*iii***), **18**-**B** (**Supplementary Figure S19*ii***), **19**-**B** (**Supplementary Figure S20*iii***), **20**-**B** (**Supplementary Figure S21*iii***), **21**-**B** (**Supplementary Figure S22*ii***), and **22**-**B** (**Supplementary Figure S23*iii***) as well as the diglycosylated saponins **1**-(**B**)$_2$, **2**-(**B**)$_2$, **3**-(**B**)$_2$, and **12**-(**B**)$_2$. Importantly, GuUGT73F15 glycosylated all 22 structurally varied triterpenes/sterols that were screened. These triterpenes/sterols contained different numbers of rings (i.e., 4 [**9**–**12**], 5 [**1**–**8**, **13**, **18**, and **20**], and 6 [**14**–**17**, **19**, and **21**–**22**]), ring sizes, and heterocyclization (i.e., *N*-heterocycles [**14**–**22**] and *O*-heterocycles [**3**, **14**, **17**, **19**, and **21**]). They also have different 3-dimensional geometry (i.e., planar [**12**] vs. non-planar [**1**–**11** and **13**–**22**] A ring, C5–C6 alkane [**1**–**9**, **11**–**12**, **15**, **19**, and **21**–**22**] vs C5–C6 alkene [**10**, **13**–**14**, **16**–**18**, and **20**] on the B ring, and different C–F ring cyclizations) and oxidation/amination numbers/locations. The number of different triterpenes/sterols that were studied was only limited by the availability of triterpenes/sterols from commercial vendors that were easily characterized by LC-UV/MS. Therefore, the substrate scope of GuUGT73F15 for triterpene/sterol sugar acceptors is expected to extend further.

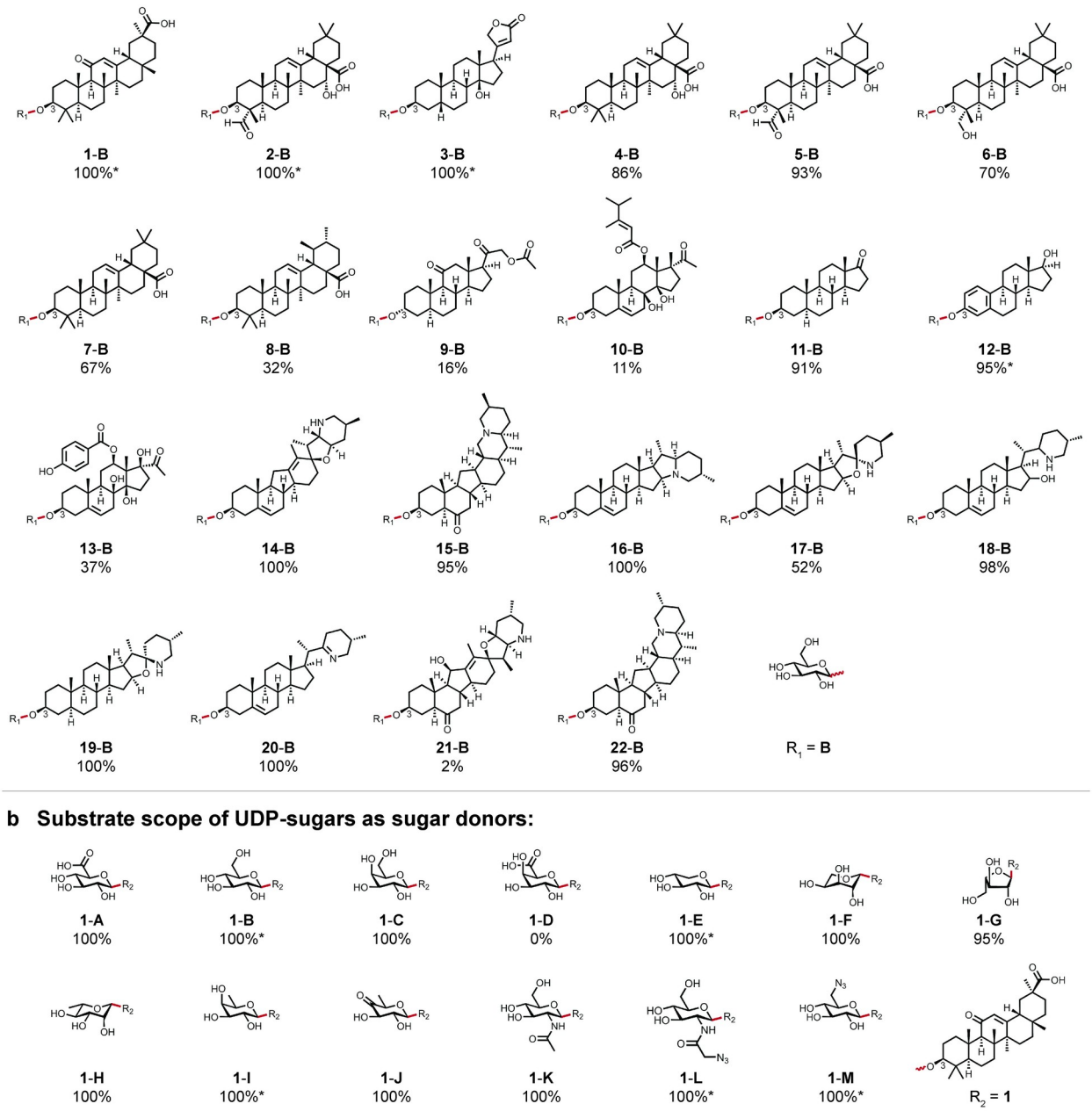

Figure 4. The substrate scope of GuUGT73F15 for (a) triterpenes/sterols as sugar acceptors and (b) UDP-sugars as sugar donors was screened via *in vitro* glycosylation reactions with UDP-D-glucose (UDP-**B**) and glycyrrhetinic acid (**1**), respectively. In the saponin chemical structures, C3 β-*O*-glycosidic bonds are highlighted in red and select carbons are numbered in gray. The saponin name abbreviation (top) and the reaction percent conversion (bottom) are listed below the saponin chemical structures. The following reaction conditions were used for conversion reactions: 2.5 mM UDP-sugar, 200 μM triterpene/sterol, 20 μM MBP-tagged GuUGT73F15, 50 mM HEPES buffer (pH = 7.5), and 2 mM $MgCl_2$ for 16 h at room temperature. Reaction mixtures were characterized by LC-UV/MS and percent conversions for reactions were quantified from absorbances at λ = 215 nm (percent conversions for reaction mixtures containing **11**, **15**,

