Supporting Information

Mechanistic Investigation of the Radical *S*-Adenosyl-L-methionine (SAM) Enzyme DesII Using Fluorinated Analogs

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Contents

1S Materials

1S.1 Instrumentation

NMR spectra were recorded using a Varian DirectDrive 600 MHz, Varian Inova 500 MHz or Varian DirectDrive 400 MHz NMR spectrometer at the Nuclear Magnetic Resonance Facility at the University of Texas at Austin. ^{13}C and ^{31}P NMR spectra were recorded with proton broad-band decoupling, and deuterated solvents were used as the internal reference. High-resolution mass spectra (HRMS) were acquired at the Mass Spectrometry Facility (MSF) in the Department of Chemistry at the University of Texas at Austin.

High-performance liquid chromatography (HPLC) was performed using a Beckman System Gold 125 Solvent Module with a 166 detector equipped with a Dionex anion exchange column (CarboPac PA1, Thermo Scientific, 4 × 250 mm or 9 × 250 mm). Silica gel column chromatography employed silica gel 60 (230–400 mesh). DEAE anion exchange chromatography employed sepharose CL-6B (GE Healthcare) resin.

1S.2 Reagents, enzymes and conditions

All chemical reagents and anhydrous solvents were purchased from commercial sources and used without further purification unless otherwise specified. Tetrahydrofuran was distilled with sodium benzophenone ketyl under argon. Dichloromethane was dried over CaH_2 and then distilled. *S*-adenosyl-L-methionine (SAM) was prepared as previously described.[1] The concentration of SAM was determined by measuring its absorption at 260 nm calibrated with the extinction coefficient for adenosine at 260 nm (ε_{260} = 17000 $cm^{-1}M^{-1}$). TDP-sugar concentrations were determined in a similar way using the extinction coefficient for thymidine at 267 nm (ε_{267} = 9600 $cm^{-1}M^{-1}$).

All enzymatic activity assays were carried out anaerobically in a Coy anaerobic glove box under an atmosphere of > 98% N_2 and ∼ 1.5% H_2 with < 1 ppm O_2. Assay buffers were prepared aerobically, degassed by bubbling with N_2 for at least 10 min prior to transfer to the the glove box, where they were stirred open to the anaerobic atmosphere overnight to allow complete equilibration. Dry reagents were allowed to equilibrate with the anaerobic atmosphere in the glove box for at least 1 h prior to use. DesII from *Streptomyces venezuelae* was expressed heterologously from *Escherichia coli* and purified as previously described.[2] The DesII [Fe-S] clusters were reconstituted as previously reported,[3] and the enzyme was maintained at ca. 10 °C under anaerobic conditions prior to use.

2S Methods

2S.1 HPLC

HPLC analysis was performed using a Dionex column (CarboPac PA1, Thermo Scientific, 4 × 250 mm) and a two-solvent system of A, H_2O, and B, 1 M NH_4OAc. Two related HPLC gradient systems were employed, both involving a flow rate of 1 mL/min and a UV-detector wavelength

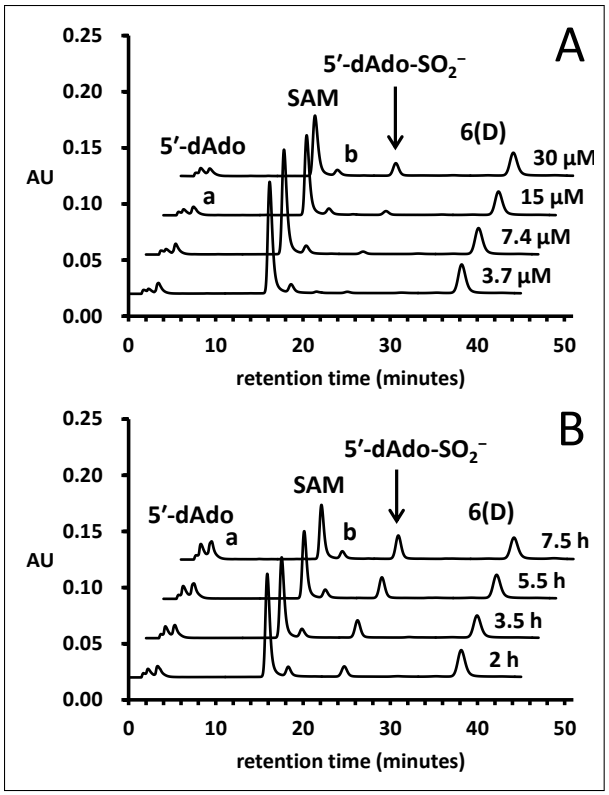

Figure 1S: HPLC traces for incubations involving **6D**, SAM and DesII in the presence of $Na_2S_2O_4$. Reactions were run anaerobically at room temperature in 25 mM EPPS buffer (pH 8.0, NaOH). (A) Constant time incubations of 2 h with variable 3.7–30 μM DesII (indicated on each trace) and 250 μM **6D**, 300 μM SAM and 1 mM $Na_2S_2O_4$. (B) Variable time incubations of 2–7.5 h (indicated on each trace) with 3.7 μM DesII, 250 μM **6D**, 300 μM SAM and 1 mM $Na_2S_2O_4$. Methylthioadenosine also contributes to the peaks at early retention times (peak **a**, < 5 min),[4,5] and the minor peaks (**b**) between 17 and 19 min correspond to thymidine-monophosphate (TMP) and impurities in the SAM preparation.

of 267 nm. Method 1 began with a linear gradient from 3.75% to 22.5% B within the first 30 min, followed by a second linear gradient from 22.5% to 35% B in the next 15 min. The system was then brought back to 3.75% B in 5 min. Using method 1, typical retention times of 5′-deoxyadenosine (5′-dAdo), SAM, **5**, and 5′-deoxyadenosylsulfinate were ca. 2, 19, 25 and 27 min, respectively. In contrast, HPLC method 2 began with a linear gradient from 3.75% to 22.5% B within the first 20 min, followed by a second linear gradient from 22.5% to 30% B in the next 20 min. The system was then brought back to 3.75% B in 5 min. Using method 2, typical retention times of 5′-dAdo, SAM, 5′-deoxyadenosylsulfinate, **5** and **6** were ca. 2, 15, 23, 21 and 38 min, respectively.

