Electronic Supplementary Material (ESI) for

Selective profiling of steviol-catalyzing UDP-glycosyltransferases with a metabolically synthesized probe

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1. Supplementary Figures and Tables

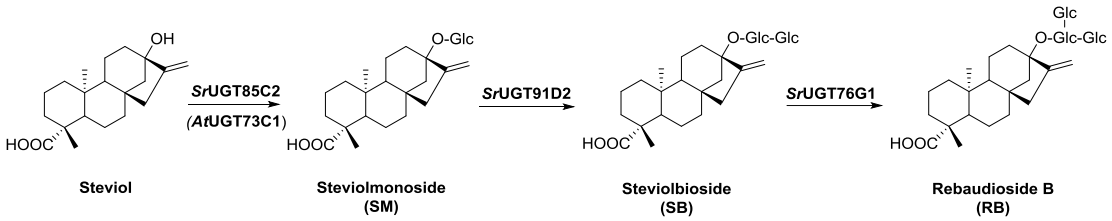


Fig. S1 Recombinant UGTs used in this study and their catalytic activities towards steviol glycosides.

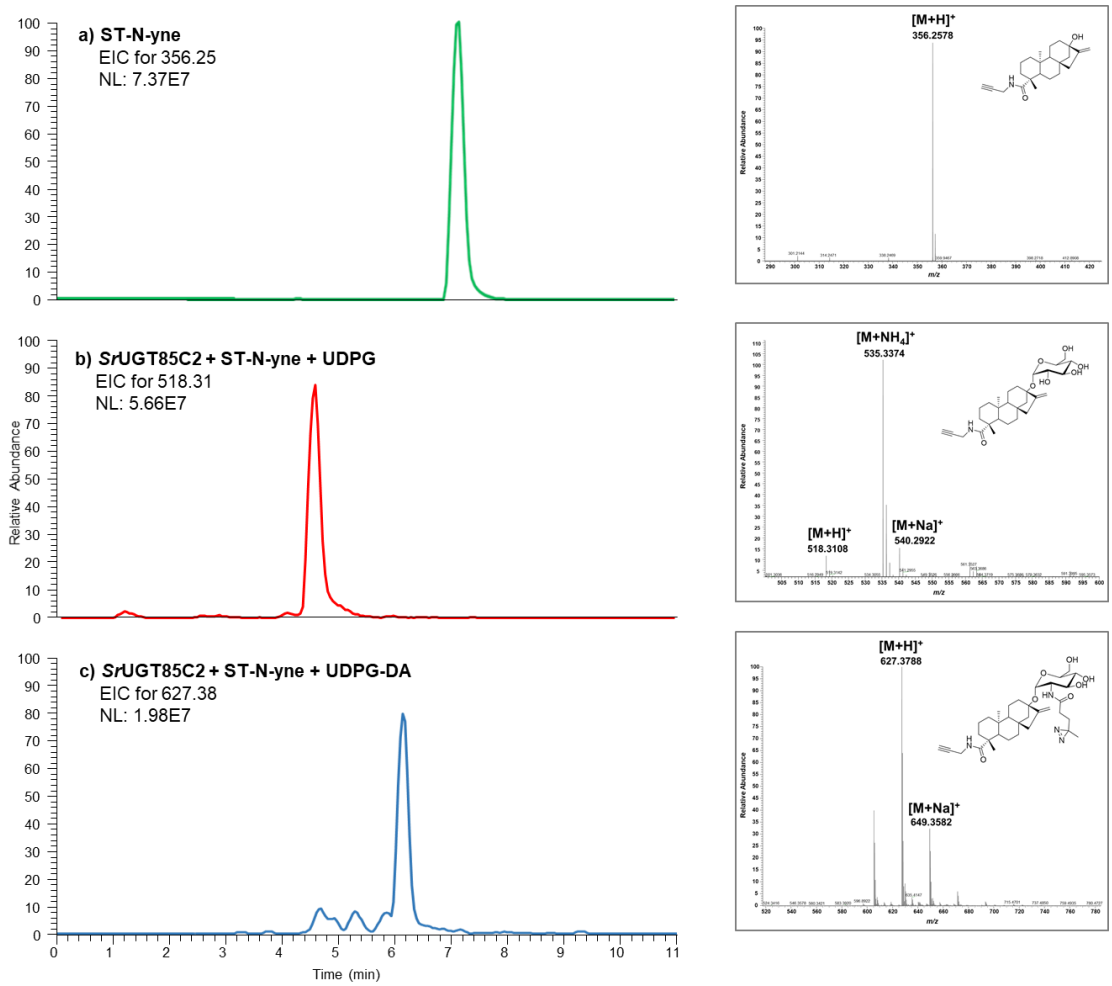


Fig. S2 (A) LC-MS analysis of **ST-N-yne**; (B) LC-MS analysis of crude **SM-yne** which was produced by recombinant *Sr*UGT85C2 with **ST-N-yne** and **UDPG** as substrates; (C) LC-MS analysis of the fully functionalized probe **P1** which was produced by recombinant *Sr*UGT85C2 with **ST-N-yne** and **UDPG-DA** as substrates.

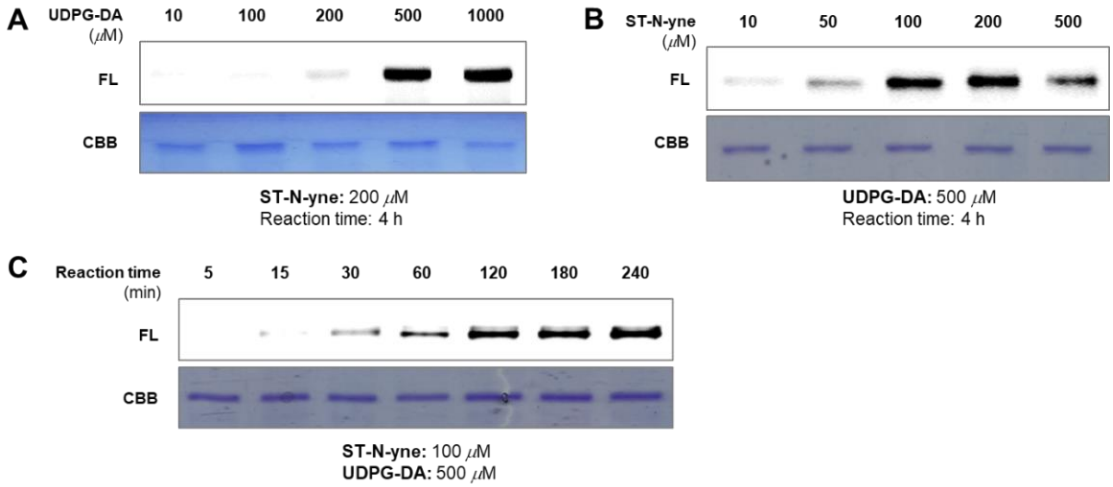


Fig. S3 Optimization of labeling conditions by using recombinant *Sr*UGT85C2 with (A) various **UDPG-DA** concentrations; (B) various **ST-N-yne** concentrations; and (C) various reaction durations.

