

## Supporting Information for

### Synthesis of 1,4-azaphosphinine pyrimidine nucleosides and their evaluation as inhibitors of human cytidine deaminase and APOBEC3A

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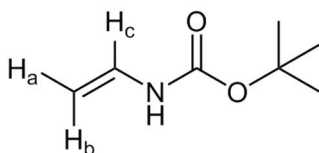
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## 1. General Methods.

Reagents from commercial suppliers were used without further purification. Pyridine and  $\text{CH}_2\text{Cl}_2$  were freshly distilled from  $\text{CaH}_2$ ;  $\text{Et}_2\text{O}$ , ethyl vinyl ether, THF and  $\text{Et}_3\text{N}$  – from Na. Hoffer's chlorosugar was prepared as previously described.<sup>1</sup> Saturated  $\text{NH}_3$  in  $\text{CHCl}_3$  was prepared by bubbling  $\text{NH}_3$  gas in an ice-cooled  $\text{CHCl}_3$  until saturation; the solution was stored at +4 °C.  $^1\text{H}$  (400, 500 or 700 MHz),  $^{13}\text{C}$  (100.6, 125.8 or 176.1 MHz) and  $^{31}\text{P}$  (202.5 MHz) NMR spectra were obtained with Bruker Avance 400, Avance 500 or Avance 700 MHz spectrometers and referenced to residual solvent signals ( $\text{CDCl}_3$ : 7.26 ppm for  $^1\text{H}$  and 77.16 ppm for  $^{13}\text{C}$ ;  $d_6$ -DMSO: 2.50 ppm for  $^1\text{H}$  and 39.52 ppm for  $^{13}\text{C}$ , according to reference <sup>2</sup>, 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionic acid ( $d_4$ -TMSP, 0.0 ppm for  $^1\text{H}$  and  $^{13}\text{C}$ ) internal standard for water samples or 85% aq.  $\text{H}_3\text{PO}_4$  external standard (0.0 ppm for  $^{31}\text{P}$ ).  $^1\text{H}$  NMR coupling constants are reported in Hertz (Hz) and refer to apparent multiplicities. The assignments of signals were done using 2D homonuclear  $^1\text{H}$ - $^1\text{H}$  COSY, NOESY and heteronuclear  $^1\text{H}$ - $^{13}\text{C}$  HMQC or HSQC, and HMBC spectra. NMR spectra were processed in Spinworks version 4.1 (developed by Dr. Kirk Marat, Department of Chemistry, University of Manitoba). High-resolution electrospray mass spectra were recorded on a Thermo Scientific Q Exactive Focus Hybrid Quadrupole-Orbitrap mass spectrometer. Ions generated by ESI were detected in positive or negative ion modes. Total ion count (TIC) was recorded in centroid mode over the  $m/z$  range of 100-3,000 and analyzed using ThermoXcalibur Qual Browser. Analytical thin-layer chromatography was performed on Kieselgel 60 F<sub>254</sub> precoated aluminum plates (Merck). Spots were visualised by UV or stained with  $\text{I}_2/\text{SiO}_2$ , aq.  $\text{KMnO}_4$ , ninhydrin solution or cerium ammonium molybdate (CAM) stain. The latter was prepared by suspending  $\text{Ce}(\text{NH}_4)_4(\text{SO}_4)_4 \cdot 2\text{H}_2\text{O}$  (4 g), ammonium molybdate (10 g) in  $\text{H}_2\text{O}$  (360 mL) followed by addition of 98%  $\text{H}_2\text{SO}_4$  (40 mL). Silica gel column chromatography was performed on silica gel 60 with size particles 40–63  $\mu\text{m}$  or 20–45  $\mu\text{m}$  (specified) using Buchi Sepacore X50 flash chromatography system. Oligonucleotide synthesis was carried out on a MerMade-4 DNA/RNA synthesizer (BioAutomation) on a 5  $\mu\text{mol}$  scale using standard manufacturer's protocol. 'Saltless buffer' used for oligonucleotide desalting consists of 10  $\mu\text{M}$  Tris-HCl (pH 8.0), 1  $\mu\text{M}$  EDTA and 0.001% w/v  $\text{NaN}_3$ .