2S.2 Enzyme assays

A typical assay was carried out by incubating **5D** or **6D**, DesII, 300 μM SAM and 1 mM of sodium dithionite ($Na_2S_2O_4$) in 25 mM EPPS buffer (pH 8.0, NaOH). Reaction volumes were 50 μL and were maintained at room temperature prior to quenching. Different enzyme concentrations were tested by using 3.7–30 μM DesII and 250 μM analog in reactions run for 2 h. Dependence on incubation times ranging from 2–8 h were tested using 3.7 μM DesII and 250 μM analog. Experiments were also conducted to look for dependence on substrate concentration by employing 0.25–2.0 mM analog and 3.7 μM DesII (with **5D**) or 14.8 μM DesII (with **6D**). In these later experiments, the reactions were allowed to incubate for 2 h. Freshly prepared $Na_2S_2O_4$ was added to reactions at 2 h intervals when they were run for longer than 2 h. The reaction mixtures were then deproteinized using YM-10 centrifugal filters (Amicon), and the filtrate was kept at -80° C before HPLC analysis. HPLC method 1 was used for the analysis of reactions with **5D**, whereas HPLC method 2 was used for assays of **6D**. Species of interest were collected, lyophilized to dryness and characterized by mass spectrometry and coinjection with an authentic standard when available.

2S.3 Measurement of deuterium exchange

Reactions were prepared and run at 30 °C as described in Sec. 2S.2 and initially contained 20 μM DesII, 600 μM **5D** or **6D**, 300 μM SAM and 500 μM $Na_2S_2O_4$ in 400 μL of EPPS buffer (25 mM, pH = 8.0). Aliquots (100 μL) were removed every 2 h, and fresh $Na_2S_2O_4$ was added to the unquenched residual reaction. Quenched aliqouts were deproteinized using YM-10 centrifugal filtration, and the residual 3-fluoro substrate analog and SAM were isolated by HPLC (method 2 for both **5D** and **6D**). The HPLC fractions were lyophilized to dryness and analyzed by mass spectrometry to determine the deuterium enrichment.

3S Supplemental results

3S.1 Reaction of DesII with 5D and 6D

The HPLC traces for **5D** are shown and discussed in the primary manuscript (see Fig. 1). HPLC traces from the incubation of **6D** with SAM, DesII and $Na_2S_2O_4$ are shown in Fig. 1S. The reaction outcomes with this analog were very similar to those of **5D**. Thus, there was no obvious product generation beyond 5′-dAdo-SO_2^- and a small amount of 5′-dAdo. The rates of formation of these two species were both enzyme concentration- (see Fig. 1SA) and time-dependent (see Fig. 1SB). Likewise, SAM was consumed together with the production of 5′-dAdo and 5′-dAdo-SO_2^-, while the concentration of **6D** remains essentially constant.

3S.2 Evidence for 3-*epi*-5

More careful inspection of the HPLC time courses following prolonged (> 8 h) incubation with elevated concentrations

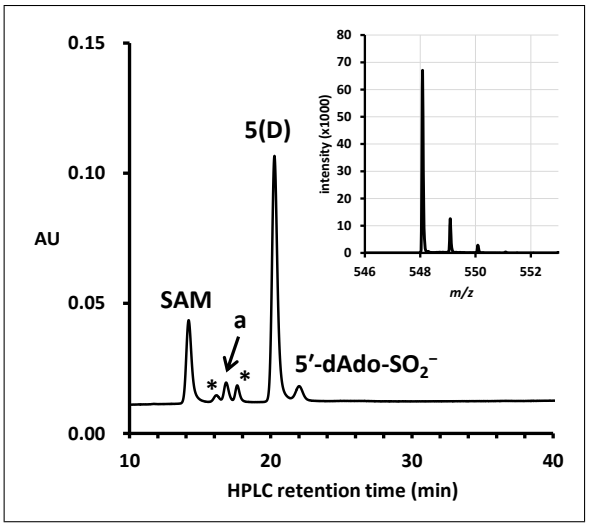

Figure 2S: HPLC chromatogram demonstrating the minor peak (peak **a**) observed at ca. 17 min that forms in a time-dependent manner during prolonged incubations with **5D**, SAM, DesII and $Na_2S_2O_4$. Peaks labeled with an asterisk correspond to TMP and contaminants in the SAM preparation. The inset shows an ESI-MS spectrum (negative ion mode) of the isolated peak. HPLC method 2 was used to produce this chromatogram.

of DesII (ca. 20 μM) demonstrated the time-dependent formation of a minor peak with a retention time of ca. 17 min (see peak **a** in Fig. 2S). This peak overlaps with minor peaks arising from TMP and contaminants in SAM. ESI-MS of the collected peak demonstrated an m/z of 548.1 (negative ion mode, see inset of Fig. 2S). This is consistent with expectations for nondeuterated **5** ($C_{16}H_{26}FN_3O_{13}P_2$, $[M-H]^-$: 548.0852). Given the HPLC retention time and its apparent mass, the new peak is tentatively assigned as the nondeuterated C3-epimer of **5**.

3S.3 Deuterium exchange results

Figs. 3SA & B plot the change in deuterium enrichment in **5(D)** and SAM as a function of reaction time in the presence of 20 μM DesII. Figs. 3SC & D similarly plot the change in deuterium content in **6(D)** and SAM as a function of incubation time in the presence of 20 μM DesII. Prior to reaction, ca. 98% of both **5D** and **6D** were monodeuterated, while $<1\%$ of SAM was either mono- or dideuterated.

4S Chemical syntheses

4S.1 TDP-(3*S*)-4-amino-3,4,6-trideoxy-3-fluoro-α-D-[3-^{2}H]allopyranose (5D)