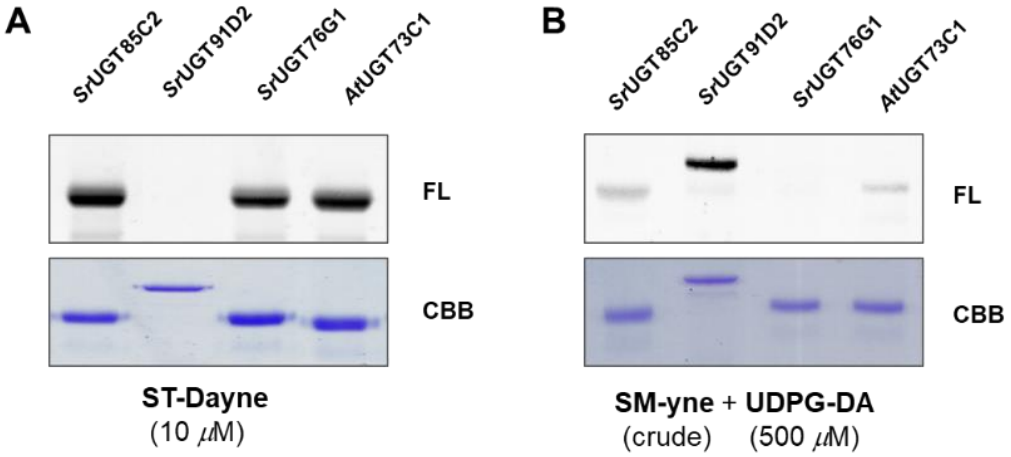


Fig. S4 (A) Labeling of recombinant UGTs with fully functionalized probe **ST-Dayne** (10 μM); (B) Labeling of recombinant UGTs of known functions with combinatorial use of crude **SM-yne** (approximately 100 μM) and **UDPG-DA** (500 μM).

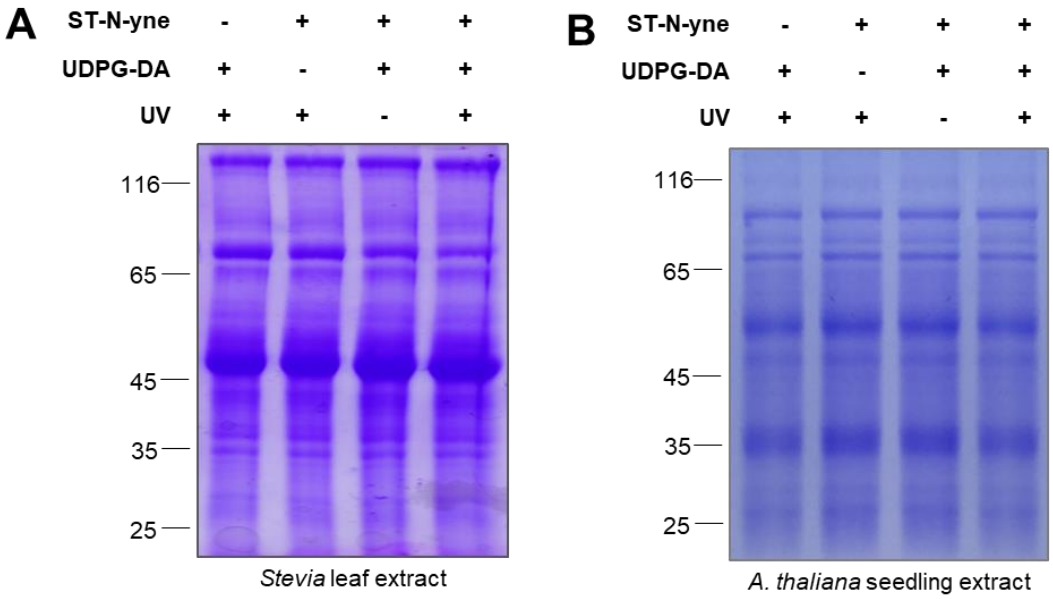


Fig. S5 Coomassie blue staining results corresponding to the fluorescent gel in (A) Fig. 2D for *Stevia* leaf extracts and (B) Fig. 3A for *Arabidopsis* seedling extracts.

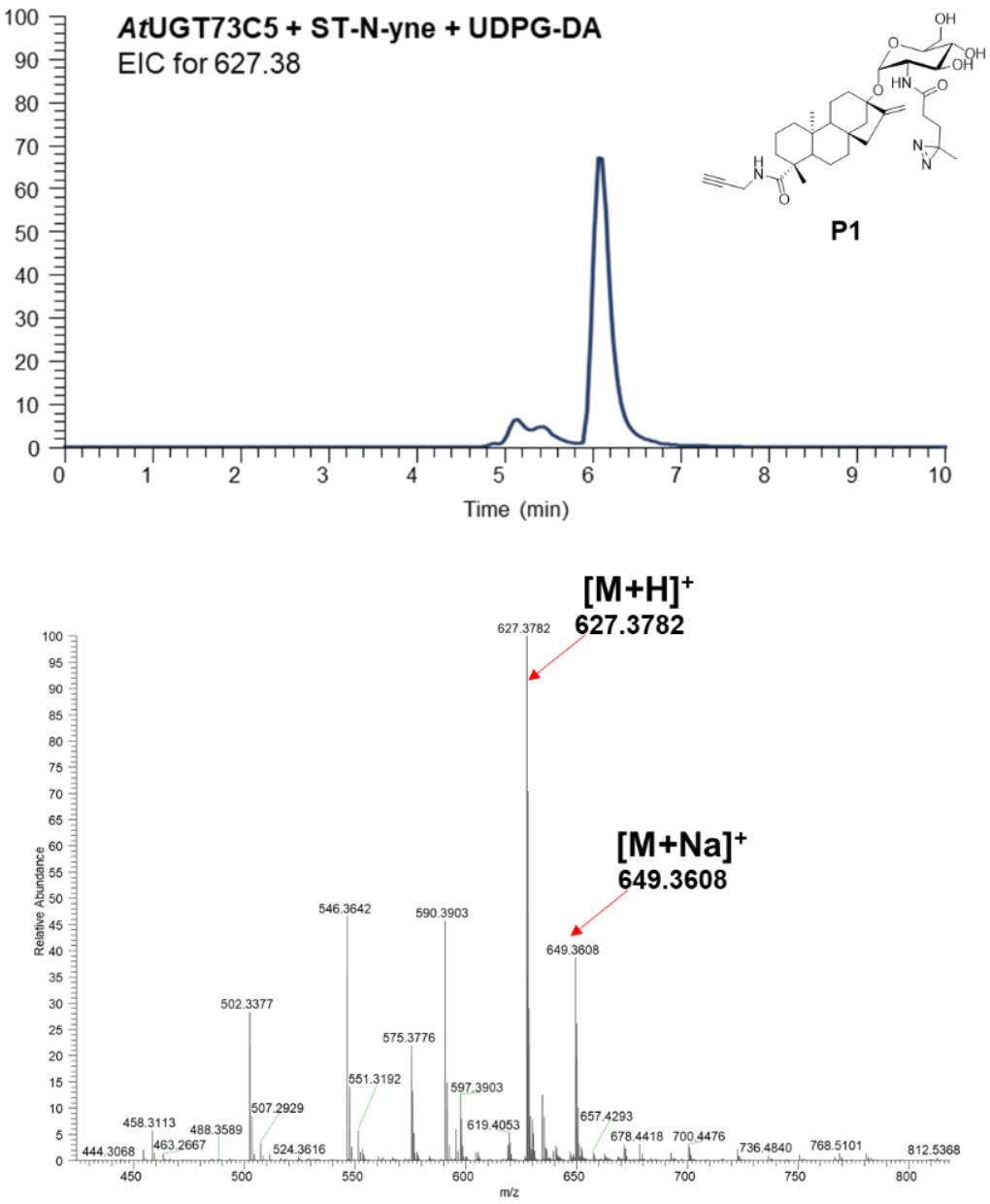


Fig. S6 LC-MS analysis of the fully functionalized probe **P1** which was produced by recombinant *At*UGT73C5 with **ST-N-yne** and **UDPG-DA** as substrates.

