**SUPPLEMENTARY DATA**

**Supplementary data includes:**

a) **Supplementary methods**
b) **Supplementary figures (Figure S1 – Figure S4)**
c) **Supplementary references**

**a) Supplementary methods**

**Synthesis of UDP-4-deoxy-4-fluoro-N-acetylglucosamine**

Synthesis of UDP-4-deoxy-4-fluoro-N-acetylglucosamine (UDP-4F-GlcNAc) was accomplished as described in the scheme in Figure S1 starting from N-acetylgalactosamine (GalNAc). The hydroxyls on commercially available GalNAc were protected at C4, C6 positions using a benzylidine protecting group and C1, C3 positions by acetylation using acetic anhydride respectively (Nishimura, S.-i., Hato, M., et al. 2012). The C4 hydroxyl was then freed up for deoxyfluorination by removing the benzylidene group using $Pd(OH)_2/C$ in the presence of triethylsilane followed by selective protection of C6 hydroxyl using a trityl chloride (Santra, A., Ghosh, T., et al. 2013). Reaction of the free hydroxyl from previous step, with (diethylamino) sulfur trifluoride (DAST) installed the C4 fluoride functionality with an equatorial configuration. De-*O*-acetylation at C1 and C3 yielded 4F-GlcNAc (**6**).

Subsequently, the synthesis was continued enzymatically using enzymes NahK and GlmU. NahK, an N-acetylhexosamine 1-phosphate kinase from *Bifidobacterium longum* was found to be quiet promiscuous to various GlcNAc 3- and 4- epimers (Cai, L., Guan, W., et al. 2009). This combined with the previous known use of *Escherichia coli* GlmU with 4F-GlcNAc as a substrate (Schultz, V.L., Zhang, X., et al. 2017) helped us in the design of a single pot reaction to directly synthesize UDP-4F-GlcNAc (**7**) from 4F-GlcNAc in high yields.

**Materials**

All reagents were purchased from commercial sources and used without any further purification. Flash chromatography was performed using silica gel (230-400 mesh, Silicycle Inc.) and reaction progress was monitored using Thin Layer Chromatography (Silica gel 60 $F_{254}$ plates, 2.5 X 7.5 cm, Sigma-Aldrich).

The chemicals used in the synthesis include: 2-acetamido-2-deoxy-D-galactopyranose (Aldrich) (GalNAc), benzaldehydedimethyl acetal (99%, Alfa Aesar), camphorsulfonic acid (98%, Alfa Aesar), anhydrous pyridine (99.8%, Sigma-Aldrich), acetic anhydride, $Pd(OH)_2$ on carbon (Sigma-Aldrich), triethylsilane (99%) (Sigma-Aldrich), triethylamine (Sigma), triphenylmethyl chloride (98%, Aldrich), dimethylaminopyridine (Sigma-Aldrich), diethylamino sulfur trifluoride (Sigma-Aldrich), acetic acid (≥99%, Sigma-Aldrich), AG® 501-X8(D) resin (Bio-Rad), Biogel P-2 (Bio-Rad), activated charcoal (500 mg, SiliCycle), *B. longum* NahK ( 5 U, Creative Enzymes), *E. coli* GlmU (5 U, Galen Laboratory Supplies), inorganic pyrophosphatase (*S. crevisiae,* Sigma), calf intestinal alkaline phosphatase (Sigma).

**Synthesis of 2-acetamido-4,6-*O*-benzylidene-2-deoxy-1,3-di-*O*-acetyl-D-galactopyranose (1) (Nishimura, S.-i., Hato, M., et al. 2012)**:

Benzaldehydedimethyl acetal (3.04 mL, 20 mmol) and a catalytic amount of camphorsulfonic acid (37.2 mg, 0.16 mmol) were added to a solution of 2-acetamido-2-deoxy-D-galactopyranose (GalNAc) (1.768 g, 8 mmol) in 40 mL acetonitrile. The reaction was stirred at room temperature for 15 h and evaporated under reduced pressure. Six milliliters of acetic anhydride (63 mmol) were added slowly to the solution of the residue in 20 mL pyridine at 0 $^{o}$C. After an overnight reaction at room temperature, the solvent was removed and the residue dissolved in 250 mL chloroform. The resulting solution was washed with 1M HCl solution, sat. $NaHCO_3$, and brine (250 mL each). The chloroform layer was further dried using anhydrous $MgSO_4$, followed by concentration and purification using flash chromatography (40:1, chloroform-methanol) to yield product **1** in the form of a white solid (2.98 g, 95.3%).