Methyl (3*R*)-4-azido-2-*O*-benzyl-4,6-dideoxy-α-D-[3-^{2}H]allopyranoside (22). DMSO (4.83 mL, 62.4 mmol) was added dropwise over 10 min to a solution of $(COCl)_2$ (2.76 mL, 31.2 mmol) in anhydrous CH_2Cl_2 (150 mL) at −78° C. The resulting sulfonium solution was stirred at −78 °C for 10 min, prior to the dropwise addition of **21** (6.58 g, 22.3 mmol) in anhydrous CH_2Cl_2 (50 mL) over 30 min. This produced a colorless, cloudy solution that was stirred at −78 °C for another 1 h before adding NEt_3 (15.5 mL, 111.2 mmol) dropwise over 30 min. The reaction mixture was then allowed to stir at −78 °C for 2 h followed by room temperature for 45 min. This produced a yellow suspension that was washed with water (150 mL), and the aqueous layer was further extracted with CH_2Cl_2 (150 mL). The combined organic layers were subsequently dried over Na_2SO_4, filtered and concentrated to afford the ketone as a yellow solid. The solid was then suspended in MeOH (100 mL) at 0 °C, to which $NaBD_4$ (1.87 g, 44.7 mmol) was added over 30 min. The resulting clear solution was stirred at 0 °C for 30 min and at room temperature for 12 h. The reaction was again cooled to 0 °C and quenched with AcOH. After extraction with CH_2Cl_2 (150 mL ×2) and water (150 mL), the organic layer was dried over Na_2SO_4, filtered and concentrated to give a yellowbrown oil. Flash chromatography with silica gel (EtOAc/Hex = 1/9 − 2/8) afforded **22** as a pale yellow oil (5.94 g, 90%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.29 (d, J = 6.4 Hz, 3H, C6–H), 2.69 (d, J = 10.0 Hz, 1H, C4–H), 3.38 (d, J = 3.6 Hz, 1H, C2–H), 3.40 (s, 3H, OMe), 3.50 (brs, 1H, OH), 4.02 (dq, J = 10.0, 6.4 Hz, 1H, C5–H), 4.55 (d, J = 12.4 Hz, 1H, OCH_2Ph), 4.69 (d, J = 3.6 Hz, 1H, C1–H), 4.74 (d, J = 12.4 Hz, 1H, OCH_2Ph), 7.25–7.37 (m, 5H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.7, 55.8, 61.2, 63.6, 68.2 (t, J = 22.7 Hz, C3), 70.3, 73.4, 98.8, 127.8, 128.0, 128.5, 137.0. HRMS (ESI): m/z calculated for $C_{14}H_{18}DN_3O_4$ ($[M+Na]^+$): 317.13305; found: 317.13305.

Methyl (3*S*)-4-azido-2-*O*-benzyl-3,4,6-trideoxy-3-fluoro-α-D-[3-^{2}H]allopyranoside (23). DAST (5.3 mL, 40.1 mmol) was added dropwise over 10 min to a mixture of **22** (5.94 g, 20.2 mmol) and 4 Å molecular sieves in anhydrous CH_2Cl_2 (200 mL) at −40 °C. The reaction was stirred at −40 °C for 30 min and then slowly warmed to 0 °C over 4.5 h. The reaction was then stirred at 0 °C for an additional 16 h and quenched with MeOH (10 mL, at 0 °C). The mixture was allowed to stir at 0 °C for another 30 min before filtration through celite and concentration to give a yellow slurry. The slurry was chromatographed using silica gel (EtOAc/Hex = 1/9) to afford **23** as a yellow oil (2.91 g, 49%). ^{1}H NMR (CDCl3, 400 MHz) δ 1.23 (dd, J = 6.2, 0.6 Hz, 3H, C6–H), 3.19 (dd, J = 13.0, 10.2 Hz, 1H, C4–H), 3.33 (s, 3H, OMe), 3.46–3.60 (m, 2H, C2–H and C5–H), 4.57 (t, J = 3.6 Hz, 1H, C1–H), 4.64 (d, J = 12.4 Hz, 1H, OCH_2Ph), 4.82 (d, J = 12.4 Hz, 1H, OCH_2Ph), 7.25–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.0 (d, J = 1.1 Hz), 55.4, 65.1 (d, J = 6.5 Hz), 66.6 (d, J = 16.2 Hz), 73.2 (d, J = 2.1 Hz), 76.5 (d, J = 15.8 Hz), 98.3 (d, J = 10.8 Hz), 128.0, 128.0, 128.5, 137.5. ^{19}F NMR ($CDCl_3$, 376 MHz) δ −193.4 (m). HRMS (ESI):

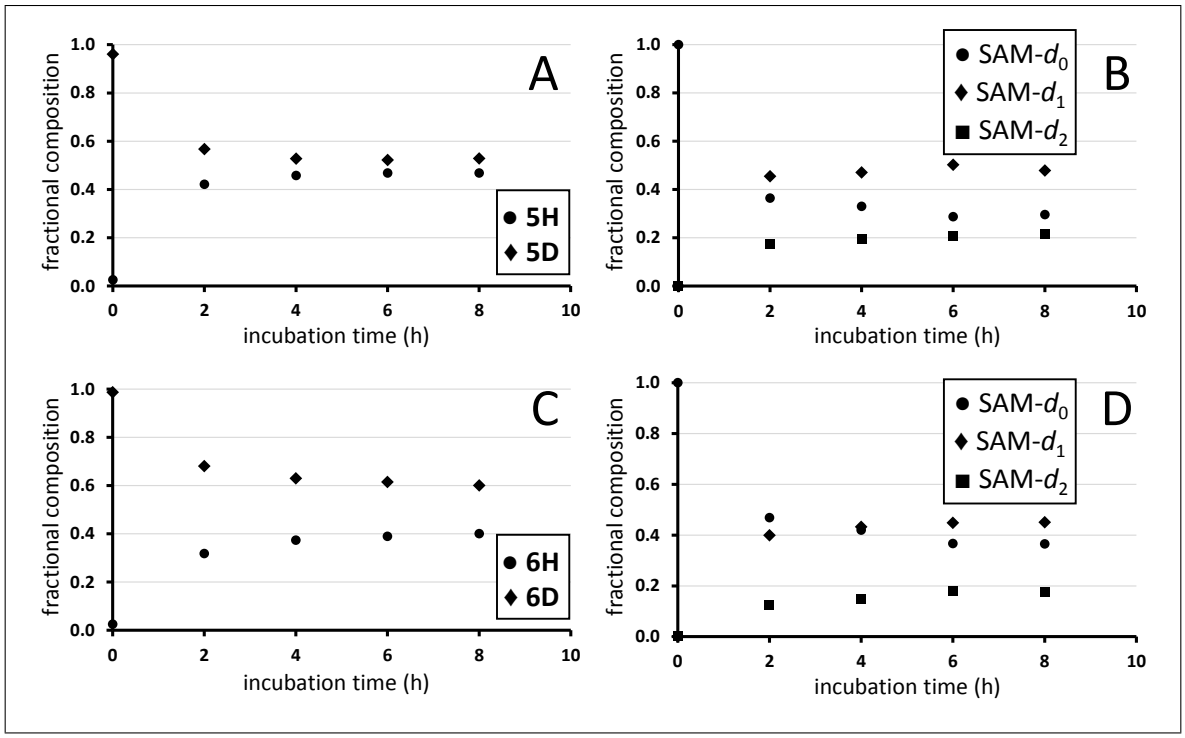

Figure 3S: Fractional composition of deuterated isotopologs for the TDP-3-flouro-sugar analogs and SAM as functions of incubation time with DesII. A & B, reaction of **5D/H** (A) and SAM (B). C & D, reaction of **6D/H** (C) and SAM (D). Reactions contained 20 μM DesII, 600 μM **5D** or **6D**, 300 μM SAM and 500 μM $Na_2S_2O_4$ in 400 μL of EPPS buffer (25 mM, pH 8.0). Reactions were run anaerobically at 30 °C.

m/z calculated for $C_{14}H_{17}DFN_3O_3$ ($[M+Na]^+$): 319.12872; found: 319.12872.