19, and **22** were estimated using total ion chromatograms (TICs) because these triterpenes/sterols do not exhibit absorbance at λ = 215 nm). *Reactions where some products that contain multiple glycosylations were observed.

The substrate scope of GuUGT73F15 was also screened against a varied set of UDP-sugars that were predominantly sourced from commercial vendors. This included UDP-D-galactose (UDP-**C**), UDP-D-galacturonic acid (UDP-**D**), UDP-D-xylose (UDP-**E**), UDP-L-arabinopyranose (UDP-**F**), UDP-L-arabinofuranose (UDP-**G**), UDP-L-rhamnose (UDP-**H**), UDP-D-fucose (UDP-**I**), UDP-4-keto-6-deoxy-D-glucose (UDP-**J**), UDP-*N*-acetyl-D-glucosamine (UDP-**K**), UDP-*N*-azidoacetyl-D-glucosamine (UDP-**L**), and UDP-6-azido-6-deoxy-D-glucose (UDP-**M**) (**Figure 4b**; **Supplementary Table S1**). In addition to **1**-**A** and **1**-**B** (described above), *in vitro* glycosylation reactions of GuUGT73F15 with **1** and each UDP-sugar produced the monoglycosylated saponins **1**-**C** (**Supplementary Figure S2*iv***), **1**-**E** (**Supplementary Figure S2*vi***), **1**-**F** (**Supplementary Figure S2*vii***), **1**-**G** (**Supplementary Figure S2*viii***), **1**-**H** (**Supplementary Figure S2*ix***), **1**-**I** (**Supplementary Figure S2*x***), **1**-**J** (**Supplementary Figure S2*xi***), **1**-**K** (**Supplementary Figure S2*xii***), **1**-**L** (**Supplementary Figure S2*xiii***), and **1**-**M** (**Supplementary Figure S2*xiv***) as well as the diglycosylated saponins **1**-(**B**)$_2$, **1**-(**E**)$_2$, **1**-(**I**)$_2$, **1**-(**L**)$_2$, and **1**-(**M**)$_2$. The 12 different UDP-sugars utilized by GuUGT73F15 to produce monoglycosylated saponins included many of the most common natural UDP-sugars as well as two azido sugars. Based on the observed endpoint conversions and the lack of observed activity with UDP-**D** (**Supplementary Figure S2*v***), the enzyme is most efficient with UDP-sugars that are not uronic acids and feature equatorial C4 hydroxyl groups.

A comprehensive pairwise screen of GuUGT73F15 with 11 different triterpenes/sterols (i.e., **1**, **2**, **3**, **12**, **13**, **14**, **15**, **17**, **19**, **20**, and **22**) and 11 different UDP-sugars (i.e., UPD-**A**, UDP-**B**, UDP-**C**, UDP-**E**, UDP-**F**, UDP-**H**, UDP-**I**, UDP-**J**, UDP-**K**, UDP-**L**, and UDP-**M**) was performed (**Figure 5**, **Supplementary Figures S2**, **S3**, **S4**, **S13**, **S14**, **S15**, **S16**, **S18**, **S20**, **S21**, and **S23**) and resulted in the enzymatic monoglycosylation to synthesize 115 unique monoglycosylated saponins. The available amounts of UDP-**D** and UDP-**G** were limited, so the activity of GuUGT73F15 with these sugars were only screened while paired with **1** (described above), **2**, **3**, and **20**. While no activity was observed when UDP-**D** was used (**Supplementary Figures S2*v***,

S3*v*, **S4*v***, and **S21*v***), GuUGT73F15 produced **1-G** (described above), **2-G** (**Supplementary Figure S3*viii***), **3-G** (**Supplementary Figure S4*viii***), and **20-G** (**Supplementary Figure S21*viii***). When counted with the products described above, 130 unique monoglycosylated saponins were produced (**Supplementary Text 1**; **Supplementary Data 1**), including more than 100 new-to-nature saponins that have, to the best of our knowledge, not previously been reported as natural products. This extremely varied set of saponins could comprise a library for pharmacological screening. Further screening of GuUGT73F15 with additional triterpenes/sterols as sugar acceptors and UDP-sugars as sugar donors is expected to significantly expand this saponin library. The exceptionally broad substrate scope of GuUGT73F15 indicates that this enzyme would be a valuable starting point for engineering/evolution campaigns to expand/narrow its reactivity and modulate its kinetics.