## 2. Synthetic procedures.



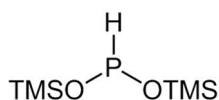
***N*-Boc-vinylamine (3)** was prepared starting from commercially available *N*-vinylformamide **1** as a stable source of vinylamine. To a solution of *N*-vinylformamide **1** (10.5 mL, 150 mmol) and  $\text{Boc}_2\text{O}$  (36.0 g, 165 mmol) in THF (150 mL), DMAP (183 mg, 1.5 mmol) was added and reaction mixture was stirred overnight at room temperature. An aliquot of the reaction mixture was evaporated, and the progress of the reaction was controlled by  $^1\text{H}$  NMR which clearly showed formation of *N*-Boc-*N*-vinylformamide **2** as a major product.

$^1\text{H}$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  9.16 (s, 1H, CHO), 6.56 (ddd, 1H,  $J_{\text{Hb,Hc}} = 16.2$  Hz,  $J_{\text{Ha,Hc}} = 9.6$  Hz,  $J = 0.9$  Hz,  $H_c$ ), 5.56 (d, 1H,  $J_{\text{Hb,Hc}} = 16.2$  Hz,  $H_b$ ), 5.04 (dd, 1H,  $J_{\text{Ha,Hc}} = 9.6$  Hz,  $J = 0.9$  Hz,  $H_a$ ), 1.51 (s, 9H,  $\text{CH}_3$ ).

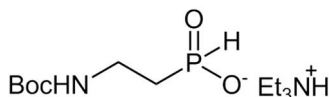
To cleave formyl group, aqueous solution of NaOH (90 mL, 5 M) was added and the mixture was stirred for 3 h. Water layer was separated, extracted with AcOEt ( $2 \times 150$  mL), combined organic layers were washed with brine (150 mL), dried over  $\text{Na}_2\text{SO}_4$ , filtered and evaporated. *N*-Boc-vinylamine **3** was purified by sublimation *in vacuo* (1 mmHg) resulting in 19.1 g (89%) of white solid.

$^1\text{H}$  NMR (400 MHz,  $d_6$ -DMSO)  $\delta$  9.20 (d, 1H,  $J = 9.4$  Hz, NH), 6.51 (m, 1H,  $H_c$ ), 4.45 (d, 1H,  $J_{\text{Hb,Hc}} = 15.8$  Hz,  $H_b$ ), 4.09 (d, 1H,  $J_{\text{Ha,Hc}} = 8.8$  Hz,  $H_a$ ), 1.41 (s, 9H,  $\text{CH}_3$ ).

$^{13}\text{C}\{^1\text{H}\}$  NMR (100.6 MHz,  $d_6$ -DMSO)  $\delta$  152.9 (CO), 130.8 (CH), 92.0 ( $\text{CH}_2$ ), 79.0 ( $\text{C}(\text{CH}_3)_3$ ), 28.0 ( $\text{CH}_3$ ).



**Bis-(trimethylsilyloxy)phosphine (BTSP, 4)** was prepared as previously described<sup>3</sup>. The mixture of ammonium hypophosphite (20.75 g, 250 mmol) with hexamethyldisilazane (HMDS, 62.4 mL, 300 mmol) was heated at 100–110°C during 2.5–3h under argon atmosphere. The resulting homogenous liquid of **4** was cooled to room temperature under argon and used without purification (**Caution! BTSP is highly pyrophoric on air thus all operations must be performed under argon**).



**2-N-Boc-aminoethylphosphinic acid, triethylammonium salt (5)**. Solution of *N*-Boc-vinylamine **3** (14.30 g, 100 mmol) and azobisisobutyronitrile (AIBN, 3.28 g, 20 mmol) in acetonitrile (200 mL) was added to BTSP (prepared from ammonium hypophosphite (20.75 g, 250 mmol) and HMDS (62.4 mL, 300 mmol)) at room temperature. The reaction mixture was stirred at 80–90 °C (oil bath) overnight under argon, quenched with a mixture of  $\text{Et}_3\text{N}$  (42.5 mL) and MeOH (25 mL), solids were filtered out and filtrate was evaporated *in vacuo*. Column chromatography on silica eluting with  $\text{H}_2\text{O}/i\text{PrOH}$  (5:95, v/v) resulted in a mixture of 2-*N*-Boc-aminoethylphosphinic acid with its triethylammonium salt. Co-evaporation with  $\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2$  led to triethylammonium salt **5** (15.6 g, 50%).