(3*S*)-4-Azido-2-*O*-benzyl-3,4,6-trideoxy-3-fluoro-α/β-D-[3-^{2}H]allopyranose (24). Concentrated sulfuric acid (1.4 mL) was added dropwise over 1 min to a solution of **23** (837 mg, 2.83 mmol) in Ac_2O/AcOH (28 mL, $v/v = 1$) at 0 °C, and the resulting yellow solution was stirred at 0 °C for 2 h. After quenching with $NaHCO_{3(aq)}$ and extraction with CH_2Cl_2 (100 mL ×2) and water (100 mL), the organic layer was dried over $MgSO_4$, filtered and co-evaporated with toluene to give glycosyl acetate as an orange oil. This crude oil was then re-suspended in MeOH (14 mL), and 0.5 M methanolic NaOMe (1.1 mL, 0.55 mmol) was added dropwise. After stirrring at room temperature for 1.5 h, the reaction was quenched with 1 N $HCl_{(aq)}$. MeOH was removed under reduced pressure, and the residue was partitioned between CH_2Cl_2 (50 mL ×2) and water (50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated to give a crude yellow oil, which was then chromatographed using silica gel to afford **24** as pale yellow oil (661 mg, 83%, $\alpha : \beta = 1 : 1$). Spectroscopic characterization of the α-anomer: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.27 (dd, J = 6.4, 0.8 Hz, 3H, C6–H), 3.18 (brd, J = 2.0 Hz, 1H, OH), 3.18–3.24 (m, 1H, C4–H), 3.57 (dd, J = 12.4, 3.6 Hz, 1H, C2–H), 3.83 (dq, J = 10.0, 6.4 Hz, 1H, C5–H), 4.66 (d, J = 12.0 Hz, 1H, OCH_2Ph), 4.83 (d, J = 12.0 Hz, 1H, OCH_2Ph), 5.12 (brtd, J = 3.6, 2.0 Hz, 1H, C1–H), 7.26–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.0 (d, J = 1.1 Hz), 65.5 (d, J = 6.4 Hz), 66.3 (d, J = 16.1 Hz), 73.3 (d, J = 2.1 Hz), 76.9 (d, J = 15.8 Hz), 91.5 (d, J = 10.8 Hz), 128.1, 128.2, 128.5, 137.2. ^{19}F NMR ($CDCl_3$, 376 MHz) δ −194.3 (m). Spectroscopic characterization of the β-isomer: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.33 (dd, J = 6.0, 0.4 Hz, 3H, C6–H), 3.16–3.30 (m, 2H, C4–H and C5–H), 3.40 (dd, J = 13.6, 7.6 Hz, 1H, C2–H), 3.64 (d, J = 4.8 Hz, 1H, OH), 4.58 (dd, J = 7.6, 4.8 Hz, 1H, C1–H), 4.81 (s, 2H, OCH_2Ph), 7.26–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.1 (d, J = 1.5 Hz), 66.9 (d, J = 16.4 Hz), 69.5 (d, J = 7.6 Hz), 74.4 (d, J = 1.5 Hz), 80.2 (d, J = 16.6 Hz), 96.0 (d, J = 12.3 Hz), 128.1, 128.2, 128.5, 137.5. ^{19}F NMR ($CDCl_3$, 376 MHz) δ −189.8 (m). HRMS (ESI): m/z calculated for $C_{13}H_{15}DFN_3O_3$ ($[M+Na]^+$): 305.11307; found: 305.11320.

Dibenzyl (3*S*)-4-azido-2-*O*-benzyl-3,4,6-trideoxy-3-fluoro-α-D-[3-^{2}H]allopyranosyl-1-phosphate (25). A mixture of **24** (287.3 mg, 1.02 mmol) and 1*H*-tetrazole (357 mg, 5.1 mmol) in a round-bottom flask was co-evaporated with anhydrous pyridine three times and further dried under high vacuum. The flask was then charged with Ar, and the mixture was resuspended in anhydrous CH_2Cl_2 (10 mL). Next, dibenzyl *N*,*N*-diisopropylphosphoramidite (0.68 mL, 2.02 mmol) was added dropwise over 10 min to the sus-

pension at −20 °C. The reaction was stirred at −20 °C for 2 h, and then at room temperature for another 1 h. The reaction mixture was then cooled to −40 °C, and *m*CPBA (684 mg, 3.05 mmol) was added portion-wise over 15 min. After stirring at −40 °C for 1 h and then at room temperature for 30 min, the reaction mixture was partitioned between CH_2Cl_2 (50 mL ×2) and $NaHCO_{3(aq)}$ (50 mL). The organic layer was then dried over $MgSO_4$, filtered and concentrated to give the crude solid ($\alpha : \beta = 2 : 1$, determined by ^{1}H NMR). The solid was repeatedly chromatographed using silica gel (EtOAc/Hex = 3/7) to afford **25** as a colorless oil (272 mg, 49%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.19 (dd, J = 6.2, 0.4 Hz, 3H, C6–H), 3.24 (dd, J = 12.8, 10.4 Hz, 1H, C4–H), 3.58–3.70 (m, 2H, C2–H and C5–H), 4.70 (d, J = 11.8 Hz, 1H, OCH_2Ph), 4.74 (d, J = 11.8 Hz, 1H, OCH_2Ph), 5.01 (d, J = 7.2 Hz, 2H, OCH_2Ph), 5.03 (d, J = 8.4 Hz, 2H, OCH_2Ph), 5.12 (brdt, J = 7.2, 3.6 Hz, 1H, C1–H), 7.21–7.37 (m, 15H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.9, 65.8 (d, J = 16.2 Hz), 67.5 (d, J = 6.4 Hz), 69.3 (d, J = 5.4 Hz), 69.5 (d, J = 5.4 Hz), 73.0 (d, J = 1.7 Hz), 76.0 (dd, J = 16.7, 7.5 Hz), 95.3 (dd, J = 11.3, 5.9 Hz), 127.7, 127.8, 128.5, 128.5, 128.5, 128.6, 128.6, 135.5 (d, J = 2.6 Hz), 135.6 (d, J = 2.6 Hz), 137.0. ^{19}F NMR ($CDCl_3$, 376 MHz) δ −195.0 (brs). ^{31}P NMR ($CDCl_3$, 162 MHz) δ −2.4 (s). HRMS (ESI): m/z calculated for $C_{27}H_{28}DFN_3O_6P$ ($[M + Na]^+$): 565.17330; found: 565.17322.