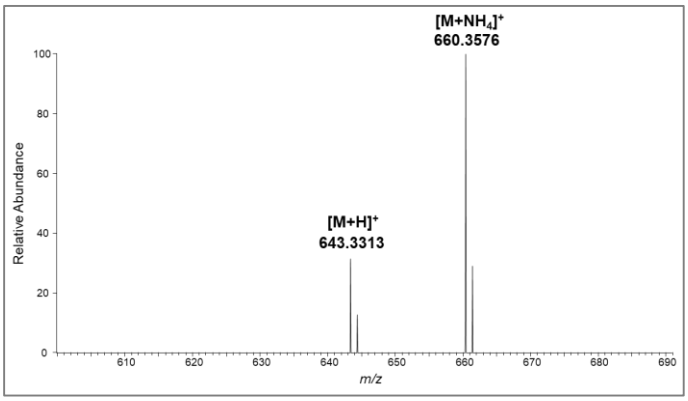
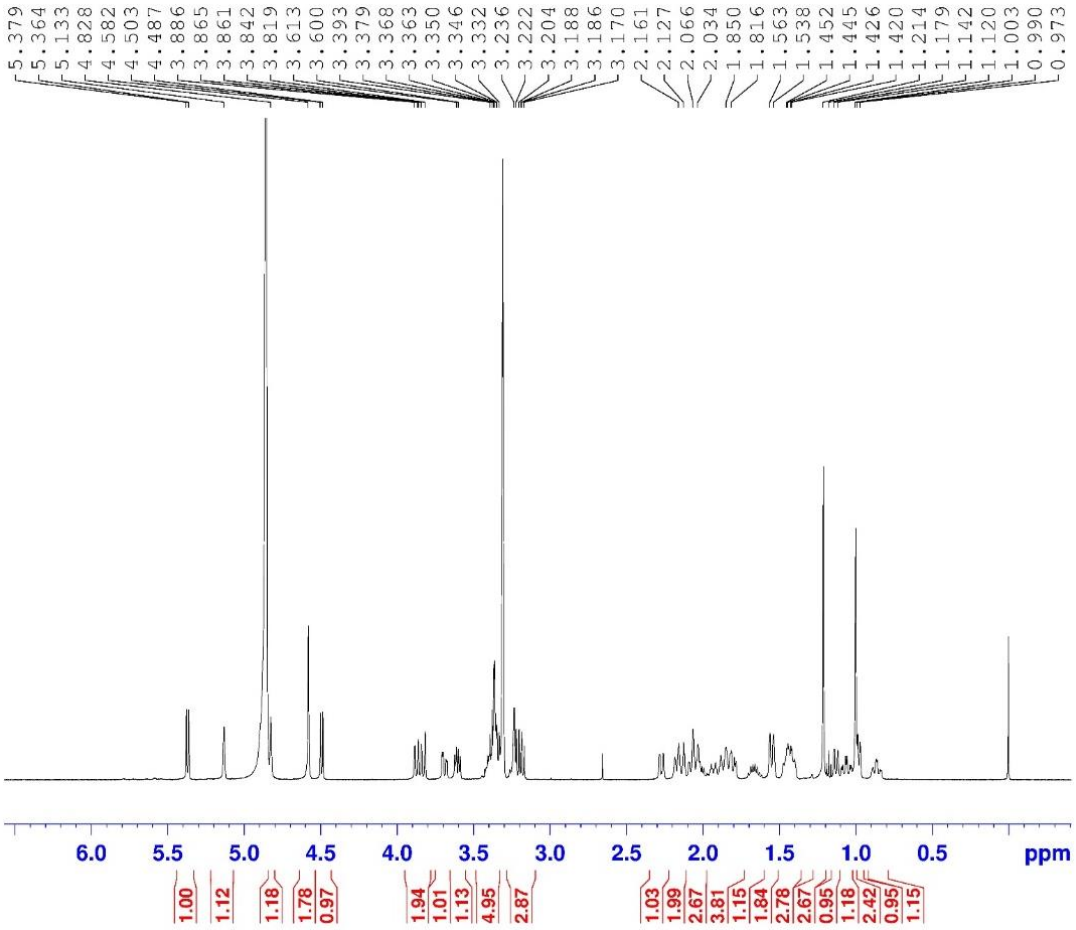


Fig. S7 LC-MS analysis reveals that a glycosylated product with molecular weight corresponding to steviol plus two glucose moieties was generated by recombinant *At*UGT73C5 with steviol and UDPG as substrates in Fig. 3E (ii).



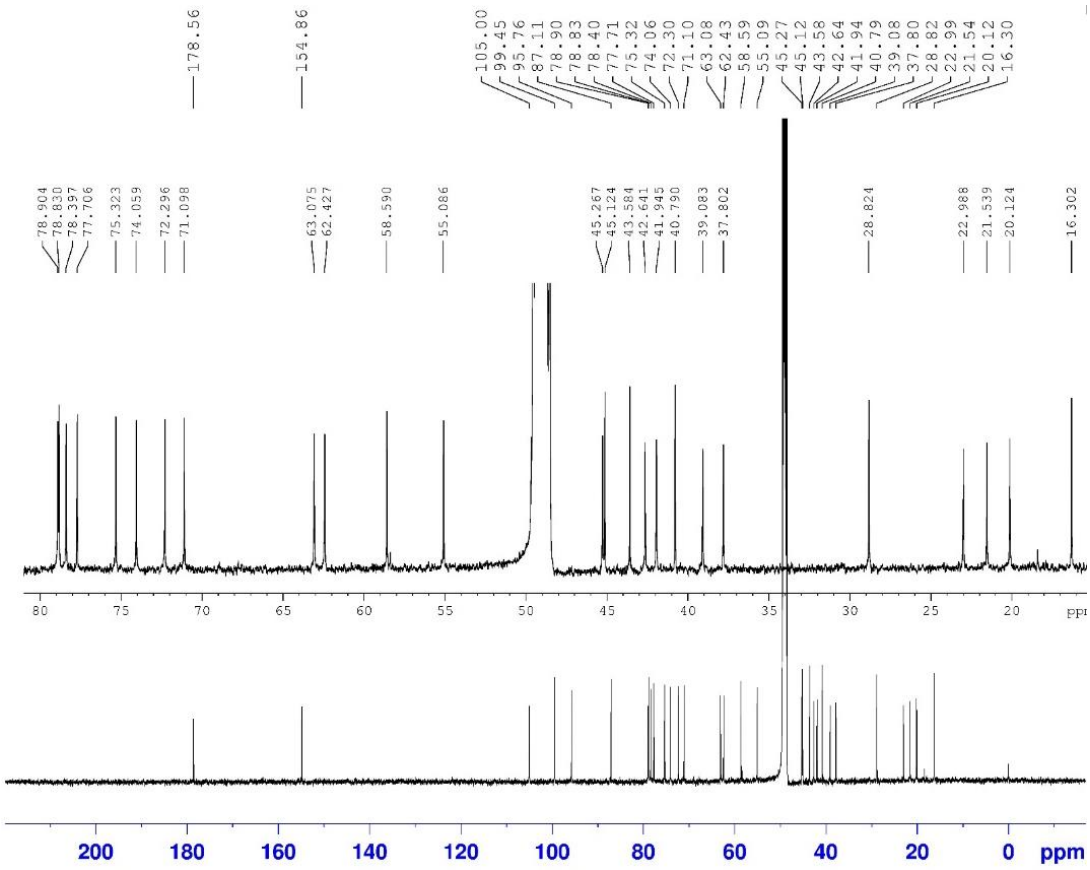


Fig. S8 Characterization of the enzyme reaction product in Fig. 3E (vii) as rububoside by ^{1}H-NMR (500 MHz, MeOD) *δ* 5.37 (d, *J*=7.5 Hz, 1H), 5.13 (brs, 1H), 4.83 (brs, 1H), 4.58 (brs 2H), 4.50 (d, *J*=8 Hz, 1H), 3.90-3.80 (m, 2H), 3.70 (dd, *J*=3.0 and 8.0 Hz, 1H), 3.60 (dd, *J*=6.0 and 12.3 Hz, 1H), 3.45-3.30 (m, 5H), 3.27-3.15 (m, 3H), 2.27 (d, *J*=11.5 Hz, 1H), 2.19-2.05(m, 3H), 2.04-1.75 (m, 4H), 1.70-1.60 (m, 1H), 1.55 (d, *J*=12.5 Hz, 2H), 1.50-1.38 (m, 3H), 1.24 (s, 3H), 1.13 (d, *J*=11 Hz, 1H), 1.06 (td, J=4 and 13.7 Hz, 1H), 1.00 (s, 3H), 0.98 (d, *J*=8.5 Hz, 1H), 0.87 (td, *J*=4 and 13.5 Hz, 1H); and ^{13}C-NMR (125 MHz, MeOD) *δ* 178.6, 154.9, 105.0, 99.5, 95.8, 87.1, 78.9, 78.8, 78.4, 77.7, 75.3, 74.1, 72.3, 71.1, 63.1, 62.4, 58.6, 55.1, 45.3, 45.1, 43.6, 42.6, 41.9, 40.8, 39.1, 37.8, 28.8, 23.0, 21.5, 20.1, 16.3.