**2-Acetamido-2-deoxy-1,3-di-*O*-acetyl-D-galactopyranose (2) (Santra, A., Ghosh, T., et al. 2013):**

To a mixture of compound **1** (2.98 g, 7.58 mmol) and 10% $Pd(OH)_2$/C (300mg) in methanol (35mL), $Et_3SiH$ (4 mL, 25 mmol) was added in 200 µl portions every 10 minutes. The reaction was monitored by TLC and was found to be completed after 4 h. The catalyst was removed by filtration with Celite and the filtrate evaporated and the residue purified by silica gel chromatography (7:1 chloroform-methanol) to afford (1.88 g, 81.2%) of product **2**.

**2-Acetamido-2-deoxy-1,3-di-*O*-acetyl-6-*O*-triphenylmethyl-D-galactopyranose (3) (Nishimura, S.-i., Hato, M., et al. 2012):**

To a solution of **2** (1.88 g, 6.2 mmol) in dichloromethane (40 mL) at 0 °C was added triethylamine (1.5 mL, 10.67 mmol), triphenylmethyl chloride (3.5 g, 12.6 mmol), and catalytic amount of 4-dimethylaminopyridine (150 mg). After stirring at room temperature overnight, the mixture was evaporated under reduced pressure and the residue was purified by silica gel chromatography (1:20 hexane-ethyl acetate) resulting in a white powdery product **3** (2.95 g, 87.5%).

**2-Acetamido-2,4-dideoxy-1,3-di-*O*-acetyl-4-fluoro-6-*O*-triphenylmethyl-D-galactopyranose (4) (Nishimura, S.-i., Hato, M., et al. 2012):**

Diethylamino sulfur trifluoride (4.1 mL, 31 mmol) was added in 200 µL portions, dropwise at 15 minute intervals to a solution of **3** (2.95 g, 5.4 mmol) and pyridine (3 mL, 35.6 mmol) in dry dichloromethane (5 mL) at −40 °C. The mixture was then allowed to warm to room temperature over 3 h, and was quenched by the addition of MeOH at 0 °C. The residue was diluted with chloroform and the solution was washed

with water, sat. $NaHCO_3$ aq., and brine and then was dried over anhydrous $MgSO_4$. The solvent was removed by evaporation and purified by flash chromatography (1:6 hexane-ethyl acetate) to yield **4** (2.2 g, 77%).