Tributylammonium (3*S*)-4-amino-3,4,6-trideoxy-3-fluoro-α-D-[3-^{2}H]allopyranosyl-1-phosphate (26). A mixture of **25** (178.0 mg, 0.328 mmol) and Pd/C (10%) in MeOH (10 mL) was stirred under H_2 pressure (1 atm) for 3 h. The reaction mixture was then filtered through celite to remove the catalyst. The collected methanolic solution was next concentrated to give a colorless oil. This oil was then dissolved in water (10 mL), and DOWEX resin (50WX8, Bu_3NH^+ form) was added. The mixture was stirred at 4 °C for one day before the resin was removed by filtration. The final solution was lyophilized to yield **26** as a white/pale-yellow solid (51.3 mg, 46%, hexose : Bu_3N = 2 : 1). ^{1}H NMR (D_2O, 400 MHz) δ 1.08–1.24 (m, 3H, C6–H, signals overlap with those from tributylammonium), 3.15 (t, J = 10.8 Hz, 1H, C4–H), 3.74 (dt, J = 12.4, 3.6 Hz, 1H, C2–H), 4.04 (dq, 1H, J = 10.8, 6.2 Hz, C5–H), 5.30 (dt, J = 7.2, 3.6 Hz, 1H, C1–H). ^{13}C NMR (D_2O, 100 MHz) δ 18.9, 57.9 (d, J = 16.5 Hz), 67.2 (d, J = 5.8 Hz), 72.3, 96.6 (dd, J = 10.7, 6.1 Hz). ^{19}F NMR (D_2O, 376 MHz) δ −195.0 (brs). ^{31}P NMR (D_2O, 162 MHz) δ −1.1 (s). HRMS (ESI): m/z calculated for $C_6H_{12}DFNO_6P$ ($[M - H]^-$): 245.04545; found: 245.04566.

TDP-(3*S*)-4-amino-3,4,6-trideoxy-3-fluoro-α-D-[3-^{2}H]allopyranose (5D). A flask containing **26** (51.3 mg, 0.16 mmol) and TMP-morpholidate (212.4 mg, 0.30 mmol) was co-evaporated with anhydrous pyridine 3-times, and the mixture was dried further under high vacuum. This same procedure was repeated in a second flask that contained 1*H*-tetrazole (0.45 M in MeCN, 3.36 mL, 1.51 mmol). Anhydrous pyridine (5 mL and 5 mL) was then added to both flasks, and the tetrazole/pyridine solution was subsequently combined with the **26**/TMP-morpholidate/pyridine solution. The resulting yellowish mixture was then stirred at room temperature for a week before adding 20 mL ether and 40 mL water. The aqueous layer was then removed, washed twice with 20 mL of ether, lyophilized to dryness and purified using a DEAE anion-exchange column with a gradient of 0–200 mM $NaHCO_3$. Fractions containing the desired product were collected, concentrated, and further purified by HPLC using a Dionex column (CarboPac PA1, 9 × 250 mm; solvent A: H_2O, B: 1 M NH_4OAc, isocratic 15% solvent B; UV detection at 267 nm; flow rate: 4 mL/min). The desired product eluted at 14 min. The collected product was concentrated and lyophilized to give **5D** as white solid (21.2 mg, 23.3%). ^{1}H NMR (D_2O, 600 MHz) δ 1.26 (d, J = 6.0 Hz, 3H, C6″–H), 1.81 (d, J = 1.2 Hz, 3H, C5–Me), 2.20–2.31 (m, 2H, C2′–H), 3.23 (t, J = 10.5 Hz, 1H, C4″–H), 3.84 (dt, J = 10.5, 3.6 Hz, 1H, C2″–H), 4.03–4.09 (m, 3H, C4′ and C5′–H), 4.17 (dq, J = 10.5, 6.0 Hz, 1H, C5″–H), 4.50 (quin, J = 3.0 Hz, 1H, C3′–H), 5.52 (dt, J = 7.2, 3.6 Hz, 1H, C1″–H), 6.23 (t, J = 7.2 Hz, 1H, C1′–H), 7.61 (d, J = 1.2 Hz, 1H, C6–H). ^{13}C NMR (D_2O, 150 MHz) δ 11.6 (s, C6″), 16.5 (s, C5–Me), 38.5 (s, C2′), 55.3 (d, J = 16.5 Hz, C4″), 65.4 (d, J = 5.7 Hz, C5 and C5′), 69.9 (dd, J = 17.0, 8.7 Hz, C2″), 71.0 (s, C3′), 85.0 (s, C1′), 85.3 (d, J = 8.9 Hz, C4′), 95.0 (dd, J = 10.8, 6.2 Hz, C1″), 111.7 (s, C5), 137.3 (s, C6), 151.7 (s, C2), 166.5 (s, C4). ^{19}F NMR (D_2O, 565 MHz) δ −200.0 (brs). ^{31}P NMR (D_2O, 243 MHz) δ −13.5 (d, J = 19.4 Hz), −11.5 (d, J = 19.4 Hz). HRMS (ESI): m/z calculated for $C_{16}H_{25}DFN_3O_{13}P_2$ ($[M - H]^-$): 549.09150; found: 549.09100.

4S.2 Synthesis of TDP-(3*S*)-3,6-dideoxy-3-fluoro-α-D-[3-^{2}H]allopyranose (6D).