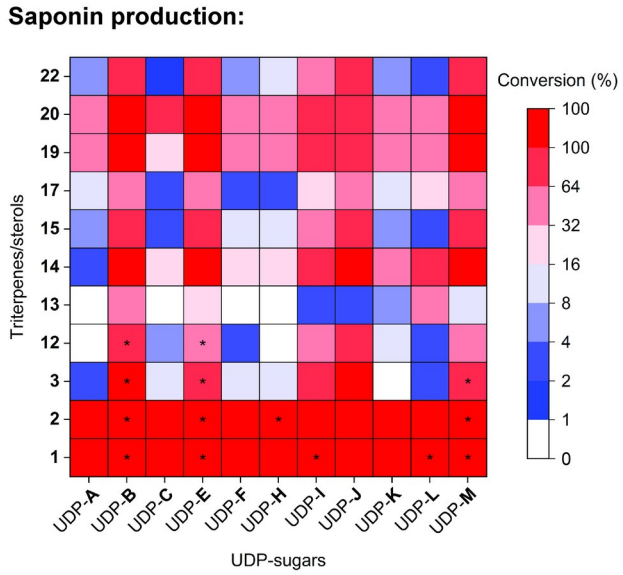

Figure 5. Heat map of percent conversions for reactions from *in vitro* glycosylation reaction with GuUGT73F15 and a pairwise screen of triterpenes/sterols and UDP-sugars. Values for entries in the heat map can be found in **Supplementary Table S6**. Reaction mixtures were characterized by LC-UV/MS and percent conversions for reactions were quantified from absorbances at λ = 215 nm (percent conversions for reaction mixtures containing **15**, **19**, and **22** were estimated using TICs or extracted ion chromatograms (EICs) because these triterpenes/sterols do not exhibit absorbance at λ = 215 nm). *Reactions where some products that contain multiple glycosylations were observed.

Investigating the regioselectivity and multiple glycosylations of GuUGT73F15

All triterpene/steroidal sugar acceptors that were tested in this work contain a C3-OH group. While this group is the *de facto* glycosylation site for sugar acceptors lacking additional suitably nucleophilic moieties (i.e., **9**, **11**, and **14**–**22**), other sugar acceptors possess plausible locations for *O*-glycosylation (i.e., **1**–**8**, **10**, **12**, and **13**). Structural elucidation of **1**-C3-**A**, **1**-C3-**B**, **2**-C3-**A**, **2**-C3-**B**, and **3**-C3-**B** suggests that GuUGT73F15 preferentially *O*-glycosylates the C3 position irrespective of other nucleophilic moieties. To further verify this observation, the *O*-glucosides of **6** (bearing a proximal C23-OH), **12** (bearing a C17-OH that is roughly symmetrical with the C3-OH along the central axis of the acceptor), and **13** (bearing three additional tertiary OH as well as a phenolic OH) were structurally characterized. Compared to **6** (**Supplementary Figures S100–S106**; **Supplementary Table S10**) and **13** (**Supplementary Figures S129–S135**; **Supplementary Table S12**), NMR spectroscopy of **6**-C3-**B** (**Supplementary Figures S107–S113**; **Supplementary Table S10**) and **13**-C3-**B** (**Supplementary Figures S136–S142**; **Supplementary Table S12**) revealed that both saponin products featured C3 *β*-*O*-glycosidic bonds.

In contrast with other tested acceptors where the monoglycoside is the prevalent product, the predominant product of **12,** UDP-**B**, and GuUGT73F15 was a saponin with a mass corresponding to the addition of three glucose residues. The chemical structure of this unique, multiglycosylated product was investigated using NMR spectroscopy. Compared to **12** (**Supplementary Figures S114–S120**; **Supplementary Table S11**), **12**-(**B**)$_3$ (**Supplementary Figures S121–S128**; **Supplementary Table S11**) contained a C3 β-*O*-glycosidic bond and C17-OH diglucosylation bearing a 4→1 linkage (**12**-C3-**B**-C17-**B**-(4←1)-**B**). LC-MS/MS analysis of the comparatively trace mono- and di-glucosylated products suggest that β-*O*-glycosylation occurs first at C17-OH and second at C3-OH (**Supplementary Figures S143**). The unique glycoside product distribution of **12** by GuUGT73F15 may reflect the quasi-symmetry of the aglycone acceptor with respect to hydroxyl groups as well as the reduced nucleophilicity of phenolates compared to alkoxides while the installation of the C17-OH disaccharide may arise from the C17-OH monosaccharide adopting an overall structure that is reminiscent of pentacyclic acceptors.

Beyond **12**, **3** was the only other tested sugar acceptor to show appreciable production of multiglycosylated products by GuUGT73F15. This multiple glycosylation was readily suppressible by reducing both the amount of catalyst loading and UDP-sugar during scaled production pursuant to structural elucidation, which determined the product to be **3**-C3-**B**. Therefore, it was reasoned that extrapolating this trend in the opposite direction (i.e., increasing catalyst loading and adding more equivalents of UDP-sugar) could afford increased production of **3**-(**B**)$_2$. Reacting **3** with 5 mM UDP-**B** in the presence of 20% mol GuUGT73F15 resulted in production of an approximately 1:1 ratio of **3**-(**B**)$_1$:**3**-(**B**)$_2$ (**Supplementary Figure S144*i***). To probe the nature of the diglycoside product **3**-(**B**)$_2$ without necessitating scaled production and structural elucidation, 14-anhydrodigitoxigenin (**3a**) was tested using identical conditions. In contrast to **3**, only the monoglucosylated product **3a**-C3-**B**, was observed (**Supplementary Figure S144*ii***), suggesting that **3**-(**B**)$_2$ is **3**-C3-**B**-C14-**B**. Taken as a whole, these data confirm that GuUGT73F15 is capable of glycosylating the C3-OH of highly varied triterpene and sterol acceptors; however, special consideration must be given if the target aglycone has features that may discourage fidelitous C3-OH glycosylation such as (quasi-)symmetrical acceptor sites and/or reduced nucleophilicity (as present in the phenolic C3-OH of **12**).