**2-Acetamido-2,4-dideoxy-1,3-di-*O*-acetyl-4-fluoro-D-glucopyranose (5) (Nishimura, S.-i., Hato, M., et al. 2012):**

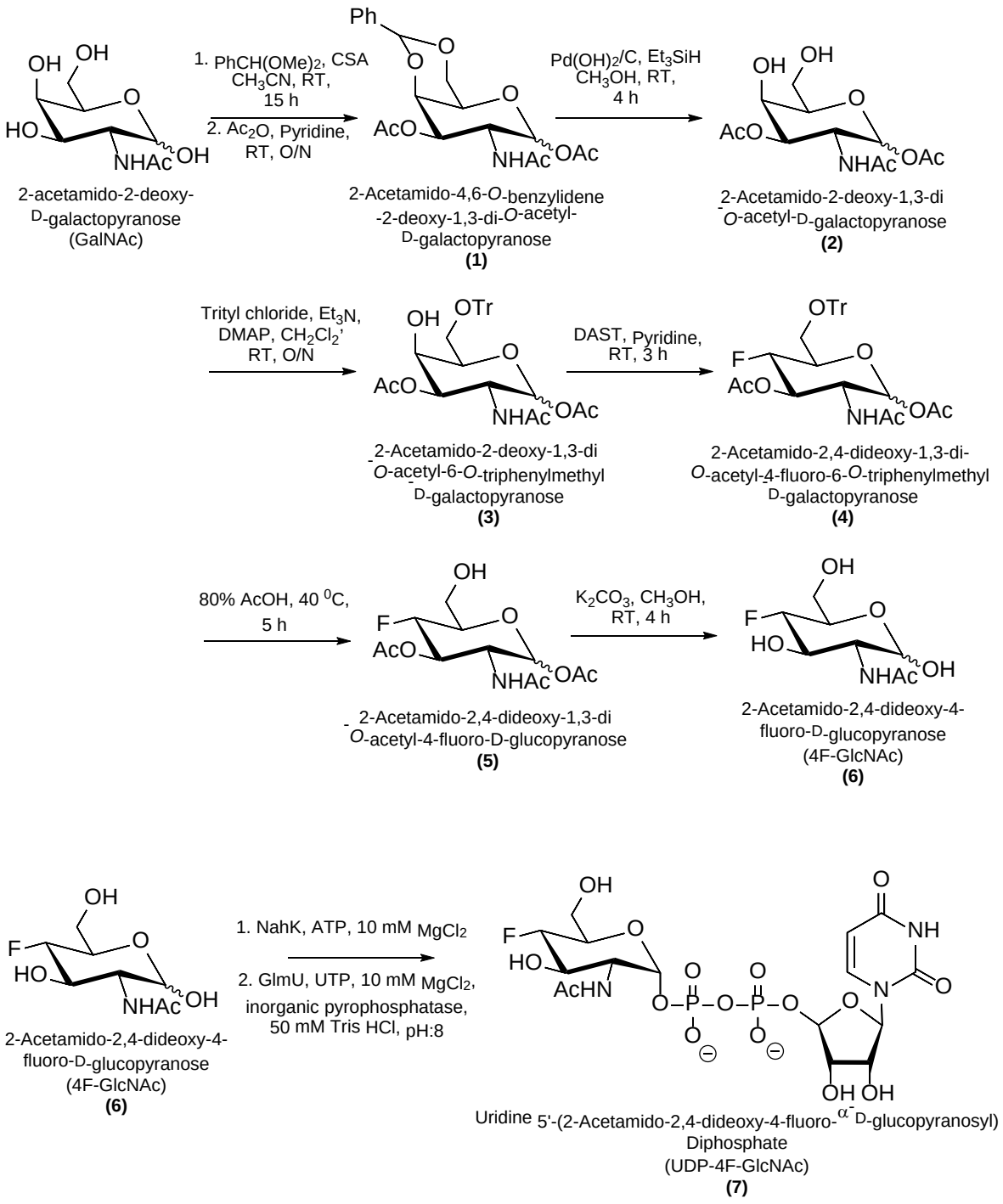
The solution of **5** (2.2 g, 4 mmol) in 80% acetic acid-water (30 mL) was stirred for 5 h at 40 °C. The solution was evaporated under reduced pressure and purified by silica chromatography (6:1 EtOAc-Hexane) to give **5** (0.91 g, 74.6%) as a white solid.

**2-Acetamido-2,4-dideoxy-4-fluoro-D-glucopyranose (6) (Nishimura, S.-i., Hato, M., et al. 2012):**

To a solution of **5** (910 mg, 2.96 mmol) in methanol (50 mL) was added at -40 °C $K_2CO_3$ (820 mg, 6 mmol). The mixture was stirred at room temperature for 4 h and neutralized by addition with AG® 501-X8(D) (H+) resin. The mixture was filtered and the solution was evaporated before purification using flash chromatography on silica gel (5:1 chloroform-methanol) to give **6** (480 mg, 72.6%) (Figure S2 - $^{1}H$, $^{13}C$-HSQC of **6**).