Table S1 Proteins identified in the ~50 kDa gel piece cut from the silver stained gel in Fig. 2E.

Accession	Description	Coverage (%)	PSMs	Unique Peptides	Score Sequest HT
Q6VAB0	**UDP-glycosyltransferase 85C2 OS=Stevia rebaudiana GN=UGT85C2 PE=1 SV=1**	**42.40**	**57**	**16**	191.29
D3PFC2	Ribulose bisphosphate carboxylase large chain (Fragment) OS=Stevia rebaudiana GN=rbcL PE=3 SV=1	40.81	94	19	172.61
Q8GV27	Elongation factor 1-alpha OS=Stevia rebaudiana PE=2 SV=1	18.70	13	9	23.59
Q8H6A3	Actin OS=Stevia rebaudiana PE=2 SV=1	19.36	7	6	12.83
A0A0P0EG81	1-deoxy-D-xylulose-5-phosphate reductoisomerase 2 OS=Stevia rebaudiana GN=DXR2 PE=2 SV=1	13.74	5	5	9.86

Table S2 Full list of proteins identified in the ~50 kDa gel piece cut from the silver stained gel in Fig. 3B.

Accession	Description	Coverage（%）	PSMs	Unique Peptides	Score Sequest HT
O03042	Ribulose bisphosphate carboxylase large chain OS=Arabidopsis thaliana OX=3702 GN=rbcL PE=1 SV=1	35.70	39	15	108.40
Q9ZQ99	**UDP-glycosyltransferase 73C1 OS=Arabidopsis thaliana OX=3702 GN=UGT73C1 PE=2 SV=1**	**33.40**	**15**	**13**	40.52
Q43127	Glutamine synthetase, chloroplastic/mitochondrial OS=Arabidopsis thaliana OX=3702 GN=GLN2 PE=1 SV=1	19.53	9	6	29.04
Q9LD57	Phosphoglycerate kinase 1, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=PGK1 PE=1 SV=1	23.08	10	4	28.90
P19366	ATP synthase subunit beta, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=atpB PE=1 SV=2	19.88	9	6	27.48
P50318	Phosphoglycerate kinase 2, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=At1g56190 PE=1 SV=3	19.04	8	2	22.31
Q9SUR6	Cystine lyase CORI3 OS=Arabidopsis thaliana OX=3702 GN=CORI3 PE=1 SV=1	21.33	7	7	19.53
Q9ZQ94	**UDP-glycosyltransferase 73C5 OS=Arabidopsis thaliana OX=3702 GN=UGT73C5 PE=1 SV=1**	**11.80**	**6**	**4**	18.36
P92549	ATP synthase subunit alpha, mitochondrial OS=Arabidopsis thaliana OX=3702 GN=ATPA PE=1 SV=2	4.54	5	2	14.57
Q8RWV0	Transketolase-1, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=TKL-1 PE=1 SV=1	9.72	5	4	12.64
P0DH99	Elongation factor 1-alpha 1 OS=Arabidopsis thaliana OX=3702 GN=A1 PE=1 SV=1	8.02	5	4	10.98
Q9LR30	Glutamate--glyoxylate aminotransferase 1 OS=Arabidopsis thaliana OX=3702	10.60	4	3	10.94

	GN=GGAT1 PE=1 SV=1				
P22954	Probable mediator of RNA polymerase II transcription subunit 37c OS=Arabidopsis thaliana OX=3702 GN=MED37D PE=1 SV=2	6.89	4	3	9.92
P42799	Glutamate-1-semialdehyde 2,1-aminomutase 1, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=GSA1 PE=1 SV=1	10.13	3	3	8.47
Q56YA5	Serine--glyoxylate aminotransferase OS=Arabidopsis thaliana OX=3702 GN=AGT1 PE=1 SV=2	9.97	3	3	8.21
Q9SLK0	Peroxisomal isocitrate dehydrogenase [NADP] OS=Arabidopsis thaliana OX=3702 GN=ICDH PE=1 SV=1	5.05	3	2	7.86
Q9LF33	UDP-glucose 6-dehydrogenase 3 OS=Arabidopsis thaliana OX=3702 GN=UGD3 PE=1 SV=1	7.08	3	3	7.44
P25819	Catalase-2 OS=Arabidopsis thaliana OX=3702 GN=CAT2 PE=1 SV=3	4.88	3	1	7.28
Q9C9W5	Glycerate dehydrogenase HPR, peroxisomal OS=Arabidopsis thaliana OX=3702 GN=HPR PE=1 SV=1	10.36	3	3	7.26
Q1WIQ6	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase OS=Arabidopsis thaliana OX=3702 GN=ALDH11A3 PE=1 SV=2	6.85	3	3	7.07
Q9CAI7	Eukaryotic initiation factor 4A-3 OS=Arabidopsis thaliana OX=3702 GN=TIF4A-3 PE=1 SV=1	7.24	3	3	6.64
O23255	Adenosylhomocysteinase 1 OS=Arabidopsis thaliana OX=3702 GN=SAHH1 PE=1 SV=1	6.18	3	3	6.48

2. Biological Experiments

2.1 Protein extraction from *Stevia* leaves and *Arabidopsis* seedlings

Preparation and purification of *Stevia* leaves extracts were carried out as described previously (*ACS Chem, Biol.*, **2018**, 13, 1944). For *Arabidopsis* proteome extraction, ten grams of *Arabidopsis* seedlings were homogenized in liquid nitrogen and transferred to 50 mL buffer A containing 50 mM HEPES pH 7.5, 10 mM DTT, 0.1% Triton X-100, 10 g PVPP, 10 g PVP, and a protease inhibitor cocktail (Roche). Insoluble substances were removed by filtering, followed by centrifugation at 18,000 g for 30 min. Soluble materials were loaded onto a DEAE column (1.5 × 20 cm, Aogma Biosciences) equilibrated in 50 mM HEPES of pH 7.5 with 10 mM DTT (buffer B) and eluted by a linear gradient from BC10 (buffer B with 10 mM NaCl) to BC300 (buffer B with 300 mM NaCl). All DEAE fractions were combined and concentrated with a 10 kDa molecular weight cut-off column (Millipore) and collected for further enzymatic assays and chemoproteomics studies.

2.2 *In vitro* labeling of recombinant UGTs with ST-N-yne and UDPG-DA

Recombinant UGTs were diluted to 0.1 mg/mL in PBS containing 10 mM $MgCl_2$. For each sample, 200 μL of UGT solution was added with **ST-N-yne** (100 μM) and **UDPG-DA** (500 μM) as indicated at 30 °C for 2 h. Samples were irradiated with UV light at 365 nm (8 Watt) on ice for 20 min and 1% SDS (final concentration) was added. Then, samples were subjected to click reaction with TAMRA-N_3 (100 μM, Lumiprobe), $CuSO_4$ (1 mM), THPTA (100 μM, Sigma) and NaVc (1 mM), followed by SDS-PAGE and in-gel fluorescence scanning by using a FUJIFILM FLA 9000 plus DAGE fluorescence scanner.