Investigating the minimal sugar acceptor of GuUGT73F15

The substrate scope of GuUGT73F15 was further explored with a varied set of minimized sugar acceptor substrates from commercial vendors, including five cyclohexanol-type molecules (i.e., cyclohexanol [**23**], decahydro-2-naphthol [**25**], decahydro-1-naphthol [**26**], *trans*-decahydro-9-naphthol [**27**], and 1,2,3,4-tetrahydro-1-phenanthrol [**33**]) and nine phenol-type molecules (i.e., phenol [**24**], 5,6,7,8-tetrahydro-2-naphthol [**28**], 2-naphthol [**29**], 1-naphthol [**30**], 2-anthrol [**31**], 2-phenanthrol [**32**], 1-phenanthrol [**34**], 9-phenanthrol [**35**], and 1-pyrenol [**36**]) (**Figure 6**; **Supplementary Table S1**). *In vitro* glycosylation reactions with GuUGT73F15 were set up with each individual sugar acceptor and UDP-**M**. These reactions produced the monoglycosylated products **23**-**M** (**Supplementary Figures S24*ii*** and **S38**), **24**-**M** (**Supplementary Figures S25*ii*** and **S39**), **25**-**M** (**Supplementary Figures S26*ii*** and **S40**), **26**-**M** (**Supplementary Figures S27*ii*** and **S41**), **28**-**M** (**Supplementary Figure S29*ii***), **29**-**M** (**Supplementary Figure S30*ii***), **30**-**M** (**Supplementary Figure S31*ii***), **31**-**M** (**Supplementary Figure S32*ii***), **32**-**M** (**Supplementary**

Figure S33*ii*), **33-M** (**Supplementary Figures S34*ii*** and **S43**), **34-M** (**Supplementary Figure S35*ii***), **35-M** (**Supplementary Figure S36*ii***), and **36-M** (**Supplementary Figure S37*ii***). No product was observed for **27-M** (**Supplementary Figures S28*ii*** and **S42**). Importantly, GuUGT73F15 glycosylated 13 structurally varied sugar acceptors that were screened to produce semi-biosynthetic products. These minimal sugar acceptors contained different numbers of rings (i.e., 1 [**23**–**24**], 2 [**25**–**26** and **28**–**30**], 3 [**31**–**35**], and 4 [**36**]) and 3-dimensional geometry (i.e., planar [**24**, **28**–**32**, and **34**–**36**] vs. non-planar [**23**, **25**–**26**, and **33**] of the A ring. While the enzyme utilized secondary alcohols (**23**–**26** and **28**–**36**), it did not utilize a tertiary alcohol (**27**). These results suggest that the minimal sugar acceptor substrate for GuUGT73F15 is a secondary hydroxyl group on a hydrophobic molecule.

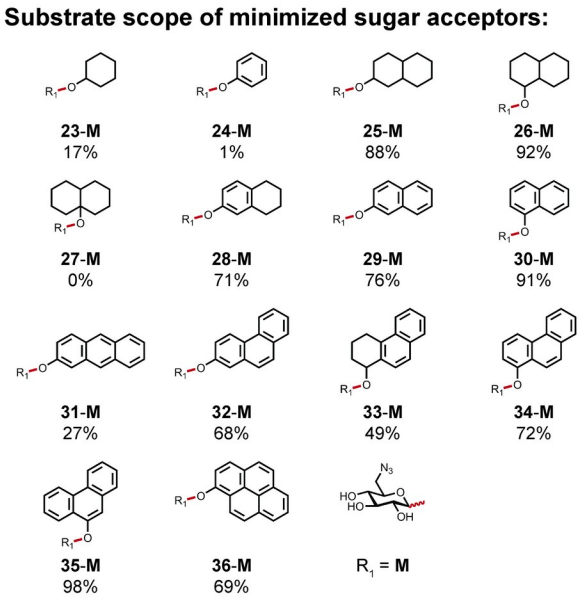

Figure 6. The substrate scope of GuUGT73F15 for minimized sugar acceptors was screened via *in vitro* glycosylation reactions with UDP-6-azido-6-deoxy-D-glucose (UDP-**M**). In the product chemical structures, β-*O*-glycosidic bonds are highlighted in red. The chemical name abbreviation (top) and the reaction percent conversion (bottom) are listed below the chemical structures. The following reaction conditions were used for conversion reactions: 2.5 mM UDP-sugar, 200 μM alcohol, 20 μM MBP-tagged GuUGT73F15, 50 mM HEPES buffer (pH = 7.5), and 2 mM $MgCl_2$ for 16 h at room temperature. Reaction mixtures were characterized by LC-UV/MS and percent conversions for reactions were quantified from absorbances at λ = 280 nm (percent conversions for reaction mixtures containing **23**, **24**, **25**, **26**, **27**, and **33** were estimated using GC-MS).