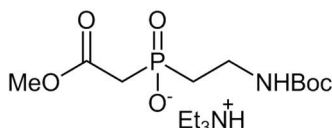
$R_f$  0.34 (MeOH/ $\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2$ , 7:5:88, v/v/v), stains with ninhydrin.

$^1\text{H}$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  6.90 (d, 1H,  $^1J_{\text{P,H}} = 484.2$  Hz, PH), 6.82 (m, 1H, NH), 3.08 (m, 2H,  $\text{CH}_2\text{NH}$ ), 3.00 (d, 6H,  $J = 6.9$  Hz,  $\text{CH}_2\text{CH}_3$ ), 1.50 (m, 2H,  $\text{CH}_2\text{CH}_2\text{P}$ ), 1.36 (s, 9H, Boc), 1.17 (t, 9H,  $J = 7.2$  Hz,  $\text{CH}_2\text{CH}_3$ ).

$^{13}\text{C}\{^1\text{H}\}$  NMR (125.7 MHz,  $d_6$ -DMSO)  $\delta$  155.3 (CO), 77.6 ( $(\text{CH}_3)_3\text{C}$ ), 45.2 (3C,  $\text{CH}_2\text{CH}_3$ ), 34.0 ( $\text{CH}_2\text{NH}$ ), 31.9 (d,  $^1J_{\text{C,P}} = 88.9$  Hz,  $\text{CH}_2\text{CH}_2\text{P}$ ), 28.2 (3C,  $(\text{CH}_3)_3\text{C}$ ), 8.4 (3C,  $\text{CH}_2\text{CH}_3$ ).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.4 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  20.2.

HRMS (ESI  $-$ ): calcd. for  $\text{C}_7\text{H}_{15}\text{NO}_4\text{P}^- [\text{M}-\text{Et}_3\text{NH}]^-$  208.0744; found 208.0752.



**(2-N-Boc-aminoethyl)(2-methoxy-2-oxoethyl)phosphinic acid, triethylammonium salt (6)** was prepared from compound **5** by alkylation with methyl chloroacetate. To the solution of phosphinic salt **5** (3.89 g, 12.5 mmol),  $\text{Et}_3\text{N}$  (3.48 mL, 25.0 mmol) and  $\text{TMSCl}$  (3.17 mL, 25.0 mmol) in  $\text{CH}_2\text{Cl}_2$  (25 mL), methyl chloroacetate (1.65 mL, 18.8 mmol) was added. The mixture was stirred under argon for 5 days at room temperature, quenched with MeOH (2 mL, heated until reflux) and volatiles were removed *in vacuo*. The residue was purified on silica gel eluting with  $\text{H}_2\text{O}/i\text{PrOH}$  5:95 (v/v) resulting in alkylated salt **6** (4.04 g, 84%) after co-evaporation with  $\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2$ .

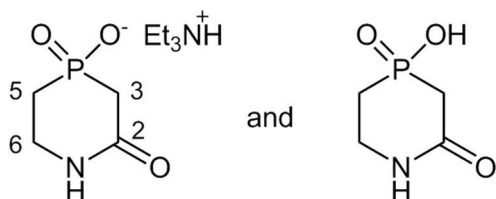
$R_f$  0.52 (MeOH/ $\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2$ , 7:5:88, v/v/v), stains with ninhydrin.

$^1\text{H}$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  6.88 (m, 1H, NH), 3.53 (s, 3H,  $\text{CH}_3\text{O}$ ), 3.12 (m, 2H,  $\text{CH}_2\text{NH}$ ), 2.96 (q, 6H,  $J = 7.2$  Hz,  $\text{CH}_2\text{CH}_3$ ), 2.51 (d, 2H,  $^2J_{\text{H,P}} = 16.4$  Hz,  $\text{CH}_2\text{CO}_2\text{Me}$ ), 1.58 (m, 2H,  $\text{CH}_2\text{CH}_2\text{P}$ ), 1.36 (s, 9H, Boc), 1.17 (t, 9H,  $J = 7.2$  Hz,  $\text{CH}_2\text{CH}_3$ ).