The 3-fluoro-4-hydroxy analog (**6D**) was prepared as shown in Fig. 4S. In this case, deuterium was first introduced at C3 of diacetone glucose (**27**) to provide **28** according to published methods.[6] Following fluorination with DAST and selective removal of the 5,6-isopropylidene protecting group (**28**→**29**→**30**),[7,8] the resulting diol (**30**) was deoxygenated at C6 using Bu_2SnO-mediated regioselective tosylation prior to reductive cleavage with LAH (**30**→**31**→**32**). Deprotection and peracetylation followed by selective cleavage of the anomeric acetyl group using hydrazine acetate[9] produced the hemiacetal **34** from which the target TDP derivative **6D** could be generated in a manner analogous to that of **5D** (i.e., **34**→**6D**). The details regarding each sythetic step follow below.

(3*S*)-3-Deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene-α-D-[3-^{2}H]allofuranose (29). DAST (15.7 mL, 119 mmol) was

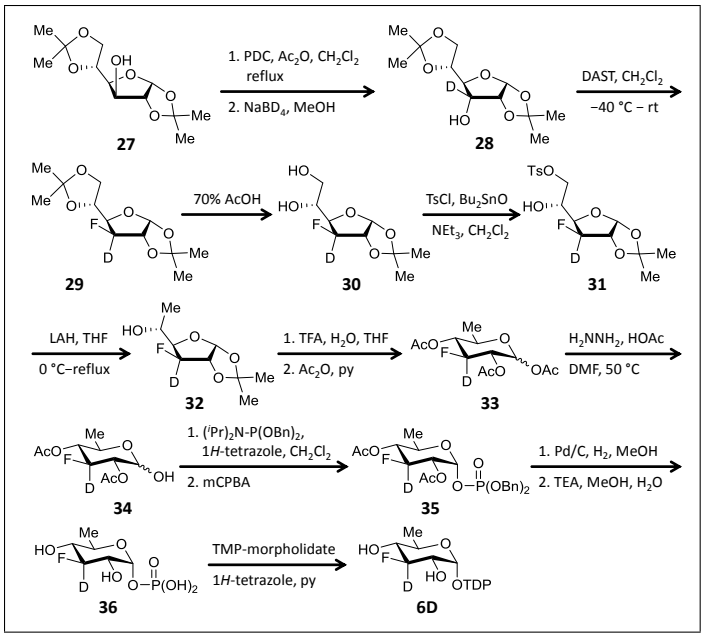

Figure 4S: Synthesis of 4-hydroxy-3-fluoro analog **6D**.

added dropwise over 15 min to a solution of **28** (16.33 g, 62.5 mmol) in anhydrous CH_2Cl_2 (300 mL) at -40 °C. The reaction mixture was stirred for 12 h, during which time the temperature was allowed to rise gradually to room temperature. The reaction mixture was then cooled to 0 °C, quenched by slowly adding methanol (10 mL), and washed with $NH_4Cl_{(aq)}$ (100 mL $\times$2). The organic layer was dried over $Na_2S_2O_4$, concentrated and chromatographed using silica gel (EtOAc/Hex = 2/8) to provide compound **29** (10.15 g, 62%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.26 (s, 3H, Me), 1.27 (s, 3H, Me), 1.34 (s, 3H, Me), 1.40 (s, 3H, Me), 3.82 (dd, J = 8.3, 5.2 Hz, 1H, C6–H), 3.99–4.06 (m, 2H, C4–H and C6–H), 4.17–4.20 (m, 1H, C5–H), 4.75 (dd, J = 11.2, 3.9 Hz, 1H, C2–H), 5.97 (d, J = 3.9 Hz, 1H, C1–H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ 25.1, 26.0, 26.4, 26.5, 66.1 (d, J = 2.3 Hz), 71.4 (d, J = 6.4 Hz), 79.8 (d, J = 18.9 Hz), 81.7 (d, J = 32.7 Hz), 104.6, 108.6, 111.5. ^{19}F NMR (DMSO-d_6, 471 MHz) δ -207.6 (ddt, J = 30.1, 11.2, 4.9 Hz). HRMS (ESI): m/z calculated for $C_{12}H_{18}DO_5F$ ($[M+H]^+$): 264.1358; found: 264.1360.

(3*S*)-3-Deoxy-3-fluoro-1,2-*O*-isopropylidene-6-*O*-(*p*-toluenesulfonyl)-α-D-[3-^{2}H]allofuranose (31). A solution of **29** (9.0 g, 34.2 mmol) in 70% $AcOH_{(aq)}$ (50 mL) was stirred at room temperature for 13 h. The solvent was then removed under reduced pressure, and the residue was partitioned between EtOAc (300 mL) and water (200 mL). The organic layer was removed, dried over $Na_2S_2O_4$, filtered, concentrated and chromatographed using silica gel (EtOAc/Hex = 1/1) to afford **30** (5.31 g, 70%). Next, a solution of **30** (4.97 g, 22.3 mmol), NEt_3 (3.1 mL, 22.3 mmol), Bu_2SnO (110 mg, 0.445 mmol) and TsCl (4.24 g, 22.3 mmol) in anhydrous CH_2Cl_2 (150 mL) was stirred at room temperature for 38 h. The reaction mixture was then washed with $NH_4Cl_{(aq)}$ (50 mL $\times$3). The organic layer was dried over $Na_2S_2O_4$, filtered, concentrated and chromatographed using silica gel (EtOAc/Hex = 2/8) to give **31** (5.99 g, 71%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.29 (s, 3H, Me), 1.44 (s, 3H, Me), 2.43 (s, 3H, Me of Ts), 4.06–4.10 (m, 3H, C4–H, C5–H and one C6–H), 4.29–4.31 (m, 1H, C6–H), 4.65 (dd, J = 10.4, 3.7 Hz, 1H, C2–H), 5.88 (d, J = 3.7 Hz, 1H, C1–H), 7.28 (d, J = 8.1 Hz, 2H, Ph), 7.33 (d, J = 8.1 Hz, 2H, Ph). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.6, 26.2, 26.6, 66.5 (d, J = 8.8 Hz), 72.2, 78.9 (d, J = 18.9 Hz), 82.1 (d, J = 32.7 Hz), 93.3 (dt, J = 181.6, 25.4 Hz), 105.2, 112.6, 128.1, 130.0, 132.5, 145.1. ^{19}F NMR ($CDCl_3$, 471 MHz) δ -209.6 to -209.5 (m); HRMS (ESI): m/z calculated for $C_{16}H_{20}DO_7FS$ ($[M+H]^+$): 378.1113; found: 378.1132.

