Supporting Information

A Fluoro Analog of UDP-α-D-Glucuronic Acid Is an Inhibitor of UDP-α-D-Apiose/UDP-α-D-Xylose Synthase

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S1. Materials and instrumentation

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise specified. Escherichia coli DH5α cells were obtained from Bethesda Research Laboratories (Muskegon, MI). The vector pET28b(+) and enzyme KOD DNA polymerase were purchased from Novagen (Madison, WI). DNA modifying enzymes (for restriction digestion and ligation), PCR primers, and the overexpression host E. coli BL21 star (DE3) were acquired from Invitrogen (Carlsbad, CA) and New England Biolabs (NEB, Beverly, MA). LB media were products of Difco (Detroit, MI) or Fisher Scientific, and pre-stained protein markers were the product of NEB. Ni-NTA agarose and kits for DNA gel extraction and spin miniprep were obtained from Qiagen (Valencia, CA). All reagents for SDS-PAGE and Amicon YM-10 filtration products were purchased from Bio-Rad (Hercules, CA) and Millipore (Billerica, MA), respectively. Analytical thin layer chromatography (TLC) was carried out on precoated TLC glass plates (Silica gel, grade 60, F254, 0.25mm layer thickness) obtained from EMD chemicals (Madison, WI). Flash column chromatography was performed on silica gel (230-400 mesh, grade 60) from Sorbent Technologies (Atlanta, GA) by elution with the specified solvents. Protein concentrations were determined by Bradford assay using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS-polyacrylamide gel electrophoresis as described. The general methods and protocols for recombinant DNA manipulations were as described by Sambrook and Russell1. DNA sequencing was performed by the Core Facilities in the Institute of Cellular and Molecular Biology of the University of Texas at Austin. The NMR spectra
were acquired on a Varian Unity Inova 500 or 600 MHz spectrometer housed in the NMR Facility of the Department of Chemistry and Biochemistry, University of Texas at Austin. The MS analyses were carried out at the Mass Spectrometry and Proteomics Facility of the Department of Chemistry and Biochemistry, University of Texas at Austin.

S2. Preparation of AXS and UDP-2F-GlcA (9)

S2.1. Preparation of AXS

Gene axs1 was identified as the coding gene for UDP-d-apiose synthase in Arabidopsis. Based on this information, RNA was extracted from the leaves of Arabidopsis, reverse transcribed into cDNA, the gene axs1 encoding UDP-apiose/UDP-xylose synthase was amplified, and cloned into the pET28b(+) vector. The Escherichia coli BL21 (DE3) cells transformed with this recombinant plasmid expressed very little soluble UDP-apiose synthase protein. The axs1 gene was further cloned into a maltose binding protein (MBP) fusion vector (MalE-pET) at NdeI and EcoRI sites after a silent mutation at nucleotide 552 of the axs1 gene to eliminate an internal NdeI site. The resulting plasmid was then transformed into Escherichia coli BL21 (DE3) cells. The cells were grown at 37 °C in 6 L of LB broth containing kanamycin (50 µg/mL) until the OD600 reached approximately 1.0. Expression of the MBP-apiose synthase fusion protein was induced by the addition of 50 µM IPTG. The cells were cultured for an additional 18 h at 15 °C, harvested by centrifugation and stored at −80 °C. Thawed cells were resuspended in lysis buffer (50 mM Tris buffer (pH 8.0), 1 M NaCl, 10 mM imidazole, 10% glycerol and 1 mM β-mercaptoethanol) and sonicated to lyse the cells. After centrifugation at 25,000 × g for 20 min, the lysate was incubated with 10 mL Ni-NTA agarose (Qiagen) for 1 to 2 h prior to extensive washing with washing buffer (50 mM Tris buffer (pH 8.0), 1 M NaCl, 20 mM imidazole, 10% glycerol and 1 mM β-mercaptoethanol). The MBP-AXS fusion protein was eluted from the Ni-NTA agarose with a buffer similar to that described above containing 250 mM imidazole, spiked with 5% (w/w) His6-tagged TEV protease to cleave off the His10-MBP fragment from the fusion protein, and dialyzed against lysis buffer at 4 °C for 1 to 2 days with 2 changes of lysis buffer. The unwanted His10-MBP and His6-TEV was removed by passing the lysate through a 10 mL Ni-NTA column. The untagged AXS was then concentrated and dialyzed against 50 mM Tris buffer (pH 8.0), 300 M NaCl, 10% glycerol and 1 mM β-mercaptoethanol prior to use.