Investigating the structural basis for the activity of GuUGT73F15

The folding and ligand binding of GuUGT73F15 were studied using Boltz-2x to predict the co-folding GuUGT73F15 with its substrates and products. The triterpenes/sterols **1**–**22** adopted variable binding poses in the GuUGT73F15 active site (**Figure 7a**). Each of **1**–**22** was predicted to bind primarily via hydrophobic interactions to different sets of amino acids, although some hydrogen bonds, salt bridges, π-π stackings, and π-cation stackings were also predicted (**Supplementary Figure S149**; **Supplementary Table S13**). In contrast, the UDP-sugars UDP-**A**–UDP-**M** adopted consistent binding poses in the GuUGT73F15 active site (**Figure 7b**). Similar sets of amino acids were predicted to bind to each of UDP-**A**–UDP-**M** mainly through hydrogen bonds with some salt bridges and π-π stackings (**Supplementary Figure S150**; **Supplementary Table S14**). The minimal sugar acceptors **23**–**36** adopted variable binding poses in the GuUGT73F15 active site (**Figure 7c**). Each of **23**–**36** was predicted to bind primarily via hydrophobic interactions to different sets of amino acids, although some hydrogen bonds, salt bridges, π-π stackings, and π-cation stackings were also predicted (**Supplementary Figure S151**; **Supplementary Table S15**). Boltz-2x was next utilized to predict the ligand binding of the substrates and products for the reactions described in **Figure 3**. For **1**-C3-**A** (**Supplementary Figure S146*i***), **1**-C3-**B** (**Supplementary Figure S146*ii***), **2**-C3-**A** (**Supplementary Figure S147*i***), **3**-C3-**A** (**Supplementary Figure S148*i***), and **3**-C3-**B** (**Supplementary Figure S148*ii***), substrates and products adopted binding poses that suggest a GT1-mediated glycosylation reaction. For substrates, the triterpene/sterol C3 hydroxyl group was oriented towards the UDP-sugar C1' anomeric carbon for substrates. In the products, the C3 sugar on the saponin was oriented towards the diphosphate on UDP. Interestingly, an exception to this trend was predicted for the triterpene substrate **2** in the presence of UDP-**B** (**Supplementary Figure S147*ii***); this prediction suggests that GuUGT73F15 may exhibit some permissivity in the orientation of triterpenes/sterols/saponins in its active site (i.e., C3 group points towards or away from the UDP group). Campaigns to engineer/evolve GuUGT73F15 may be able to refine this binding site permissivity to selectively glycosylate triterpenes/sterols/saponins at non-C3 sites. Together, the Boltz-2x analysis of GuUGT73F15 is the first step towards understanding the structural basis of the substrate permissivity of this enzyme and provides hints for the bioinformatic identification of other permissive GT1s for the glycosylation of non-C3 sites on triterpenes/sterols/saponins.

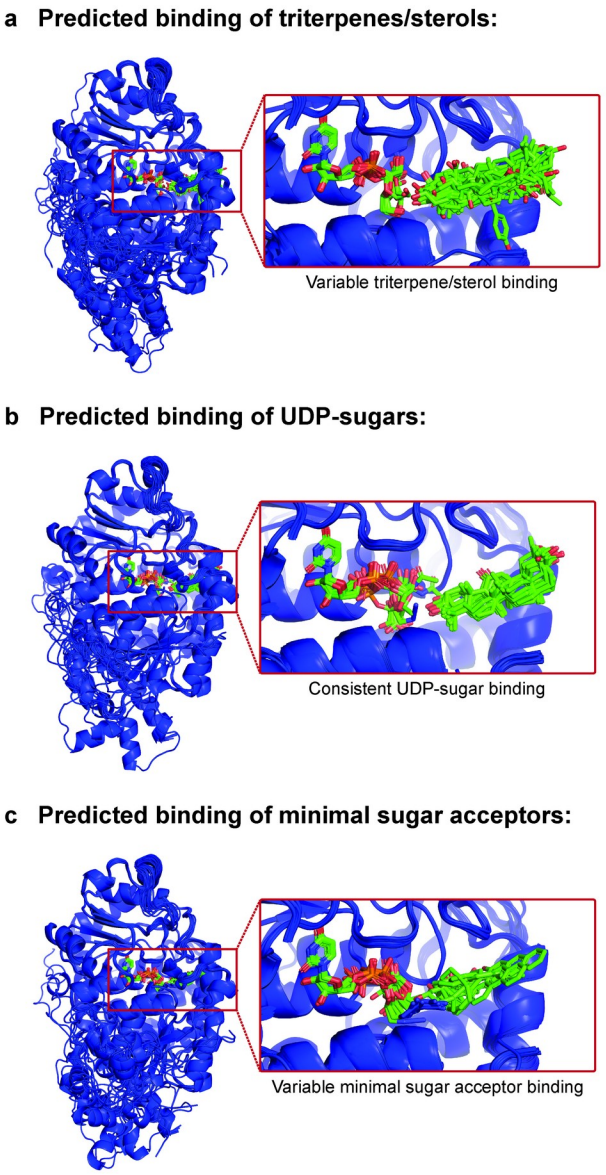

Figure 7. (a) Boltz-2x predicted protein folding and ligand binding of GuUGT73F15 for **1**–**22** and UDP-**B**. (b) Boltz-2x predicted protein folding and ligand binding of GuUGT73F15 for **1** and UDP-**A**–UDP-**M**. (c) Boltz-2x predicted protein folding and ligand binding of GuUGT73F15 for **23**–**36** and UDP-**M**. The ligands involved in substrate binding are detailed in **Supplementary Figures S149**–**S151** and **Supplementary Tables S13**–**S15**.

Functionalizing triterpenes/sterols with azides to access potential antibody-drug conjugates via click chemistry

The utility of GuUGT73F15 in the synthesis of antibody-drug conjugates (ADCs) was studied (**Figure 8a**). The anti-PD-1 antibody (1 equiv.) was first reduced into light chains (L) and

heavy chains (H) with excess TCEP for 0.5 h. Reduced thiolate moieties on cysteine residues were functionalized with bifunctional maleimide-polyethylene glycol 4-dibenzocyclooctyne (maleimide-PEG_4-DBCO) linkers (~125 equiv.) for 1 h. Characterization of products via matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) indicated that ~1 linker was attached to L (**Figure 8b**) and ~4 linkers were attached to H (**Figure 8c**). After removal of excess maleimide-PEG_4-DBCO linkers via buffer exchange, the GuUGT73F15-produced azido saponin **3-M** (~5 equiv.) was added to the reaction mixture and left to react for 18 h via strain-promoted azide-alkyne cycloaddition reaction. MALDI-TOF-MS characterization identified a mass shift corresponding to ~1 saponin per L (**Figure 8b**) and ~1 saponin per H (**Figure 8c**), confirming the successful production of antibody-saponin conjugates. The utilization of the 24 different azido saponins produced in this work, along with well developed antibody production/bioconjugation chemistry, enables the rapid production of libraries of antibody-saponin conjugates for applications as drug candidates.