(3*S*)-3,6-Dideoxy-3-fluoro-1,2-*O*-isopropylidene-α-D-[3-^{2}H]allofuranose (32). LAH (1 M in THF, 43 mL, 43 mmol) was added dropwise over 40 min to a solution of **31** (5.97 g, 15.8 mmol) in anhydrous THF (250 mL) in an ice-water bath. The ice-water bath was then removed, and the reaction mixture was refluxed for 2 h before quenching by the slow addition of $NH_4Cl_{(aq)}$ (20 mL) at 0 °C. The resulting slurry was filtered through celite and washed with CH_2Cl_2 (200 mL). The filtrate was subseqently dried over $Na_2S_2O_4$, filtered, concentrated and chromatographed using silica gel (EtOAc/Hex = 2/8) to afford compound **32** (2.25 g, 69%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.31 (s, 3H, Me), 1.35 (d, J = 6.7 Hz, 3H, C6–H), 1.48 (s, 3H, Me), 3.94 (dd, J = 29.9, 8.0 Hz, 1H, C4–H), 4.06 (dq, J = 8.0, 6.7 Hz, 1H, C5–H), 4.67 (dd, J = 11.4, 3.8 Hz, 1H, C2–H), 5.96 (d, J = 3.8 Hz, 1H, C1–H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 20.7, 26.2, 26.7, 65.0 (d, J = 7.8 Hz), 82.6 (d, J = 33.0 Hz), 83.6 (d, J = 18.5 Hz), 93.8 (dt, J = 182.3, 25.2 Hz) 105.0, 112.2. ^{19}F NMR ($CDCl_3$, 565 MHz) δ -209.4 (ddt, J = 29.9, 11.4, 7.7 Hz); HRMS (ESI) calculated for $C_9H_{14}DO_4F$ ($[M+H]^+$): 208.1095; found: 208.1096.

(3*S*)-1,2,4-*O*-Triacetyl-3,6-dideoxy-3-fluoro-α/β-D-[3-^{2}H]allopyranose (33). TFA (20 mL) was added dropwise over 10 min to a solution of **32** (2.10 g, 10.1 mmol) in THF/H_2O (10 mL, v/v = 1) followed by H_2O (5 mL). The reaction mixture was stirred at room temperature for 12 h before concentration and co-evaporation with anhydrous toluene (30 mL $\times$2) to remove the residual TFA. The residue was then dried under high vacuum and resuspended in anhydrous pyridine (30 mL), to which Ac_2O (15 mL) was added. The reaction mixture was stirred for 8 h at room temperature. After being concentrated under reduced pressure, the residue was dissolved in EtOAc (100 mL) and washed with

water (40 mL ×3). The organic layer was subsequently dried over $Na_2S_2O_4$, filtered, concentrated and chromatographed using silica gel (EtOAc/Hex = 2/8) to give **33** (2.56 g, 86%) with an $\alpha : \beta$ ratio of 1.3 : 1. Spectroscopic characterization of the α-anomer: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.20 (d, J = 6.2 Hz, 3H, C6–H), 2.06 (s, 3H, CH_3 of OAc), 2.11 (s, 3H, CH_3 of OAc), 2.13 (s, 3H, CH_3 of OAc), 3.91 (br dq, J = 10.0, 6.2 Hz, 1H, C5–H), 4.95–5.00 (m, 1H, C4–H), 5.09–5.14 (m, 1H, C2–H), 6.26 (t, J = 4.0 Hz, 1H, C1–H). ^{13}C NMR ($CDCl_3$, 125 MHz) 17.2, 20.5, 20.7, 20.8, 67.7 (d, J = 6.4 Hz), 70.0 (d, J = 8.1 Hz), 72.6–73.0 (m), 89.0 (dt, J = 181.3, 24.9 Hz), 89.4 (d, J = 9.5 Hz), 168.8, 169.4, 169.6. ^{19}F NMR ($CDCl_3$, 471 MHz) δ −200.9 to −200.8 (m). Spectroscopic characterization of the β-anomer: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.23 (d, J = 6.5 Hz, 3H, C6–H), 2.07 (s, 3H, CH_3 of OAc), 2.09 (s, 3H, CH_3 of OAc), 2.10 (s, 3H, CH_3 of OAc), 3.59 (dqd, J = 10.0, 6.5, 1.0 Hz, 1H, C5–H), 4.95–5.00 (m, 1H, C4–H), 5.11–5.23 (m, 1H, C2–H), 5.61 (d, J = 8.0 Hz, 1H, C1–H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 17.1, 20.6, 20.7, 20.8, 70.2 (d, J = 7.3 Hz), 70.7 (d, J = 18.8 Hz), 72.6–73.0 (m), 89.0 (dt, J = 181.3, 24.9 Hz), 91.2 (d, J = 13.5 Hz), 169.0, 169.2, 169.4. ^{19}F NMR ($CDCl_3$, 471 MHz) δ −197.2 to −197.1 (m). HRMS (ESI) calculated for $C_{12}H_{16}DFO_7$ ($[M+H]^+$): 294.1094; found: 294.1092.