S2.2. Preparation of UDP-2F-GlcA

Synthesis of compound 19

![Image](image_url)

To a flask containing compound 18 (α/β mixture, 14.89 g, 34.3 mmol), 1H-tetrazole solution in acetonitrile (0.45 M, 114 mL, 51.3 mmol) was added, and the mixture was concentrated in vacuo and dried under high vacuum for 2 h. Anhydrous CH2Cl2 (300 mL) was added, the reaction mixture was cooled to −20 °C, and dibenzyl N,N-diisopropylphosphoramidite (12.3 mL, 51.4 mmol) was added dropwise over 15 min. The reaction was stirred for 2 h at –
20 °C and then warmed to room temperature. After stirring for 4 h at room temperature, the reaction mixture was cooled to −40 °C and mCPBA (41.0 g, 60% purity, 120 mmol) was added portionwise over 30 min. After stirring for 3 h at −40 °C, the reaction mixture was warmed to room temperature and stirred for an additional 5 h. A saturated, aqueous solution of NaHCO₃ (300 mL) was slowly added, and the reaction mixture was extracted with CH₂Cl₂ (200 mL × 2). The combined organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by flash column chromatography to afford compound 19 (14.52 g, 20.9 mmol, 61%).

¹H NMR (CDCl₃, 500 MHz) δ 7.35 − 7.32 (m, 10H, Ph), 5.95 (dd, 1H, J = 3.7, 6.8 Hz, 1-CH), 5.54 (dd, 1H, J = 9.65, 9.64, 11.5 Hz, 3-CH), 5.09 (d, 2H, J = 7.6, CH₂ of Bn), 5.08 (d, 1H, J = 8.8 Hz, CH₂ of Bn), 5.11 − 5.05 (m, 1H, 4-CH), 4.55 (dddd, 1H, J = 2.8, 3.5, 9.6, 48.8 Hz, 2-CH), 3.99 − 3.89 (m, 2H, 5-CH + 6-CH₂), 1.17 (s, 9H, CH₃ of Piv), 1.16 (s, 9H, CH₃ of Piv), 1.13 (s, 9H, CH₃ of Piv).

¹³C NMR (CDCl₃, 125 MHz) δ 177.79 (C=O of Piv), 177.07 (C=O of Piv), 176.22 (C=O of Piv), 135.38 (d, J = 6.4 Hz, Ph-C), 135.29 (d, J = 7.4 Hz, Ph-C), 128.71 (Ph), 128.67 (Ph), 128.64 (Ph), 128.61 (Ph), 128.16 (Ph), 127.93 (Ph), 93.80 (dd, J = 5.5, 22.1 Hz, 1-CH), 87.21 (dd, J = 7.8, 196.5 Hz, 2-CH), 69.87 − 69.50 (m, 5-CH, 2 x CH₂ of Bn, + 3-CH), 66.15 (d, J = 6.9 Hz, 4-CH), 60.77 (6-CH₂), 38.81 (C of Piv), 38.79 (C of Piv), 38.72 (C of Piv), 27.03 (CH₃ of Piv), 27.02 (CH₃ of Piv), 26.99 (CH₃ of Piv).

¹⁹F NMR (CDCl₃, 564 MHz) δ −201.3 (dd, J = 11.6, 48.8 Hz).

³¹P NMR (CDCl₃, 242 MHz) δ −2.77.