2.3 Preparation of crude SM-yne by *Sr*UGT85C2

Recombinant *Sr*UGT85C2 (0.5 mg/mL, 300 μL) in PBS was added with **ST-N-yne** (100 μM final concentration) and **UDPG** (500 μM final concentration). The reaction was initiated by addition of $MgCl_2$ (10 mM), followed by incubation at 30 °C for 3 h. Upon completion, the reaction mixture was extracted with 100 μL water saturated *n*-butanol three times with vigorous vortex. The organic phase was combined and evaporated completely by a rotavapor. The residue was dissolved in 10 μL CH_3OH/H_2O (30%/70%, v/v) and used as crude **SM-yne** for *in vitro* labeling experiments of *Sr*UGT91D2 described above.

2.4 Chemoproteomic profiling in plant extracts with bi-substrate probe modules

Native proteomes (~2.0 mg/mL, 300 μL) extracted from *Stevia* leaves or *Arabidopsis* seedlings were added with 100 μM **ST-N-yne** and 500 μM **UDPG-DA**. The reaction was initiated by addition of $MgCl_2$ (10 mM), followed by incubation at 30 °C with gentle shaking for 1 h. Protein samples were transferred to a 12-well plate and photo-irradiated with UV light at 365 nm (8W) for 20 min on ice. Resultant samples were collected into new tubes

and precipitated with CH_3OH (600 μL)/$CHCl_3$ (150 μL)/H_2O (300 μL) sequentially followed by vortex. After centrifugation at 14,000 g for 3 min, the protein disk was washed twice with CH_3OH (500 μL), air-dried and re-dissolved in 200 μL of click buffer (50 mM HEPES pH 8.0, 1% SDS) by sonication. Precipitates were re-suspended in 220 μL of click buffer (50 mM HEPES pH 8.0, 1% SDS). 20 μL of the samples was subjected to click reaction with TAMRA-N_3 (100 μM, Lumiprobe), $CuSO_4$ (1 mM), THPTA (100 μM) and NaVc (1 mM), followed by in-gel fluorescence scanning by a FUJIFILM FLA 9000 plus DAGE fluorescence scanner. The remaining samples (200 μL each) were subjected to click reaction with biotin-N_3 (100 μM, Biomatrick Inc.), $CuSO_4$ (1 mM), THPTA (100 μM) and NaVc (1 mM). Samples were precipitated again with CH_3OH/$CHCl_3$/H_2O and re-dissolved in 200 μL of binding buffer (50 mM HEPES pH 8.0, 0.5% SDS) by sonication. 50 μL of streptavidin-sepharose (GE Healthcare) beads were added to each sample, which were then incubated at room temperature with continuous rotation for 1 h. The beads were washed sequentially with PBS with 0.5% SDS (w/v) for three times, 4 M urea in PBS for three times, and H_2O once. Each wash step was performed on a rotator for 30 min. Bound proteins were eluted by boiling in SDS-PAGE sample loading buffer (~50 μL) and subjected to SDS-PAGE. The resultant gel was visualized by silver staining and a consequent ~50 kDa stained band was subjected to in-gel tryptic digestion and identified by mass spectrometry.

2.5 In-gel digestion

Silver-stained gel pieces (45~65 kDa, as indicated by MW ladder) were excised, destained with 100 μL of 50 mM $K_3[Fe(CN)]_6$, 50 mM $Na_2S_2O_3$, and subjected to three cycles of dehydration with 50% acetonitrile followed by rehydration with 50 mM NH_4HCO_3. The colorless gel pieces were added with 5 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 1 h at 50 °C and dehydrated with 50% acetonitrile, followed by incubation with 20 mM iodoacetamide for 30 min in dark at 37 °C. After dehydration with acetonitrile, gels were dried completely in a fume hood and digested with trypsin (Promega, 0.02 μg/μL in 50 mM ammonium bicarbonate) at 37 °C overnight. Peptides were extracted by addition of acetonitrile and the solution was shaken for 15 min. The supernatant was collected, completely dried by a Speedvac and dissolved in 10 μL 0.1% formic acid for mass spectrometry analysis.

2.6 Nano LC-MS/MS analysis of digests

Five microliters of peptide sample were loaded onto a Thermo C18 PepMap100 precolumn (300 μM × 5 mm) and eluted on a Thermo Acclaim PepMap RSLC analytical column (75 μM × 15 cm). Mobile phase A (0.1% formic acid in H_2O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish an 80-min gradient comprising 55 min of 4−30% B, 7 min of 30−50% B, and 5 min of 50−90% B 5 min of 90% B, followed by re-equilibrating at 4% B for 8 min. The flow rate was 0.3 μL/min. Peptides were then analyzed on a Thermo Orbitrap Fusion Lumos proteomic mass spectrometer in a data-dependent manner, with automatic switching between MS and MS/MS scans by using a cycle time 3 s. MS spectra

were acquired at a resolution of 120,000 with an AGC target value of 4×10^5 ions or a maximum integration time of 50 ms. The scan range was set to be 375 to 1500 m/z. Peptide fragmentation was performed via high energy collision dissociation (HCD) with the energy level set at 38 NCE. MS/MS spectra were acquired at a resolution of 50,000 with an AGC target value of 1×10^5 ions or a maximum integration time of 10^5 ms. The fixed first *m/z* was 120, and the isolation window was 0.7 *m/z*.

2.7 Protein identification

Data processing was performed by using Proteome Discoverer 2.1 software (Thermo Scientific). Peptide sequences and protein identities were determined by matching protein databases (*Arabidopsis thaliana* or *Stevia rebuadiana*) with the acquired fragmentation pattern by SEQUEST HT algorithm using a target false discovery rate (FDR) of 1%. Precursor mass tolerance was set to 10 ppm and fragment ion mass tolerance to 0.02 Da. One missed cleavage site of trypsin was allowed. Carbamidomethyl (C) was used as a fixed modification. Oxidation (M) was used as variable modification. Identified proteins were filtered by at least three spectral counts and one unique peptide. Data are available via ProteomeXchange with the identifier PXD021170.

2.8 Recombinant expression and purification of plant UGTs

Methods for expression and purification of *Sr*UGT85C2, *Sr*UGT76G1, and *At*UGT73C1 have previously been reported (*ACS Chem, Biol.*, **2018**, 13, 1944; *Chem. Commun.*, **2018**, 54, 7179). For expression and purification of *Sr*UGT91D2, the *Sr*UGT91D2 cDNA was cloned into a pGEX-4T1 expression vector to obtain GST-tag fusions. Expression was induced at an OD_{600} of 0.6 by addition of IPTG (final concentration 0.5 mM) and carried out 20 hours at 16°C in *E. coli* BL21-(DE3) cells. The cells were harvested, resuspended in buffer (25 mM Tris-HCl, pH 8.0; 150 mM NaCl) and lysed by sonication. The lysate was clarified by centrifugation (35,000 *g*, 30 min, and 4°C), which was then subjected to affinity chromatography by using GSH resin with a 10 CV (column volume) washing step (PBS pH7.2, 1 mM DTT, 1 mM EDTA) and a 5 CV elution step (50 mM Tris-HCl pH 8.0, 0.4 M NaCl, 50 mM reduced GSH, 0.1 % Triton X-100, and 1 mM DTT). Protein concentration was determined by the Bradford protein quantification kit (Sangon Biotech, Shanghai) with bovine serum albumin as the standard.

For expression and purification of *At*UGT73C5, *At*UGT73C5 cDNA was cloned into a pET28a expression vector to obtain His-tag fusions. Expression was induced in *E. coli* BL21-(DE3) cells at an OD_{600} of 0.6 by addition of IPTG (final concentration 0.5 mM), followed by further culture for 20 h at 16 °C. The cells were harvested, resuspended in buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl) and lysed by sonication. Lysate was clarified by centrifugation (35,000 *g*, 30 min, and 4°C). The clarified lysate was subjected to His-affinity chromatography by using Ni-NTA resin with a 10 CV (column volume) washing step (25 mM Tris-HCl, pH 8.0; 150 mM NaCl; 20 mM imidazole) and a 5 CV elution step (25 mM Tris-HCl, pH 8.0; 150 mM NaCl; 50 ~ 250 mM imidazole). Protein identity was confirmed by SDS-PAGE. Protein concentration was determined by the Bradford assay

with bovine serum albumin as the standard.