**Uridine 5'-(2-Acetamido-2,4-dideoxy-4-fluoro-α-D-glucopyranosyl) Diphosphate (7) (Zhai, Y., Liang, M., et al. 2012):**

UDP-4F-GlcNAc was enzymatically synthesized in a 5 mL reaction containing 25 mM 4F-GlcNAc (**6**), 30 mM UTP, 30 mM ATP, 10 mM MgCl, 2 U each of *B. longum* NahK and *E.coli* GlmU, 10 U inorganic pyrophosphatase in 50 mM Tris/HCl buffer (pH 8.0). The pH was monitored and adjusted using a 1 M solution of KOH. Once the pH stabilized, 4 h, an aliquot of GlmU was added and reaction continued for 4 h. Once the reaction was completed and confirmed by $^{1}H$ NMR the solution was passed through a 10 KDa centrifugal filter followed by treatment with 10 U of calf intestinal alkaline phosphatase to completely hydrolyze nucleotides, (2 h at 37 °C). The mixture was again passed through a centrifugal filter to remove all the protein content. UDP-4FGlcNAc was finally purified from other reaction components using two rounds of gel filtration on a P-2 column (1 x 100 cm) and its identity/purity confirmed using NMR (Figure S3 and S4) and MALDI calculated for $C_{17}H_{24}FN_3O_{16}P_2Na_2$ ($[M-2Na-H]^-$) 608.340, found 608.249.




2-acetamido-2-deoxy-D-galactopyranose (GalNAc)

2-Acetamido-4,6-*O*-benzylidene-2-deoxy-1,3-di-*O*-acetyl-D-galactopyranose **(1)**

2-Acetamido-2-deoxy-1,3-di-*O*-acetyl-D-galactopyranose **(2)**

2-Acetamido-2-deoxy-1,3-di-*O*-acetyl-6-*O*-triphenylmethyl-D-galactopyranose **(3)**

2-Acetamido-2,4-dideoxy-1,3-di-*O*-acetyl-4-fluoro-6-*O*-triphenylmethyl-D-galactopyranose **(4)**

2-Acetamido-2,4-dideoxy-1,3-di-*O*-acetyl-4-fluoro-D-glucopyranose **(5)**

2-Acetamido-2,4-dideoxy-4-fluoro-D-glucopyranose (4F-GlcNAc) **(6)**

2-Acetamido-2,4-dideoxy-4-fluoro-D-glucopyranose (4F-GlcNAc) **(6)**

Uridine 5'-(2-Acetamido-2,4-dideoxy-4-fluoro-α-D-glucopyranosyl) Diphosphate (UDP-4F-GlcNAc) **(7)**