HRMS (ESI, positive mode) Calcd for C₃₅H₄₆O₁₁FNaP⁺ 717.2811, Found 717.2812.

Synthesis of compound 20

To an anhydrous CH₂Cl₂ solution of compound 19 (7.35 g, 10.6 mmol), DIBAL-H (1M in CH₂Cl₂, 12.7 mL) was added dropwise over 40 min at −78 °C. The reaction was stirred for 4 h at that temperature and quenched by addition of a saturated, aqueous solution of NH₄Cl (100 mL). The reaction mixture was stirred for an additional 30 min at room temperature and filtered using a celite pad. The filtrate was extracted with CH₂Cl₂ (150 mL × 2). The combined organic layers were dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by flash column chromatography to afford compound 20 (2.76 g, 4.52 mmol, 40%).

¹H NMR (CDCl₃, 500 MHz) δ 7.36 − 7.31 (m, 10H, Ph), 5.95 (dd, 1H, J = 3.7, 6.4 Hz, 1-CH), 5.62 (dd, 1H, J = 9.58, 9.64, 11.9 Hz, 3-CH), 5.09 (d, 2H, J = 7.7, CH₂ of Bn), 5.086 (d, 1H, J = 8.6 Hz, CH₂ of Bn), 5.01 (dd, 1H, J = 9.89, 9.94 Hz, 4-CH), 4.54 (dddd, 1H, J = 2.9, 3.6, 9.5, 48.7 Hz, 2-CH), 3.81 (dddd, 1H, J = 2.1, 3.5, 10.3 Hz, 5-CH), 3.49 (dd, 1H, J = 2.1, 13.1 Hz, 6-CH₂), 3.35 (dd, 1H, J = 3.9, 13.1 Hz, 6-CH₂), 1.17 (s, 9H, CH₃ of Piv), 1.15 (s, 9H, CH₃ of Piv).

¹³C NMR (CDCl₃, 125 MHz) δ 177.93 (C=O of Piv), 177.00 (C=O of Piv), 135.40 (d, J = 7.0 Hz, Ph-C), 135.27 (d, J = 7.5 Hz, Ph-C), 128.72 (Ph), 128.70 (Ph), 128.64 (Ph), 128.17 (Ph), 127.96 (Ph), 93.80 (dd, J = 5.0, 21.9 Hz, 1-CH), 87.21 (dd, J = 7.8, 196.5 Hz, 2-CH), 69.87 − 69.50 (m, 5-CH, 2 x CH₂ of Bn, + 3-CH), 66.15 (d, J = 6.9 Hz, 4-CH), 60.77 (6-CH₂), 38.81 (C of Piv), 38.79 (C of Piv), 38.72 (C of Piv), 27.03 (CH₃ of Piv), 27.02 (CH₃ of Piv), 26.99 (CH₃ of Piv).
87.27 (dd, $J = 8.5$, 196.5 Hz, 2-CH), 71.71 (5-CH), 69.92 (d, $J = 5.5$ Hz, CH$_2$ of Bn), 69.732 (d, $J = 5.3$ Hz, CH$_2$ of Bn), 69.11 (d, $J = 19.4$ Hz, 3-CH), 67.04 (d, $J = 7.5$ Hz, 4-CH), 60.34 (6-CH$_2$), 38.89 (C of Piv), 38.84 (C of Piv), 27.05 (CH$_3$ of Piv), 27.02 (CH$_3$ of Piv).

$^{19}$F NMR (CDCl$_3$, 470 MHz) $\delta$ –200.74 (dd, $J = 12.0$, 48.7 Hz).

$^{31}$P NMR (CDCl$_3$, 202 MHz) $\delta$ –2.03.

HRMS (ESI, positive mode) Calcd for C$_{30}$H$_{40}$O$_{10}$FNaP$^+$ 633.2235, Found: 633.2235.