2.9 Enzymatic assay

The reaction was carried out in a total volume of 500 μL containing 50 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 10 μg/mL BSA, 1 mM **UDPG** as sugar donors, along with 100 μM sugar acceptors [steviol, steviolmonoside (**SM**), 19-O-β glucopyranosol steviol (**S19G**)]. After addition of 100 μg recombinant UGTs, the reaction proceeded for 4 h at 30 °C and was quenched by adding 200 μL of water saturated *n*-butanol with vigorous vortex. The sample was extracted three times with 200 μL of water-saturated *n*-butanol. The combined organic phase was evaporated completely by a rotavapor and the residue was dissolved in CH_3OH/H_2O (50%/50%, v/v) for LC-MS analysis by means of an Agilent 6520 Accurate-Mass Q-TOF instrument.

2.10 UPLC-MS analysis of enzymatic assay products

LC-MS analysis was performed on a Waters Acquity UPLC tandem with a Q-Exactive mass spectrometer (Thermo Scientific). Chromatographic separation was carried out at 30 °C on a Waters Acquity UPLC BEH C18 column (1.7 μm, 50 mm × 2.1 mm, 130 Å, Waters). Mobile phase A (0.1% formic acid in H_2O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish a 12 min gradient elution containing: 8 min of 20−95% B, and 1.5 min of 95% B, 0.5 min of 95−20% B followed by re-equilibrating at 20% B for 2 min. The flow rate was 300 μL/min. Full mass spectra were acquired at a resolution of 70,000 with a target value of 3×10^6 ions or a maximum integration time of 100 ms. Mass acquisition was performed in positive ionization mode with scan range of 200 to 1600 *m/z*.

2.11 Purification of enzymatic reaction products

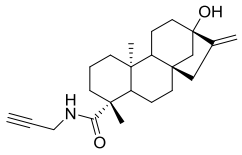
Large-scale enzymatic reaction for NMR analyses was performed in 40 mL of enzymatic reactions in conditions identical to the enzymatic assays. The reaction mixtures utilizing various substrates (steviol, steviolmonoside, and S19G) were individually extracted with 10 mL water-saturated *n*-butanol for three times. All organic layers were combined (~90 mL) and evaporated to dryness under vacuum. The residues were re-dissolved in 500 μL $MeOH/H_2O$ (50%/50%, v/v) and subjected to HPLC analyses (Agilent 1260 Series LC system, Agilent Technologies, Santa Clara, California) by using a Luna C18(2) column (5 μm, 250 × 10 mm, 100 Å, Phenomenex). A linear gradient elution was performed with mobile phases containing mobile phase A (0.1% formic acid in H_2O) and mobile phase B (acetonitrile): 30% to 80% A in B for 20 min, 100% B maintained for 10 min. Products were monitored at 210 nm.

3. Chemistry

Materials

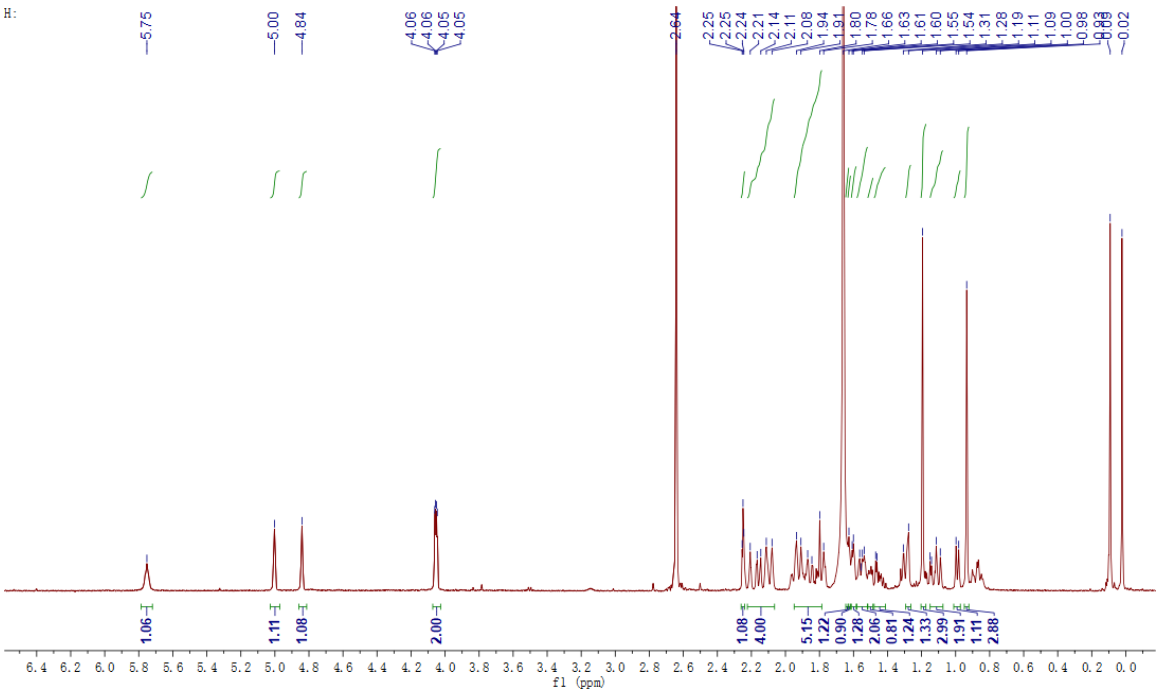
All the reagents were purchased commercially and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Huanghai silica gel plates (HSGF254) by using UV light as a visualizing agent. Flash column chromatography was carried out by using Yantai Xinnuo silica.
^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker Ultrashield™ 400 PLUS (^{1}H: 400 MHz, ^{13}C: 100 MHz) with chemical shift values in ppm relative to TMS (δ_H 0.00 and δ_C 0.00) and residual *D*-chloroform (δ_H 7.26 and δ_C 77.16) as a standard. HRMS was obtained by means of an Agilent 6520 Accurate-Mass Q-TOF instrument.