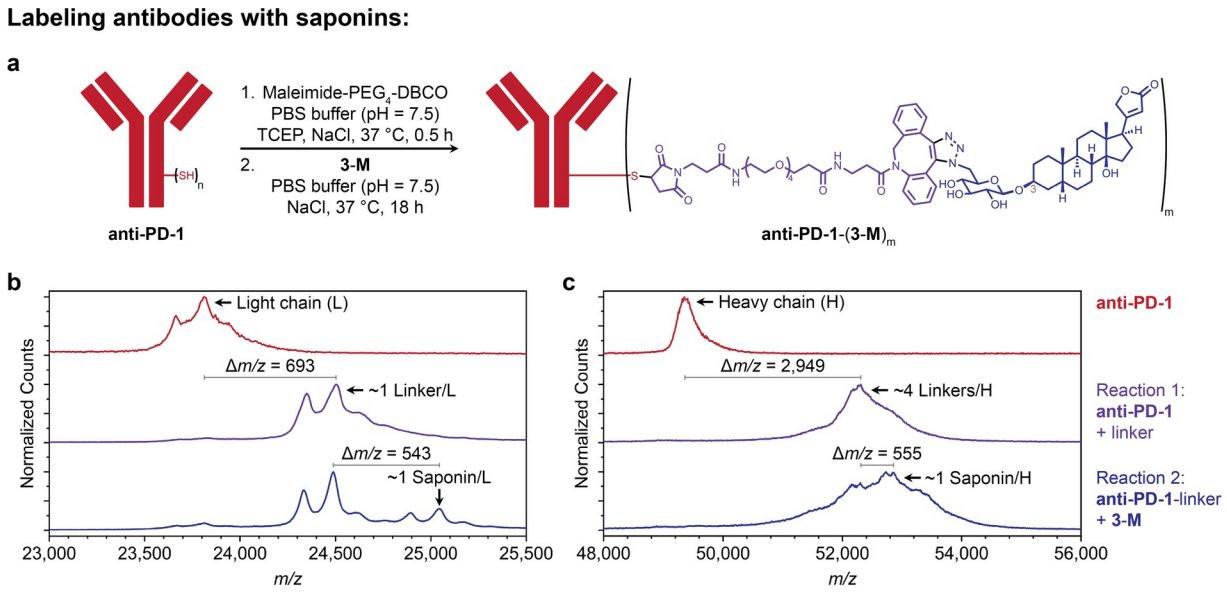

Figure 8. (a) After reduction with TCEP, reduced thiols on antibody light and heavy chains (red) were functionalized with maleimide-PEG_4-DBCO linkers (purple). The following *in vitro* reaction conditions were used: 13 µM antibody, 1.6 mM maleimide-PEG_4-DBCO, 2 mM TCEP, 10 mM PBS buffer (pH = 7.5), and 137 mM NaCl. Next, DBCO-modified antibodies were functionalized with the azide-modified saponin **3-M** (blue). The following *in vitro* reaction conditions were used: 10 µM antibody-linker, ~50 µM **3-M**, 10 mM PBS buffer (pH = 7.5), and 137 mM NaCl. Antibody (b) light and (c) heavy chains were characterized by MALDI-TOF-MS at each step of the reaction. The

expected mass change for functionalization per linker is $\Delta m/z_{linker} = 676$ and the expected mass change per **3**-**M** saponin is $\Delta m/z_{saponin} = 562$.

In vivo microbial glycosylation of triterpenes/sterols using GuUGT73F15

The ability of GuUGT73F15 to produce new-to-nature quillaic acid derivatives (i.e., **2**-**B** and **2**-**C**) *in vivo* in engineered microorganisms was investigated next. GuUGT73F15 was integrated into the genome of **2**-producing yeast YL-10 to generate the derivative strain PWGH-1. In both strains, all heterologous genes are under galactose-inducible promoters and, therefore, robust expression necessitates culturing in media containing galactose in absence of glucose. In contrast to the parent strain, PWGH-1 produced a new metabolite with an observed retention time and *m/z* congruent with **2**-C3-**B** and **2**-C3-**C** that was produced via *in vitro* enzymatic synthesis (**Figure 9**; **Supplementary Figure S3*iii***). Given that both UDP-**B** and UDP-**C** are present in appreciable quantities during galactose fermentation[61] and that GuUGT73F15 generally exhibited more complete conversion utilizing UDP-**B** vs. UDP-**C** (**Figure 5**), it is hypothesized that the observed metabolite is predominantly **2**-C3-**B**.

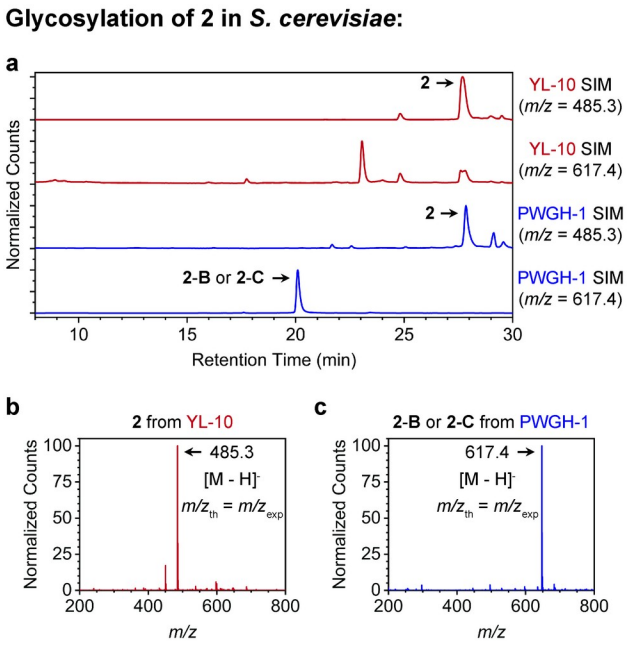

Figure 9. *S. cerevisiae* strain PWGH-1 (blue) was constructed by integrating a gene for GuUGT73F15 into the strain YL-10 (red) which produces **2**, UDP-**B**, and UDP-**C**. Strains were grown for 2 days in 1× YPD then grown for 2 days in 1× YPGal. Triterpenes and saponins were extracted from cell pellets and analyzed by LC-UV/MS. (a) Chromatograms