Figure S1

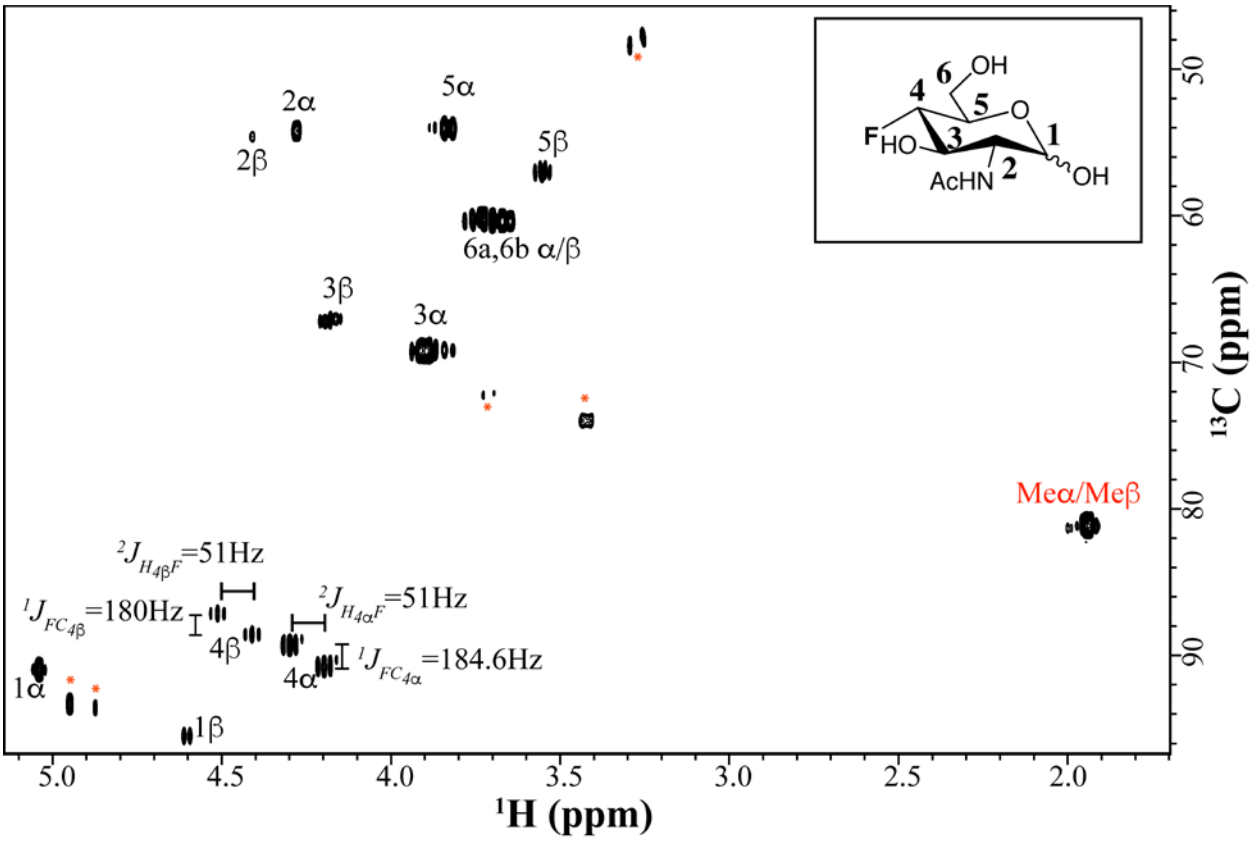



**Figure S2.** $^1$H, $^{13}$C-HSQC of 4F-GlcNAc (inset) dissolved in $D_2O$ collected on a Bruker Avance III 500 MHz spectrometer, equipped with a Z-gradient QXI probe, at 298K. Arabic numerals indicate atom assignments. 4F-GlcNAc in solution alternates between the alpha (α) and beta (β) configuration at the anomeric end. 4F-GlcNAc exists in ca. 70% alpha (α) and 30% beta form. Signals corresponding to the □ and β anomers are indicated with Greek letters. The methyl (Me) signal of the NHAc group, is shown in red to indicate signal folding. Red asterisks denote impurities. GlcNAc $^1$H, $^{13}$C-4 signals are split in the proton and carbon dimensions due to $^1$H-$^{19}$F and $^{13}$C-$^{19}$F coupling.