Dibenzyl (3*S*)-2,4-*O*-diacetyl-3,6-dideoxy-3-fluoro-α-D-[3-^{2}H]allopyranosyl-1-phosphate (35). Hydrazine acetate (0.92 g, 10.0 mmol) was added to a solution of **33** (2.41 g, 8.20 mmol) in anhydrous DMF (5 mL) at 50 °C. The reaction mixture was stirred at the same temperature for 30 min, at which time it was then poured into 50 mL of water. The aqueous mixture was then extracted with EtOAc (50 mL ×3). The combined organic layer was dried over $Na_2S_2O_4$, filtered, concentrated, and chromatographed using silica gel (EtOAc/Hex = 1/2) to give compound **34** (1.71 g, 83%). A mixture of **34** (1.71 g, 6.81 mmol) and 1*H*-tetrazole (0.45 M in MeCN, 37.8 mL, 17.0 mmol) was then concentrated and dried under high vacuum before being redissolved in anhydrous CH_2Cl_2 (80 mL) and cooled to −20 °C. Dibenzyl *N*,*N*-diisopropylphosphoramidite (4.7 mL, 17.0 mmol) was next added to the solution dropwise over 15 min. The reaction was stirred at −20 °C for 2 h and then at room temperature for 4 h. The reaction mixture was subsequently cooled to −40 °C, and *m*CPBA (8.3 g, 70%, 33.7 mmol) was added portion-wise over 30 min. After stirring at −40 °C for 3 h, the reaction mixture was gradually warmed to room temperature and stirred at room temperature for another 5 h. The reaction was then quenched with $NaHCO_{3(aq)}$ (80 mL), and extracted with CH_2Cl_2 (80 mL ×2). The combined organic layer was dried over $Na_2S_2O_4$, filtered, concentrated, and chromatographed using silica gel (EtOAc/Hex = 1/2) to afford compound **35** (2.33 g, 67%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.11 (d, J = 6.2 Hz, 3H, C6–H), 1.94 (s, 3H, CH_3 of OAc), 2.09 (s, 3H, CH_3 of OAc), 3.89 (dq, J = 10.1, 6.2 Hz, 1H, C5–H), 4.93 (dd, J = 12.4, 10.1 Hz, 1H, C4–H), 4.96 (dt, J = 12.0, 3.2 Hz, 1H, C2–H), 5.01–5.05 (m, 4H, OCH_2Ph), 5.81–5.83 (dt, J = 6.6, 3.2 Hz, 1H, C1–H), 7.31–7.36 (m, 10H, Ph). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 17.0, 20.4, 20.7, 67.2 (d, J = 6.3 Hz), 69.6 (d, J = 5.4 Hz), 69.7 (d, J = 5.7 Hz), 70.6 (dd, J = 17.6, 7.5 Hz), 72.7 (d, J = 17.4 Hz), 94.2 (dd, J = 9.8, 5.6 Hz), 127.9, 128.0, 128.0, 128.6, 128.7 128.7, 128.7, 128.8, 128.8, 128.9, 135.4 (d, J = 7.1 Hz), 135.6 (d, J = 6.2 Hz), 169.4, 169.8. ^{19}F NMR ($CDCl_3$, 565 MHz) δ −201.5 (brs). ^{31}P NMR ($CDCl_3$, 243 MHz) δ −2.7 (s). HRMS (ESI) calculated for $C_{24}H_{27}DFO_9P$ ($[M+H]^+$): 512.1591; found: 512.1593.

(3*S*)-3,6-Dideoxy-3-fluoro-α-D-[3-^{2}H]allopyranosyl-1-phosphate (36). A mixture of **35** (1.28 g, 2.51 mmol) and Pd/C (10%, 200 mg) in MeOH (50 mL) was first stirred under 1 atm of H_2 for 12 h. The catalyst was then filtered off, and the filtrate was concentrated. The residue was redissolved in $MeOH/H_2O/NEt_3$ (45 mL, 1 : 1 : 1) and stirred at room temperature for another 24 h. The reaction mixture was then concentrated and purified using a DEAE anion-exchange column with a gradient of 0–500 mM $NaHCO_3$. Fractions containing the desired product were collected and concentrated. To the residue was added 30 mL of water and lyophilized (×2) to give the compound **36**. Compound **36** was dissolved in water (5 mL), filtered through a DOWEX column (50WX8, Et_3NH^+ form) and lyophilized (×2) to afford the NEt_3 salt of **36** (648 mg, 69%, hexose : Et_3N = 1 : 1.27). ^{1}H NMR (D_2O, 500 MHz) δ 1.12 (d, J = 6.2 Hz, 3H, C6–H), 3.28 (dd, J = 13.9, 9.8 Hz, 1H, C4–H), 3.62 (ddd, 1H, J = 12.7, 3.8, 1.9 Hz, C2–H), 3.83 (dq, 1H, J = 9.8, 6.2 Hz, C5–H), 5.25–5.28 (dt, J = 7.5, 3.8 Hz, 1H, C1–H). ^{13}C NMR (D_2O, 125 MHz) δ 16.7, 67.2, 70.9 (dd, J = 16.5, 7.4 Hz), 73.7 (d, J = 15.5 Hz), 93.8 (dd, J = 10.0, 5.5 Hz), 95.3 (t, J = 23.8 Hz). ^{19}F NMR (D_2O, 471 MHz) δ −199.0 (brs). ^{31}P NMR (D_2O, 202 MHz) δ 2.5. HRMS (ESI) calculated for $C_6H_{11}DFO_7P$ ($[M-H]^-$): 246.0295; found: 246.0293.

TDP-(3*S*)-3,6-dideoxy-3-fluoro-α-D-[3-^{2}H]allopyranose (6D). 1*H*-tetrazole (83.4 mg, 1.20 mmol) and TMP-morpholidate (434 mg, 0.635 mmol) were first added to a solution of **36** (149 mg, 0.397 mmol) in anhydrous pyridine (10 mL). After stirring at room temperature for 3 days, the reaction mixture was concentrated and partitioned between Et_2O (10 mL) and water (10 mL). The aqueous layer was concentrated and purified by DEAE anion-exchange chromatography using a gradient of 0–500 mM $NaHCO_3$. Fractions containing the product were collected, concentrated and lyophilized (×2) to afford **6D** (84 mg, 38%). ^{1}H NMR (D_2O, 600 MHz) δ 1.14 (d, J = 6.2 Hz, 3H, C6″–H), 1.78 (d, J = 1.3 Hz, 3H, C5–CH_3), 2.21–2.24 (m, 2H, C2′–H), 3.23 (dd, J = 13.7, 10.0 Hz, 1H, C4″–H), 3.69 (dt, 1H, J = 12.6, 3.1 Hz, C2″–H), 3.84 (dq, 1H, J = 10.0, 6.2 Hz,

C5″–H), 4.01–4.05 (m, 3H, C4′–H and C5′–H), 4.43–4.47 (m, 1H, C3′–H), 5.42–5.44 (m, 1H, C1″–H), 6.18 (t, J = 7.0 Hz, 1H, C1′–H), 7.59 (d, J = 1.3 Hz, 1H, C6–H). ^{13}C NMR (D_2O, 150 MHz) δ 11.6 (C5), 16.5 (C6″), 38.5 (C2′), 68.4 (d, J = 7.8 Hz, C5″), 70.2 (dd, J = 16.8, 9.5 Hz, C2″), 70.7 (C3′), 73.1 (d, J = 16.9 Hz, 1H, C4″), 85.0 (C1′), 85.1 (C5′), 85.2 (C4′), 95.3 (dd, J = 11.2, 5.6 Hz, C1″),137.3 (C6), 137.4 (C5), 151.8 (C2), 166.6 (C4). ^{19}F NMR (D_2O, 565 MHz) δ −201.5 (s). ^{31}P NMR (D_2O, 243 MHz) δ −12.5 (d, J = 20.5 Hz), −10.7 (d, J = 20.5 Hz). HRMS (ESI) calculated for $C_{16}H_{24}DFN_2O_{14}P_2$ ($[M-H]^-$): 550.0755; found: 550.0752.

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