of products from YL-10 and PWGH-1 with single-ion monitoring (SIM) for **2** and **2-B/2-C**. The assignment of **2-B/2-C** was corroborated by products of *in vitro* reactions (Supplementary **Figure S3*iii*,*iv***). The peak in the YL-10 SIM chromatogram for *m/z* = 617.4 is a minor impurity that appears in the plotted trace due to normalization. (b) Mass spectrum for **2** from YL-10. (c) Mass spectrum for **2-B** and/or **2-C** from PWGH-1.

Characterization of QS-21,[13,14,20] vaccaroside,[52] saponarioside,[12] and yossoside[58] biosynthetic pathways has revealed that C3 β-*O*-glucuronosylation of **2**, performed by integral transmembrane glucuronosyltransferases like Csl enzymes, must strictly occur prior to other subsequent glycosylation reactions. This stringency is observed in both plant and microbial heterologous chassis and is thought to arise because the aglycone **2** is insufficiently hydrophilic to escape the endoplasmic reticulum (ER) membrane and access the downstream cytoplasmic GT1s. This biosynthetic logic could represent a checkpoint that drives biosynthetic flux to favor the fully oxidized aglycone over under-oxidized intermediates. The observation that GuUGT73F15 can glycosylate **2** in engineered yeast motivated investigations to characterize the localization of GuUGT73F15 via confocal fluorescence microscopy. GuUGT73F15 bearing a C-terminal mCherry fusion was expressed from a plasmid in *S. cerevisiae* strain PWGH-2. Studying the localization of mCherry-tagged GuUGT73F15 revealed that it is robustly expressed and dispersed throughout the cytosol as opposed to being embedded in the ER membrane (**Figure S152**). These data suggest that GuUGT73F15 may be able to interact with or utilize substrates embedded in membranes. Similar cytosolic protein-ER membrane substrate interactions have been hypothesized to be mechanistically important in the biosynthesis of mogrosides.[62] Further investigation will be required for in-depth characterization of the *in vivo* biophysical interactions of GuUGT73F15.

GuUGT73F15 provides a semi-synthetic route to advanced saponin intermediates

The chemical synthesis of saponins remains challenging and requires careful utilization of orthogonal protecting groups to achieve site-selective glycosylation. Enzymatic glycosylation offers an alternative approach to efficiently install sugars on commercially-available aglycones; however, scaled enzymatic semi-synthesis using cognate enzymes remains challenging for saponins like QS-21 that require integral transmembrane glycosyltransferases. These systems

would require the effort-intensive expression into and isolation of membrane organelles (e.g., yeast microsomes). The discovered activity of the cytosolic GuUGT73F15 in this work led to the hypothesis that GuUGT73F15 could be leveraged to bypass this limitation in the *in vitro* biosynthesis of QS-21. Therefore, the activity of GuUGT73F15 was utilized in an enzymatic semi-biosynthesis of the advanced QS-21 C3 trisaccharide intermediate, **2** bearing a branched trisaccharide consisting of GlcA (**A**), Gal (**C**), and Xyl (**E**) (**2**-C3-**A**(-**C**)-**E** [also known as QA-TriX]).

Given the ability of GuUGT73F15 to utilize many UDP-sugar donors and empirically higher affinity for UDP-Xyl versus UDP-GlcA (**Figure 5**), it was deemed necessary to temporally separate the initial C3 β-*O*-glucuronosylation from the subsequent glycosylation steps (**Figure 10a**). Overnight incubation of **2** with 1 mM UDP-**A** in the presence of GuUGT73F15 led to the complete consumption of **2** and the production of **2**-C3-**A**. Subsequently, UDP-**C** and UDP-**E** and their cognate glycosyltransferases from the QS-21 biosynthetic pathway were added to the reaction mixture and the products analyzed after three hours. The **2**-C3-**A** intermediate was completely consumed and a new species was formed with a mass consistent with that predicted for **2**-C3-**A**(-**C**)-**E** (**Figure 10b**,**d**). Importantly, the retention time and *m/z* were also consistent with **2**-C3-**A**(-**C**)-**E** produced via base-hydrolysis of QS-21 (**Figure 10b**,**c**). This semi-biosynthesis of **2**-C3-**A**(-**C**)-**E** may be used to generate substrates to investigate subsequent tailoring steps in the QS-21 biosynthetic pathway. Together, this landmark result viably utilized GuUGT73F15 to access advanced semi-biosynthetic glycosylated saponin intermediates, facilitating the future semi-biosynthesis of products like QS-21.