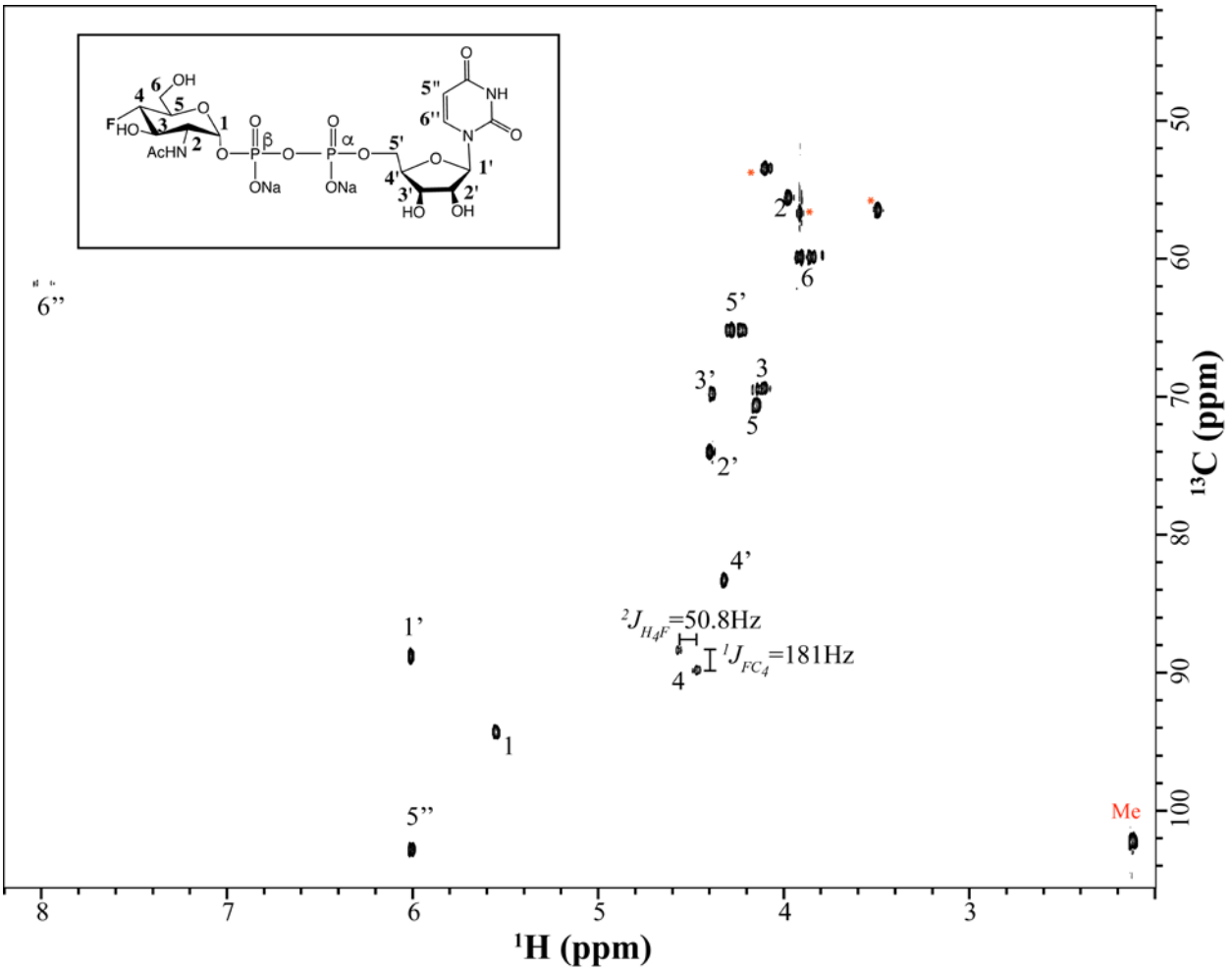



**Figure S3.** $^{1}$H, $^{13}$C-HSQC of the chemo-enzymatically synthesized UDP-4F-GlcNAc (inset) dissolved in 1:1 $H_2O$:$D_2O$ collected on a Bruker Avance III 500 MHz spectrometer, equipped with a Z-gradient QXI probe, at 310K. TSP was used as $^{1}$H internal reference, $^{13}$C resonances were referenced indirectly. Arabic numerals indicate atom assignments. The methyl of the NHAc group is depicted in red to indicate signal folding. Red asterisks denote BIS-TRIS buffer signals. Note that GlcNAc $^{1}$H,$^{13}$C-4 cross-peak due to $^{1}$H-$^{19}$F and $^{13}$C-$^{19}$F coupling confirming that the product is fluorinated at C4. Additionally, aiding atom assignments, the two-bond $^{19}$F-$^{13}$C scalar couplings to C3 and C5 were measured with values of $^{2}J_{F\text{-}C4}$=22Hz and $^{2}J_{F\text{-}C4}$=18.6Hz, respectively.