Synthesis

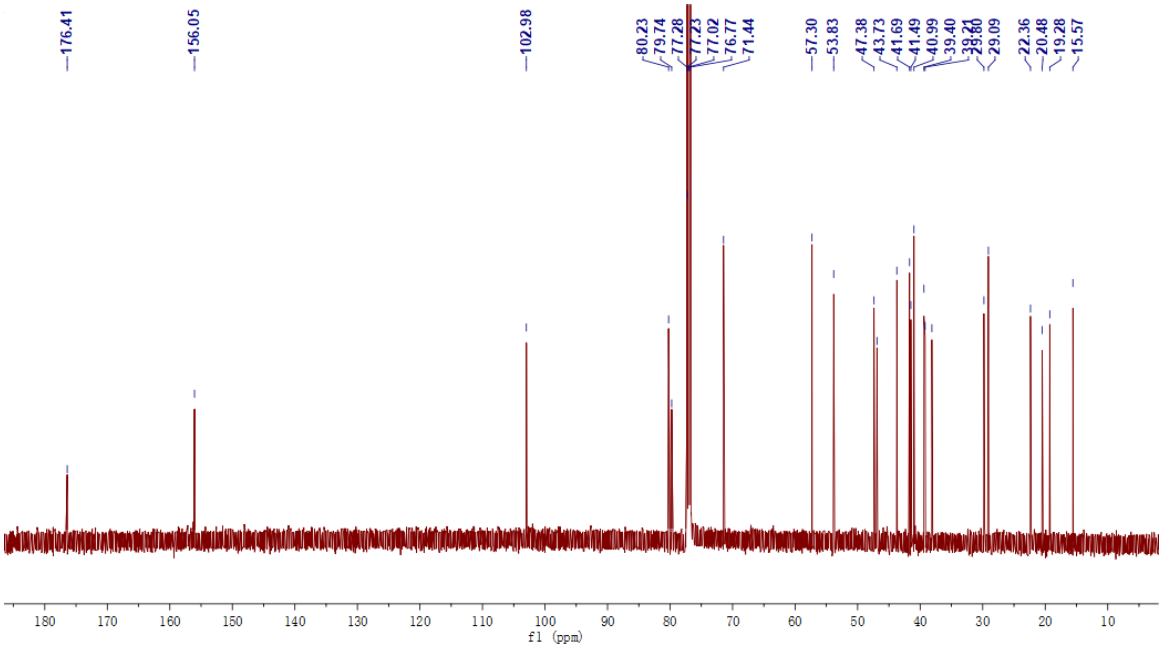


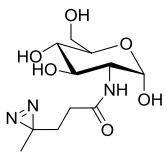
ST-N-yne Steviol (33 mg, 0.1 mmol, 1 eq) was dissolved in 2 mL anhydrous dichloromethane (DCM) and cooled to 0 °C in an ice bath. Oxalyl chloride (130 μL, 1.5 mmol, 15 eq) dissolved in 500 μL anhydrous DCM was added dropwise. The reaction proceeded with stirring at room temperature for 2 h and the solvent was evaporated under vacuum. Then the crude product was directly dissolved in 2 mL anhydrous DCM and cooled to 0 °C, followed by addition of DIPEA (17 μL, 0.15 mmol, 1.5 eq) and prop-2-yn-1-amine (22 mg, 0.4 mmol, 4 eq). The reaction proceeded with stirring at room temperature overnight and was quenched by ice-cold water (10 mL). The organic phase was washed with saturated *aq.* $NaHCO_3$, brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified with reversed-phase semi-preparative HPLC by using a C18 chromatographic column (YMC-Pack-ODS-A 5 μm, 4.6 mm × 250 mm) to afford ST-N-yne (23 mg). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.75 (brs, 1H), 5.00 (s, 1H), 4.84 (s, 1H), 4.05 (dd, J = 2.4 and 5 Hz, 2H), 2.25 (t, J = 2.4 Hz, 1H), 2.14-2.08 (m, 4H), 1.94-1.78 (m, 5H), 1.78-1.31 (m, 7H), 1.31-1.19 (m, 1H), 1.19(s, 3H), 1.13-1.00 (m, 2H), 0.98 (m, 1H), 0.93 (s, 3H), 0.85 (m, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ176.4, 156.1, 102.9, 80.2, 79.7, 71.4, 57.3, 53.8, 47.4, 46.9, 43.7, 41.7, 41.5, 41.0, 39.4, 39.2, 38.1, 29.8, 29.1, 22.4, 20.5, 19.3, 15.6. HRMS-ESI calcd. for $C_{23}H_{34}NO_2$ $[M+H]^+$: 356.2584; Found: 356.2578.

^{1}H-NMR:



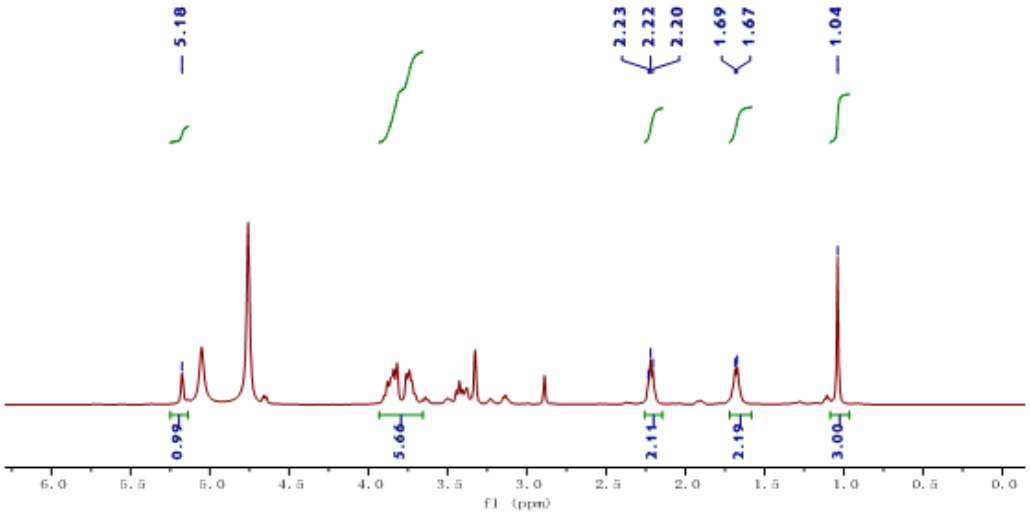
^{13}C-NMR



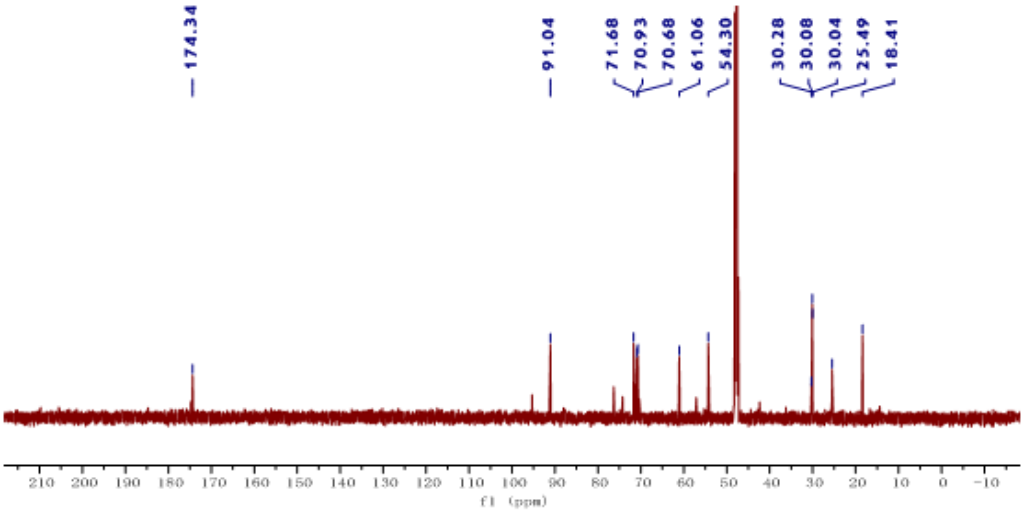


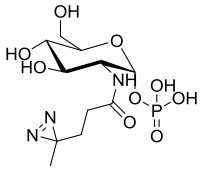
Glc-DA Diazirine-acid (104 mg, 0.8 mmol), 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (110 mg, 0.6 mmol), hydroxyl-benzotriazole (HOBt) (80 mg, 0.6 mmol), and triethylamine (80 μL) were dissolved in anhydrous CH_3OH. The solution was stirred at room temperature in dark for 2 h. *D*-(+)-glucosamine hydrochloride (104 mg, 0.6 mmol) dissolved in a mixed solvent containing 1 mL CH_3OH and 2 mL H_2O was added to the reaction mixture followed by further incubation at room temperature in dark overnight. The reaction mixture was diluted with 15 mL H_2O and extracted with 10 mL DCM for three times. The aqueous phase was concentrated under reduced pressure. The residue was purified with column chromatography on silica gel eluting with DCM/ CH_3OH (5:1 ~ 3:1) to afford a sugar fraction. The sugar fraction was dissolved in ethyl acetate and recrystallized as the titled compound. ^{1}H-NMR (400 MHz, D_2O) δ 5.18 (s, 1H), 4.0-3.5 (m, 5H), 2.21(d, J = 8 Hz, 2H), 1.68 (d, J = 8 Hz, 2H), 1.04 (s, 3H); ^{13}C-NMR (100 MHz, D_2O) δ 174.3, 91.0, 71.7, 70.9, 70.7, 61.1, 54.3, 30.08, 30.04, 25.5, 18.4. HRMS-ESI calcd. for $C_{11}H_{20}N_3O_6$ $[M+H]^+$: 290.1352; Found: 290.1348.