Synthesis of compound 23

To CrO$_3$ (1.28 g, 12.8 mmol) in H$_2$O (20 mL), conc. H$_2$SO$_4$ (1.2 mL, 21.6 mmol) was added dropwise over 5 min at 0°C. The CrO$_3$/H$_2$SO$_4$ mixture was added to a solution of compound 19 (1.40 g, 2.29 mmol) in acetone (80 mL) at 0°C, and the reaction mixture was stirred overnight while allowing the temperature to rise to room temperature. The reaction mixture was then evaporated to remove the acetone and extracted with CHCl$_3$ (50 mL × 7). The combined CHCl$_3$ layers were dried over Na$_2$SO$_4$ and concentrated. The crude compound 21 was dissolved in MeOH (70 mL) and 10 % Pd/C (300 mg) was added and stirred under a hydrogen atmosphere (1 atm) for 3 h. The reaction mixture was filtered using a celite pad and washed with MeOH (30 mL). The resulting filtrate was concentrated in vacuo and purified by DEAE ion exchange resin column chromatography (6 × 25 cm, total 2 L of 0 ~ 0.5 M NH$_4$HCO$_3$ gradient). The fractions containing the product were collected and concentrated in vacuo. To the residue was added 30 mL of water and lyophilized (× 2) to give the compound 22 (340 mg, 0.77 mmol, 33%).

Compound 22 (220 mg, 0.50 mmol) was dissolved in anhydrous MeOH (30 mL), cooled to 0°C, and treated with a 4.37 M solution of NaOMe in MeOH (1.6 mL, 7.0 mmol). After 7 h at room temperature, the reaction mixture was concentrated and purified by DEAE ion exchange resin column chromatography (2.5 × 20 cm, total 1 L of 0 ~ 0.5 M NH$_4$HCO$_3$ gradient). The fractions containing the product were collected and concentrated in vacuo. To the residue was added 25 mL of water and lyophilized (× 2) to give compound 23 (119 mg, 0.43 mmol, 86%). In order to change the salt form, compound 23 (110 mg, 3.98 mmol) was dissolved in 1:1 mixture of 10 mL of water and triethylamine. After stirring overnight at room temperature, the reaction mixture was concentrated to afford 151 mg of TEA salt of compound 22 (1.63 eq. TEA salt).

$^1$H NMR (D$_2$O, 600 MHz) $\delta$ 5.57 (dd, 1H, $J = 3.6$, 7.8 Hz, 1-CH), 4.35 (ddddd, 1H, $J = 2.2$, 3.7, 8.8, 43.7 Hz, 2-CH), 4.03 (d, 1H, $J = 10.2$ Hz, 5-CH), 3.91 (ddd, 1H, $J = 9.39$, 9.39, 12.9 Hz, 3-CH), 3.46 (dd, 1H, $J = 9.7$, 9.8 Hz, 4-CH).

$^{13}$C NMR (D$_2$O, 150 MHz) $\delta$ 175.26 (6-C=O), 91.77 (dd, $J = 5.2$, 22.5 Hz, 1-CH), 89.29 (dd, $J = 7.7$, 188.0 Hz, 2-CH), 72.54 (5-CH), 71.38 (d, $J = 8.0$ Hz, 4-CH), 70.78 (d, $J = 17.9$ Hz, 3-CH).

$^{19}$F NMR (D$_2$O, 564 MHz) $\delta$ –200.16 (dd, $J = 12.6$, 49.5 Hz).

$^{31}$P NMR (D$_2$O, 242 MHz) $\delta$ –1.40.
HRMS (ESI, negative mode) Calcd for C_{6}H_{9}FO_{9}P^{-} 274.9974, Found: 274.9976.