$^{13}\text{C}\{^1\text{H}\}$  NMR (125.7 MHz,  $d_6$ -DMSO)  $\delta$  169.4 ( $\text{CO}_2\text{Me}$ ), 155.3 (CONH), 77.3 ( $(\text{CH}_3)_3\text{C}$ ), 51.3 ( $\text{OCH}_3$ ), 44.9 (3C,  $\text{CH}_2\text{CH}_3$ ), 38.8 ( $\text{MeO}_2\text{CCH}_2\text{P}$ , from HMQC), 35.3 ( $\text{CH}_2\text{NH}$ ), 31.2 (d,  $^1J_{\text{C,P}} = 93$  Hz,  $\text{CH}_2\text{CH}_2\text{P}$ ), 28.2 (3C,  $(\text{CH}_3)_3\text{C}$ ), 8.4 (3C,  $\text{CH}_2\text{CH}_3$ ).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.4 MHz,  $d_6$ -DMSO)  $\delta$  24.4.

HRMS (ESI  $-$ ): calcd. for  $\text{C}_{10}\text{H}_{19}\text{NO}_6\text{P}^- [\text{M}-\text{Et}_3\text{NH}]^-$  280.0955; found 280.0971.



**4-Hydroxy-1,4-azaphosphinane-2,4-dione, triethylammonium salt (7).** To the solution of phosphinic salt **6** (3.89 g, 10.2 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL), trifluoroacetic acid (10 mL) was added and the mixture was stirred at room temperature overnight. All volatiles were evaporated *in vacuo*, and the residue was dissolved in pyridine (20 mL) followed by the addition of  $\text{Et}_3\text{N}$  (7.1 mL, 51 mmol). The resulting mixture was refluxed 5h, solvents were evaporated, and the residue was purified using silica gel column chromatography eluting with a step gradient of  $\text{H}_2\text{O}$  (10→50%) in *i*PrOH. Fractions containing the product were evaporated, co-evaporated with  $\text{H}_2\text{O}/\text{Et}_3\text{N}$  and dried *in vacuo* resulting in saturated nucleobase **7** as a triethylammonium salt (2.13 g, 84%).

$R_f$  0.25 ( $\text{H}_2\text{O}/i\text{PrOH}$ , 1:4, v/v), stains with CAM.

$^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ , intn. ref. *d4*-TMSP)  $\delta$  3.49 (m, 2H, H-6), 3.20 (q, 6H,  $J = 7.3$  Hz,  $\text{CH}_2\text{CH}_3$ ), 2.71 (d, 2H,  $^2J_{\text{H}3,\text{P}} = 16.0$  Hz, H-3), 1.86 (m, 2H, H-5), 1.28 (t, 9H,  $J = 7.3$  Hz,  $\text{CH}_2\text{CH}_3$ ).

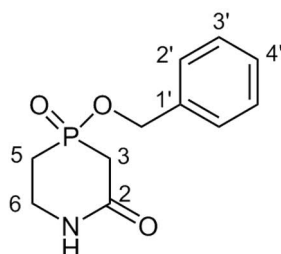
$^{13}\text{C}\{^1\text{H}\}$  NMR (125.7 MHz,  $\text{D}_2\text{O}$ , *d4*-TMSP)  $\delta$  176.5 (C2), 49.6 (3C,  $\text{CH}_2\text{CH}_3$ ), 40.4 (d,  $^1J_{\text{C}3,\text{P}} = 74.0$  Hz, C3), 40.2 (d,  $^2J_{\text{C}6,\text{P}} = 5.6$  Hz, C6), 29.7 (d,  $^1J_{\text{C}5,\text{P}} = 94.3$  Hz, C5), 11.2 (3C,  $\text{CH}_2\text{CH}_3$ ).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.4 MHz,  $\text{D}_2\text{O}$ , extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  34.4.

HRMS (ESI -): calcd. for  $\text{C}_4\text{H}_7\text{NO}_3\text{P}^-$  [ $\text{M}-\text{Et}_3\text{NH}^+$ ] 148.0169; found 148.0171.

Analytical amount of heterocycle **7** was obtained in the form of free acid by passing aq. solution of its triethylammonium salt through the column with Dowex-50W×8 (H-form) and eluting with water. After evaporation of water the resulting free acid **7** slowly solidifies at room temperature.