^{1}H-NMR:

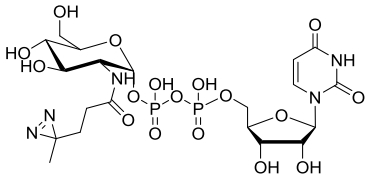


^{13}C-NMR



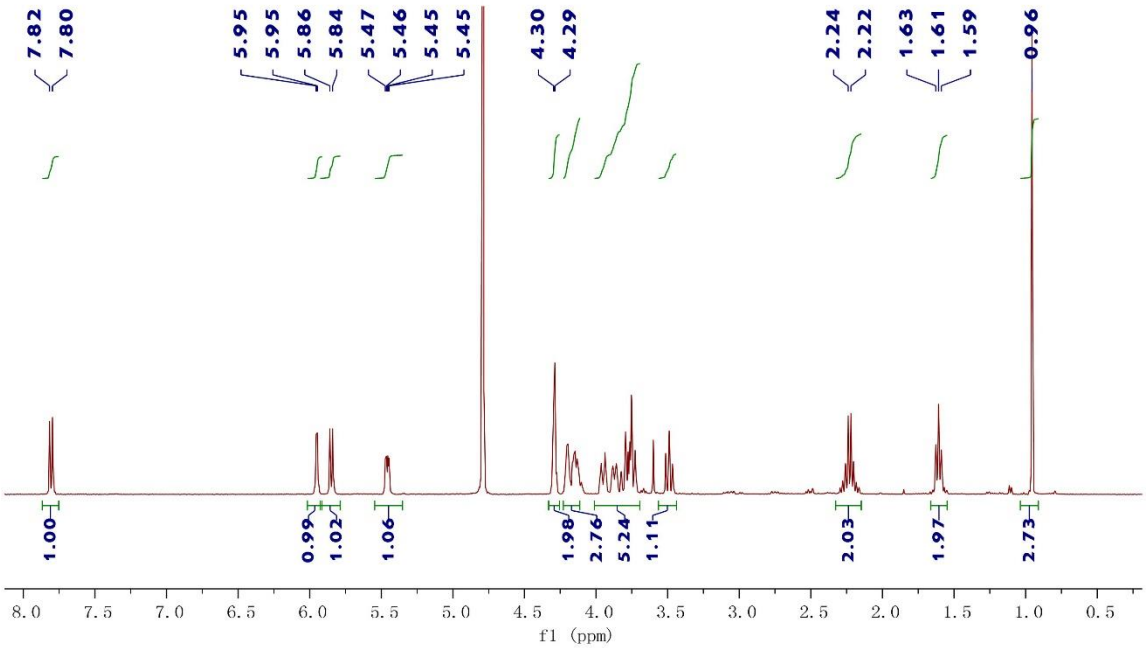


Glc-DA-1-P A reaction mixture (10 mL) was prepared by adding 40 mM Glc-DA, 50 mM ATP, 10 mM $MgCl_2$, and 1.5 mg/mL recombinant *NahK* in 100 mM Tris-HCl, pH 9.0. After incubation at 37°C for 24 h, the mixture was briefly boiled for 2 min and then centrifuged to remove proteins. The supernatant was concentrated under reduced pressure. The residue was purified by silica gel column chromatography. Fractions containing the products were monitored by thin layer chromatography. Product-containing fractions were combined, concentrated under reduced pressure, validated by LC-MS and directly applied to the next step. HRMS-ESI calcd. for $C_{11}H_{21}N_3O_9P$ $[M+H]^+$: 370.1015; Found: 371.0984.

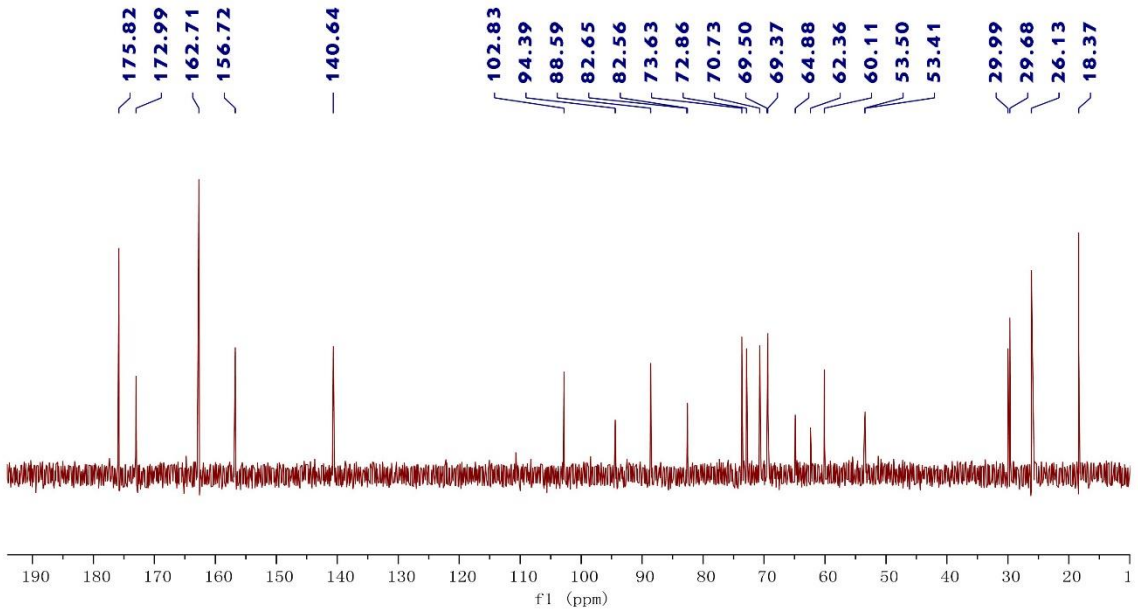


UDPG-DA Preparative-scale synthesis of UDPG-DA was performed in 5 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 160 mM Glc-DA-1-P, 25 mM UTP, 10 mM $MgCl_2$, 1 mg/mL recombinant *GlmU*, and 1 U/mL organic pyrophosphatase (PPase). The reactions were allowed to proceed at 40 °C and monitored by TLC. After reaching equilibrium, the reactions were quenched by boiling for 3 min and 10 U/mL calf intestinal alkaline phosphatase (CIAP) was added. After all nucleotides were hydrolyzed, the reaction mixture was boiled for 5 min and clarified by centrifugation. UDPG-DA was purified by means of a cellulose column. ^{1}H-NMR (400 MHz, D_2O) δ 7.81 (d, J = 8 Hz, 1H), 5.95(s, 1H), 5.85(d, J = 8 Hz, 1H), 5.46(dd, J = 4 and 8 Hz,1H), 4.30(s, 2H), 4.25-4.10 (m, 3H), 4.00-3.70 (m, 5H), 3.49 (t, J = 8 Hz,1H), 2.30 -2.10 (m, 2H), 1.61 (t, J = 8 Hz, 2H), 0.96 (s, 3H); ^{13}C-NMR (100 MHz, D_2O) δ 175.8, 162.7, 156.2, 140.6, 102.8, 94.4, 88.6, 82.7, 73.6, 72.9, 70.7, 69.5, 64.9, 62.4, 60.1, 53.5, 30.0, 29.7, 26.1, 18.4; ^{31}P-NMR (160 MHz, D_2O) δ -11.37, -11.50, -13.15, -13.28. HRMS-ESI calcd. for $C_{20}H_{32}N_5O_{17}P_2$ $[M+H]^+$: 676.1268; Found: 676.1262.

^{1}H-NMR:



^{13}C-NMR



^{31}P-NMR