**Synthesis of compound 9**

To the TEA salt of compound 23 (105 mg, 0.277 mmol) in dry pyridine (10 mL) were added 1H tetrazole (50 mg, 0.710 mmol) and UMP-morpholidate (247 mg, 0.360 mmol). After 3 days at room temperature, the reaction mixture was concentrated in vacuo. Diethyl ether (10 mL) and H_{2}O (10 mL) were added to the residue, and the water layer was concentrated and purified by DEAE ion exchange resin column chromatography (2.5 × 20 cm, total 1 L of 0 ~ 0.4 M NH_{4}HCO_{3} gradient). Fractions containing the product were collected and dried in vacuo, and lyophilized (∗ 2) to afford the compound 9 (35 mg, 0.0584 mmol, 21 %).

^{1}H NMR (D_{2}O, 600 MHz) δ 7.85 (d, 1H, J = 8.1 Hz, 6'-CH), 5.88 (d, 1H, J = 4.8 Hz, 1'-CH), 5.86 (d, 1H, J = 8.1 Hz, 5'-CH), 5.69 (dd, 1H, J = 3.5, 11.4 Hz, 1''-CH), 4.37 (dddd, 1H, J = 2.4, 3.5, 9.5, 49.1 Hz, 2''-CH), 4.26 – 4.23 (m, 2H, 2'-CH + 3'-CH), 4.12 – 4.03 (m, 2H, 5'-CH_{2}), 4.17 – 4.14 (m, 1H, 4'-CH), 4.12 – 4.03 (m, 2H, 5'-CH_{2}), 4.06 (d, 1H, J = 10.1 Hz, 5''-CH), 3.91 (dd, 1H, J = 9.4, 9.4, 12.8 Hz, 3''-CH), 3.44 (dd, 1H, J = 9.6, 9.9 Hz, 4''-CH).

^{13}C NMR (D_{2}O, 150 MHz) δ 175.72 (6''-C=O), 166.19 (4-C=O), 151.83 (2-C=O), 141.61 (6-CH), 102.69 (5-CH), 92.31 (dd, J = 5.5, 22.4 Hz, 1''-CH), 89.19 (dd, J = 8.0, 188.2 Hz, 2''-CH), 88.12 (1'-CH), 83.39 (d, J = 9.2 Hz, 4'-CH), 73.73 (2'-CH), 72.79 (5''-CH), 71.37 (d, J = 8.0 Hz, 4''-CH), 70.72 (d, J = 17.9 Hz, 3''-CH), 69.72 (3'-CH), 64.90 (d, J = 5.5 Hz, 5'-CH_{2}).

^{19}F NMR (D_{2}O, 564 MHz) δ –200.73 (dd, J = 12.8, 49.1 Hz).

^{31}P NMR (D_{2}O, 242 MHz) δ –1.54 (d, J = 17.9 Hz), –13.39 (d, J = 17.9 Hz).

HRMS (ESI, negative mode) Calcd for C_{15}H_{20}FN_{2}O_{17}P_{2}^{-} 581.0227, Found: 581.0228.

**S3. In vitro enzyme activity assays**

S3.1. Activity assay of AXS using compound 9 as a substrate

To a solution containing compound 9 (400 µM), and NAD^{+} (400 µM) in 50 mM Tris buffer (pH 8.0) at room temperature was added AXS (10 µM) to initiate the enzyme reaction (total volume 250 µL). The reaction mixture was incubated for 40 h at which time a 40 µL aliquot of the reaction mixture was taken and filtered using a YM10 microcentrifugal filter in order to remove AXS. The filtrate (20 µL) was subjected to HPLC analysis using a Dionex CarboPacTM PA1 4 × 250 mm column. The column was eluted using a linear gradient of 5 ~ 30% of solvent B over 40 min and then 30 ~ 100% of solvent B over another 5 min (flow rate; 1 mL/min, solvent A: H_{2}O, solvent B: 1M NH_{4}OAc). The result is shown in Figure S1.
**Figure S1.** HPLC traces of AXS reaction. The reaction mixture containing 10 µM AXS, 400 µM NAD\(^+\) and 400 µM UDP-2F-GlcA (9) in 50 mM Tris buffer (pH 8.0) was incubated for (a) 0 h, (b) 40 h.