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  3.48 (m, 2H, H-6), 2.85 (d, 2H,  $^2J_{\text{H}3,\text{P}} = 16.7$  Hz, H-3), 1.99 (m, 2H, H-5).



**4-(Benzyloxy)-1,4-azaphosphinane-2,4-dione (8)** was obtained by esterification of phosphinic acid **7** with benzyl alcohol in the presence of *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU).<sup>4</sup> Nucleobase **7** as a free acid (1.20 g, 8.05 mmol) was suspended in DCE (32 mL) and TBTU (5.17 g, 16.1 mmol),  $\text{Et}_3\text{N}$  (2.24 mL, 16.1 mmol), and  $\text{BnOH}$  (1.66 mL, 16.1 mmol) were added. The mixture was refluxed for 3 h until homogeneous solution was obtained, evaporated *in vacuo* and purified on silica gel (40 × 75 mm column) eluting with acetone over 30 min with a flow rate of 50 mL/min. Fractions containing the product were evaporated *in vacuo* to result in oil **8** (1.24 g, 65%) that solidifies upon standing.

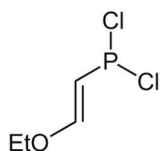
$R_f$  0.13 (acetone), UV-active, stains with  $\text{I}_2/\text{SiO}_2$ .

$^1\text{H}$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  7.88 (br. s, 1H, NH), 7.42-7.31 (m, 5H, H-2',3',4'), 5.06-4.97 (m, 2H,  $\text{CH}_2\text{Ph}$ ), 3.48-3.20 (m, 2H, H-6), 3.08-2.88 (m, 2H, H-3), 2.12-1.89 (m, 2H, H-5).

$^{13}\text{C}\{^1\text{H}\}$  NMR (125.8 MHz,  $d_6$ -DMSO)  $\delta$  167.06 (d,  $^2J_{\text{C}2,\text{P}} = 4.5$  Hz, C2), 136.6 (d,  $^3J_{\text{C}1',\text{P}} = 6.5$  Hz, C1'), 127.85 (2C, C2'), 128.2 (C4'), 128.45 (2C, C3'), 65.3 (d,  $^2J_{\text{CPh},\text{P}} = 5.9$  Hz,  $\text{CH}_2\text{Ph}$ ), 35.90 (d,  $^1J_{\text{C}3,\text{P}} = 72.5$  Hz, C3), 35.57 (d,  $^2J_{\text{C}6,\text{P}} = 4.9$  Hz, C6), 25.9 (d,  $^1J_{\text{C}5,\text{P}} = 92.7$  Hz, C5).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  50.7.

HRMS (ESI +): calcd. for  $\text{C}_{11}\text{H}_{14}\text{NNaO}_3\text{P}^+$  [ $\text{M}+\text{Na}$ ]<sup>+</sup> 262.0609; found 262.0603.



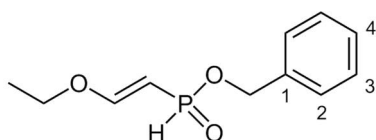
**(E)-Dichloro(2-ethoxyvinyl)phosphane (9)**<sup>5</sup> was prepared as follows. To a solution of ethyl vinyl ether (36.0 g, 0.50 mol) and Et<sub>3</sub>N (50.5 g, 0.50 mol) in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) freshly distilled PCl<sub>3</sub> (43.7 mL, 0.50 mol) was added dropwise and the mixture was stirred for 2 days at room temperature under argon. The mixture was filtered, solids were washed with hexane and all solvents were evaporated *in vacuo*. The residue was stirred with hexane (0.5 L) for 1 h, filtered and evaporated *in vacuo*. Remaining liquid was distilled *in vacuo* resulting in 46.3 g (54 %) of colourless compound **9**.

B.p. 90-100 °C (30 mbar).