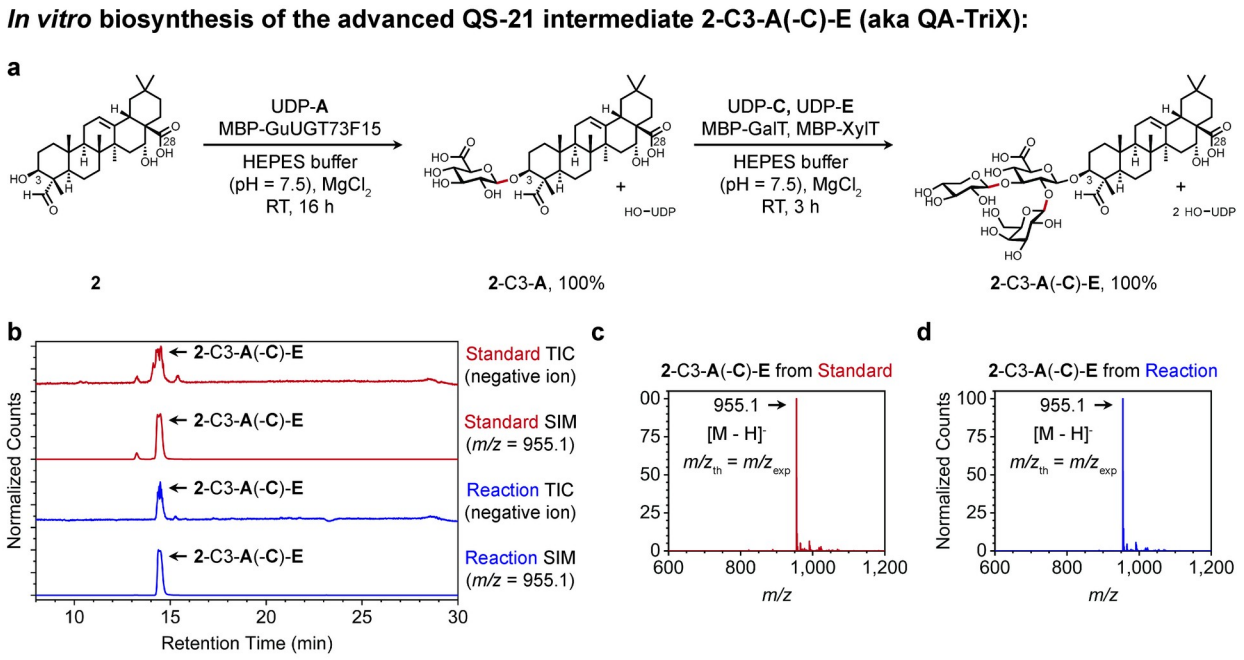

Figure 10. (a) **2**-C3-**A**(-**C**)-**E** (aka QA-TriX) was biosynthesized from **2**, UDP-**A**, UDP-**C**, and UDP-**E** through sequential *in vitro* enzymatic reactions. In the saponin chemical structures, C3 β-*O*-glycosidic bonds formed by GuUGT73F15 are highlighted in red and select carbons are numbered in gray. The following reaction conditions were used: 1 mM UDP-sugar, 200 μM triterpene/saponin, 20 μM MBP-tagged enzyme, 50 mM HEPES buffer (pH = 7.5), 2 mM $MgCl_2$ for 16 h for the first reaction followed by 3 h for the second reaction at room temperature. Reaction mixtures were characterized by LC-UV/MS and percent conversions for reactions were quantified from absorbances at λ = 215 nm. (b) Total ion chromatograms (TIC) and single-ion monitoring (SIM) chromatograms of **2**-C3-**A**(-**C**)-**E** from the standard (hydrolyzed derivative of QS-21) and the *in vitro* product. (c) Mass spectrum for **2**-C3-**A**(-**C**)-**E** from the standard. (d) Mass spectrum for **2**-C3-**A**(-**C**)-**E** from the *in vitro* product.

CONCLUSION

This report describes the discovery that GuUGT73F15, a GT1 from *Glycyrrhiza uralensis*, can utilize an extremely wide substrate scope of triterpenes/sterols as sugar acceptors and UDP-sugars as sugar donors in C3 β-*O*-glycosylation reactions. This one enzyme was used to produce 130 unique monoglycosylated saponins, 14 unique diglycosylated saponins, 1 unique triglycosylated saponin, and 14 semi-biosynthetic products. Computational predictions of GuUGT73F15 folding and substrate/product binding provide first clues into the structural basis of its substrate permissivity. The activity of GuUGT73F15 enables the rapid production of a saponin-functionalized antibody-drug conjugate as well as the recapitulation of an *in vitro*/*in vivo* biosynthesis of advanced QS-21 intermediates. Together, this GuUGT73F15 is a valuable

biocatalytic tool for producing libraries of saponin natural products and their new-to-nature analogs, enabling the future discovery and scalable production of novel saponin drugs.

SUPPORTING INFORMATION

Supporting information includes: Materials and methods, a list of saponins produced in this work, gene sequences and oligonucleotide primers, plasmid genotypes, yeast strain genotypes, SDS-PAGE of purified MBP-GuUGT73F15, additional LC-UV/MS chromatograms and associated mass spectra, NMR spectra and associated tables of assigned resonances, additional computational modeling of GuUGT73F15 binding to substrates and products, and confocal microscope images of GuUGT73F15-mCherry localization in yeast (PDF).

Supporting data includes: chemical structures and reaction conversions for saponins produced in this work (PDF).

DECLARATION OF INTERESTS

J.D.K. has financial interests in Ansa Biotechnologies, Apertor Pharma, Berkeley Yeast, BioMia, Cyklos Materials, Demetrix, Lygos, Napigen, ResVita Bio, and Zero Acre Farms. All other authors declare no competing interests.

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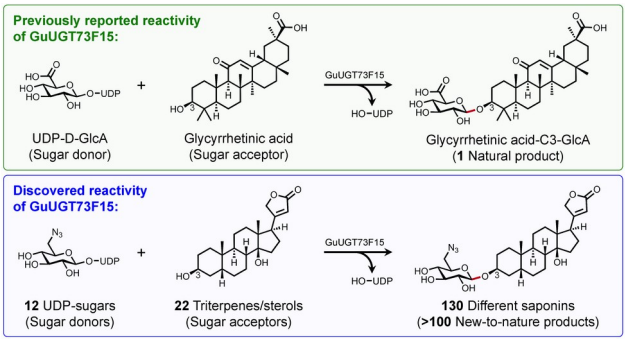

TOC Graphic.