**S3.2. AXS inhibition assay using compound 9 as an inhibitor**

The reaction mixtures containing AXS (10 µM), NAD\(^+\) (400 µM), UDP-GlcA (3) (400 µM) and different concentrations of 9 (0, 400 µM, or 4 mM) in 50 mM Tris buffer (pH 8.0) (total volume 250 µL) were incubated at room temperature. At each given time point (0, 2, 6, 10, 14, and 20 h), reaction aliquots were analyzed by HPLC as described above. The results are shown in **Figure S2 – S4**.
**Figure S2.** HPLC traces of AXS reaction. The incubation mixture containing 10 µM AXS, 400 µM NAD⁺ and 400 µM UDP-GlcA (3) in 50 mM Tris buffer (pH 8.0) was incubated for (a) 0 h, (b) 2 h, (c) 6 h, (d) 10 h, (e) 14 h and (f) 20 h.

![HPLC traces of AXS reaction](image)

**Figure S3.** HPLC traces of AXS reaction. The incubation mixture containing 10 µM AXS, 400 µM NAD⁺, 400 µM UDP-GlcA (3) and 400 µM UDP-2F-GlcA (9) in 50 mM Tris buffer (pH 8.0) was incubated for (a) 0 h, (b) 2 h, (c) 6 h, (d) 10 h, (e) 14 h and (f) 20 h.
Figure S4. HPLC traces of AXS reaction. The incubation mixture containing 10 µM AXS, 400 µM NAD+, 400 µM UDP-GlcA (3) and 4 mM UDP-2F-GlcA (9) in 50 mM Tris buffer (pH 8.0) was incubated for (a) 0 h, (b) 2 h, (c) 6 h, (d) 10 h, (e) 14 h and (f) 20 h.

S3.3. Examining the stability of UDP-GlcA (3), UDP-xylose (8), and UDP-2F-GlcA (9)

Individual reaction mixtures containing 400 µM of 3, 8, or 9 in 50 mM Tris buffer (pH 8.0) were incubated for 44 h, respectively (total volume 100 µL). At each time point (0 h, 20 h, and 44 h), 20 µL of the each reaction mixture was subjected to HPLC analysis as described above. The results are shown in Figure S5.

Figure S5. HPLC trace showing the stability of 3, 8, and 9. The reaction mixtures containing 400 µM of (A) UDP-GlcA (3), (B) UDP-xylose (8), or (C) UDP-2F-GlcA (9) were incubated in 50 mM Tris buffer (pH 8.0) for 44 h.
S3.4. Structural characterization of the oxime derivative of UDP-4-KX

A reaction mixture containing 10 µM AXS, 400 µM NAD\(^+\), and 400 µM UDP-GlcA (3) in 50 mM Tris buffer (pH 8.0) was incubated for 12 h. An aliquot of the reaction mixture was then subjected to HPLC analysis as described above. A second 40 µL aliquot of the reaction mixture was mixed with 40 µL of 400 mM H\(_2\)NOH in water. The mixture was filtered using a YM10 centrifugal filter as described above and incubated for an additional 3 h before being subjected to HPLC analysis (Figure 1). The peak at 35 min was collected, lyophilized, and subjected to MS analysis. The result is shown in Figure S6.

![Figure S6](T:\Byoung Joon\Other\CSH\Oxime full MS_1)

**Figure S6.** ESI (negative mode) MS data for the oxime adduct of UDP-4-KX, which is the peak with retention time of 35 min in HPLC trace.
S4. References


S5. Spectroscopic data

Compound 19 ('H NMR)

![NMR spectrum of Compound 19](image)
Compound 20 (H NMR)

\[ \text{PivO} \]
\[ \text{OH} \]
\[ \text{F} \]
\[ \text{O} \]
\[ \text{O} \]
\[ \text{P} - \text{OBn} \]
\[ \text{OBn} \]
Compound 20 (HRMS)
Compound 23 (HRMS)
Compound 9 (1H NMR)