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.11 (dd, 1H, *J*<sub>H,H</sub> = 14.4 Hz, <sup>3</sup>*J*<sub>H,P</sub> = 12.5 Hz, CHOEt), 5.79 (1H, *J*<sub>H,H</sub> = 14.4 Hz, <sup>2</sup>*J*<sub>H,P</sub> = 4.6 Hz, CHP), 3.97 (q, 2H, *J* = 7.1 Hz, CH<sub>2</sub>CH<sub>3</sub>), 1.36 (t, 3H, *J* = 7.1 Hz, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C{<sup>1</sup>H} NMR (125.7 MHz, CDCl<sub>3</sub>) δ 159.6 (d, <sup>2</sup>*J*<sub>C,P</sub> = 89.3 Hz, CHOEt), 108.0 (d, <sup>1</sup>*J*<sub>C,P</sub> = 46.8 Hz, CHOP), 66.4 (CH<sub>2</sub>CH<sub>3</sub>), 14.3 (CH<sub>2</sub>CH<sub>3</sub>).

<sup>31</sup>P{<sup>1</sup>H} NMR (202.5 MHz, CDCl<sub>3</sub>, ref. 85% H<sub>3</sub>PO<sub>4</sub>) δ 167.2.



**Benzyl (E)-(2-ethoxyvinyl)phosphinate (10)**. To a cooled (-78 °C) solution of dichlorophosphane **9** (52.0 g, 0.30 mol) in abs. Et<sub>2</sub>O (300 mL), pyridine (24.2 mL, 0.3 mol) followed by solution of BnOH (32.5 g, 0.3 mol) in abs. Et<sub>2</sub>O (200 mL) were added dropwise over 20 min. The reaction mixture was stirred 1 h at -78 °C then heated to 0 °C and stirred 1 h at 0 °C. A solution of H<sub>2</sub>O (6.50 mL, 0.36 mol) and pyridine (29.0 mL, 0.36 mol) in THF/Et<sub>2</sub>O (30/30 mL) was quickly added to the reaction mixture with manual shaking. The mixture was stirred overnight at room temperature, filtered, solids were washed with Et<sub>2</sub>O, combined organic layers were evaporated *in vacuo* resulting in phosphinate **10** as yellowish oil (67.6 g, quant.). Phosphinate **10** was about 90% pure according to <sup>31</sup>P NMR and was used without further purification.

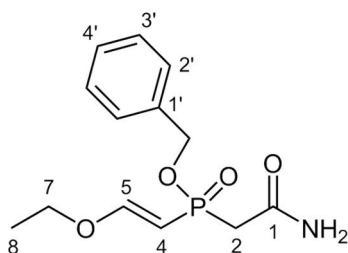
*R*<sub>f</sub> 0.31 (acetone/CH<sub>2</sub>Cl<sub>2</sub>, 1:9, v/v), UV-active, stains with KMnO<sub>4</sub>.

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.41-7.29 (m, 5H, PhH), 7.30 (d, 1H, <sup>1</sup>*J*<sub>H,P</sub> = 567.5 Hz, PH), 7.18 (dd, 1H, *J*<sub>H,H</sub> = 13.7 Hz, <sup>3</sup>*J*<sub>H,P</sub> = 12.3 Hz, OCH), 5.06 (m, 2H, CH<sub>2</sub>Ph), 4.79 (dd, 1H, *J*<sub>H,H</sub> = 13.7 Hz, <sup>2</sup>*J*<sub>H,P</sub> = 15.2 Hz, PCH), 3.89 (q, 2H, *J* = 7.1 Hz, CH<sub>2</sub>), 1.32 (t, 3H, *J* = 7.1 Hz, CH<sub>3</sub>).

<sup>13</sup>C{<sup>1</sup>H} NMR (125.7 MHz, CDCl<sub>3</sub>) δ 164.32 (d, <sup>2</sup>*J*<sub>C,P</sub> = 19.8 Hz, OCH), 136.03 (d, <sup>3</sup>*J*<sub>C,P</sub> = 6.7 Hz, C1), 128.66, 127.96 (4C, C2,3), 128.49 (C4), 90.73 (d, <sup>1</sup>*J*<sub>C,P</sub> = 142.1 Hz, PCH), 66.57 (d, <sup>2</sup>*J*<sub>C,P</sub> = 5.7 Hz, CH<sub>2</sub>Ph), 66.56 (CH<sub>2</sub>), 14.24 (CH<sub>3</sub>).