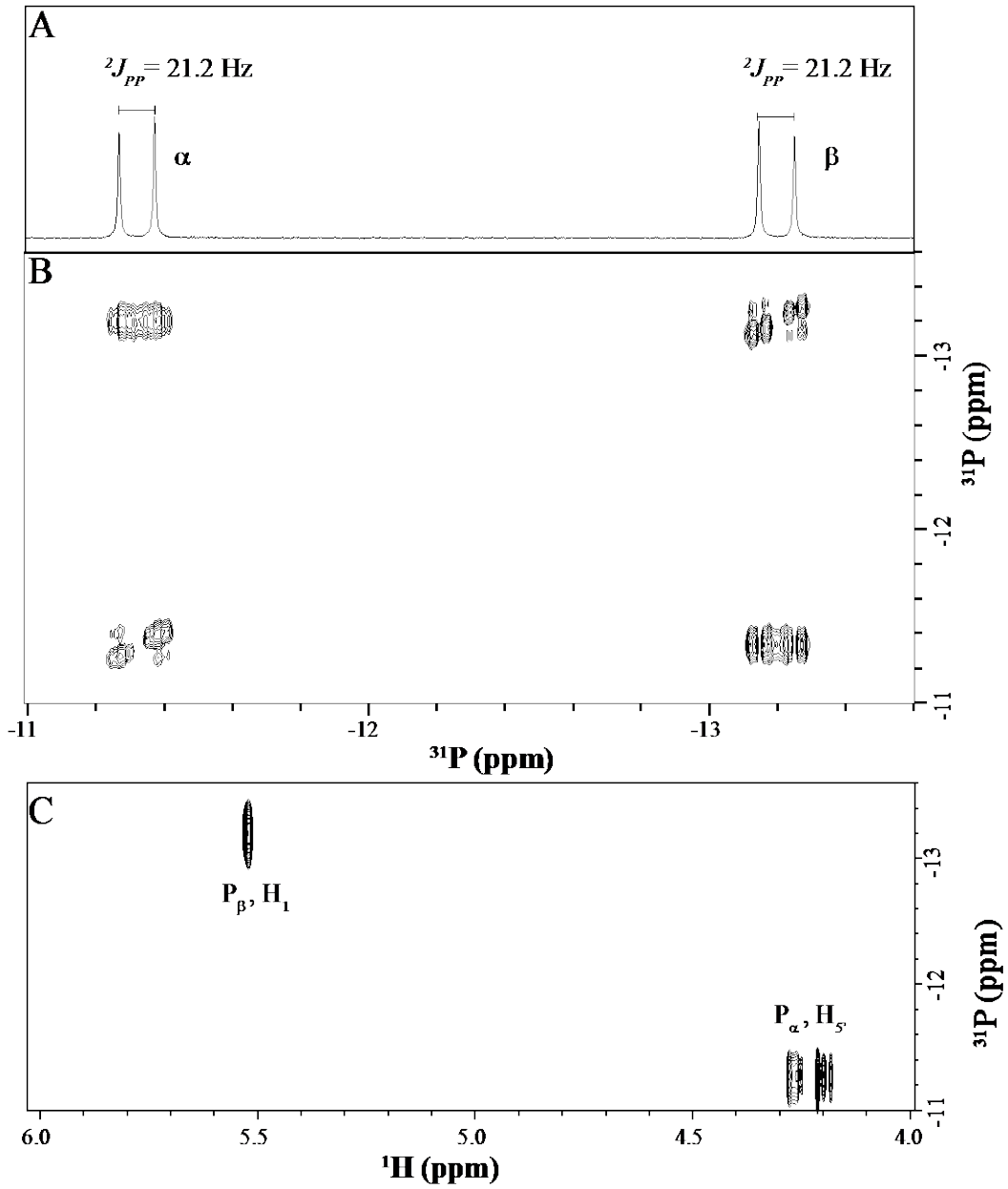



**Figure S4.** All $^{31}$P 1-dimensional (1D) and 2-dimensional (2D) experiments of UDP-4F-GlcNAc were collected on a Bruker Avance III 500 MHz spectrometer, equipped with a Z-gradient QXI probe, at 310K. **Panel A.** 1D-$^{31}$P experiment depicting the signal splitting due to $^{31}$P-$^{31}$P scalar coupling between α and β phosphorus nuclei. **Panel B.** $^{31}$P-DQF-COSY spectrum, showing cross-peaks due to $^{31}$P-$^{31}$P scalar coupling. **Panel C**. $^1$H, $^{31}$P-HSQC spectrum. The spectrum shows a cross-peak between $^{31}$Pβ and the $^1$H$_1$ of GlcNAc as well as a cross-peak $^{31}$Pα and the uridine H5' from the ribose ring confirming the connection between uridine and diphosphate 4F-GlcNAc.

**References:**

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