<sup>31</sup>P NMR (202.5 MHz, CDCl<sub>3</sub>, ref. 85% H<sub>3</sub>PO<sub>4</sub>) δ 26.17 (dm, <sup>1</sup>*J*<sub>H,P</sub> = 567.5 Hz).

HRMS (ESI +): calcd. for C<sub>11</sub>H<sub>15</sub>NaO<sub>3</sub>P<sup>+</sup> [M+Na]<sup>+</sup> 249.0656; found 249.0651.



**Benzyl (E)-(2-amino-2-oxoethyl)(2-ethoxyvinyl)phosphinate (11)**. To a solution of phosphinate **10** (3.84 g, 17.0 mmol) in ACN (17 mL), a chloroacetamide (2.07 g, 22.1 mmol) followed by HMDS (11.0 mL, 51 mmol) were added and the mixture was stirred 48 h at 70 °C (oil bath) under argon. The



reaction was quenched with MeOH (5 mL, stirred for 30 min at 70 °C), filtered and concentrated *in vacuo*. The compound was purified by column chromatography on silica gel (40 × 75 mm column) eluting with a gradient of MeOH (0→10%) in CH<sub>2</sub>Cl<sub>2</sub> over 7.5 min at a flow rate of 100 mL/min that resulted in a linear amide **11** (2.28 g, 47%).

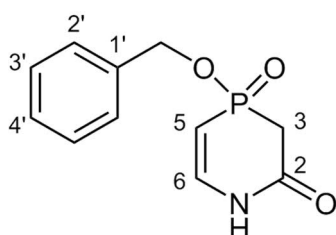
$R_f$  0.22 (MeOH/CH<sub>2</sub>Cl<sub>2</sub>, 5:95, v/v), UV-active, stains with KMnO<sub>4</sub>.

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.37-7.28 (m, 5H, H-2',3',4'), 7.22 (dd, 1H, <sup>3</sup> $J_{4,5}$  = 13.6 Hz, <sup>3</sup> $J_{H,P}$  = 11.0 Hz, H-5), 6.88, 5.83 (2br s, 2H, NH<sub>2</sub>), 5.00 (m, 2H, CH<sub>2</sub>Ph), 4.75 (dd, 1H, <sup>2</sup> $J_{H,P}$  = 15.8 Hz, <sup>3</sup> $J_{4,5}$  = 13.6 Hz, H-4), 3.92-3.79 (m, 2H, H-7), 2.96-2.78 (m, 2H, H-2), 1.30 (t, 3H,  $J_{7,8}$  = 7.1 Hz, H-8).

<sup>13</sup>C{<sup>1</sup>H} NMR (125.8 MHz, CDCl<sub>3</sub>) δ 167.1 (C1), 164.4 (d, <sup>2</sup> $J_{C5,P}$  = 17.6 Hz, C5), 136.1 (d, <sup>3</sup> $J_{C1',P}$  = 6.6 Hz, C1'); 128.6 (2C), 128.4 (1C), 127.8 (2C) (C2',3',4'); 89.4 (d, <sup>1</sup> $J_{C4,P}$  = 143.2 Hz, C4), 66.5 (C7), 66.0 (d, <sup>2</sup> $J_{C,P}$  = 5.9 Hz, CH<sub>2</sub>Ph), 39.6 (d, <sup>1</sup> $J_{C2,P}$  = 93.2 Hz, C2), 14.3 (C8).

<sup>31</sup>P{<sup>1</sup>H} NMR (202.5 MHz, CDCl<sub>3</sub>, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>) δ 40.4.

HRMS (ESI +): calcd. for C<sub>13</sub>H<sub>18</sub>NNaO<sub>4</sub>P<sup>+</sup> [M+Na]<sup>+</sup> 306.0871; found 306.0866.



**4-(Benzyloxy)-3-hydro-1,4-azaphosphinin-2(1H)-one 4-oxide (12).** To a solution of linear amide **11** (2.83 g, 10.0 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL), trifluoroacetic acid (7.66 mL, 100 mmol) was added and reaction was stirred overnight at room temperature, evaporated and purified by column chromatography on silica gel (21 × 129 mm column) eluting with a gradient of acetone (0→50%) in CH<sub>2</sub>Cl<sub>2</sub> over 20 min at a flow rate of 25 mL/min, that resulted in the cyclic amide **12** (1.60 g, 68%).

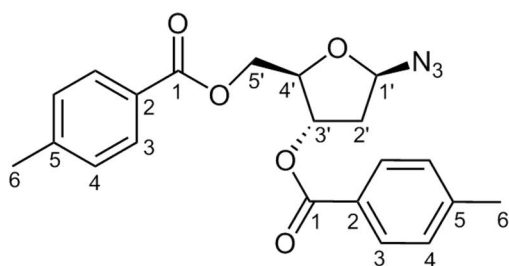
$R_f$  0.34 (acetone/CH<sub>2</sub>Cl<sub>2</sub>, 1:1, v/v).

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.52 (br s, 1H, NH), 7.40-7.30 (m, 5H, H-2',3',4'), 6.75 (ddd, 1H,  $J$  = 39.7 Hz,  $J$  = 10.8 Hz,  $J$  = 5.7 Hz, H-6), 5.30 (br dd, 1H,  $J$  = 10.8 Hz,  $J$  = 7.0 Hz, H-5), 5.06 (m, 2H, CH<sub>2</sub>Ph), 3.18-2.99 (m, 2H, H-3).

<sup>13</sup>C{<sup>1</sup>H} NMR (125.8 MHz, CDCl<sub>3</sub>) δ 166.8 (d, <sup>2</sup> $J_{C2,P}$  = 5.0 Hz, C2), 140.0 (d, <sup>2</sup> $J_{C6,P}$  = 2.5 Hz, C6), 135.8 (d, <sup>3</sup> $J_{C1',P}$  = 5.7 Hz, C1'); 128.7 (3C), 128.1 (2C) (C2',3',4'); 96.7 (d, <sup>1</sup> $J_{C5,P}$  = 127.7 Hz, C5), 66.8 (d, <sup>2</sup> $J_{C,P}$  = 6.0 Hz, CH<sub>2</sub>Ph), 37.2 (d, <sup>1</sup> $J_{C3,P}$  = 83.8 Hz, C3).

<sup>31</sup>P{<sup>1</sup>H} NMR (202.5 MHz, CDCl<sub>3</sub>, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>) δ 32.1.

HRMS (ESI +): calcd. for C<sub>11</sub>H<sub>12</sub>NNaO<sub>3</sub>P<sup>+</sup> [M+Na]<sup>+</sup> 260.0452; found 260.0447.



**2-Deoxy-3,5-bis[O-(4-methylbenzoyl)]-β-D-erythro-pentofuranosyl azide (15).**<sup>6</sup> To a stirring solution of sodium azide (97.5 g, 1.5 mol) and tetrabutylammonium hydrogen sulfate (101.7 g, 0.3 mmol) in saturated sodium bicarbonate solution (1 L), chloroform (1 L) was added and the mixture was vigorously stirred for about 5 min until a milky emulsion was formed. Hoffer's chloro-sugar **13** (116.7 g, 0.3 mol) was rapidly added to the emulsion and the mixture was stirred 20 min. After the disappearance of starting material, the organic layer was washed with satd. sodium bicarbonate (1 L), water (2 × 1 L), dried over anhydrous sodium sulfate and was filtered. Solvent was evaporated *in*

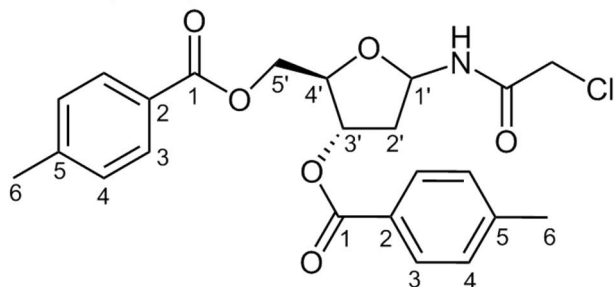
*vacuo* and the product was recrystallized from ethanol (450 mL) to yield compound **2** (105 g, 88%,  $\beta$ : $\alpha$  ratio of 19:1).

$R_f$  0.47 (CH<sub>2</sub>Cl<sub>2</sub>).

<sup>1</sup>H NMR is fully consistent with the data reported in <sup>7</sup>. <sup>1</sup>H NMR (500 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  7.94-7.84 (m, 4H, H-3), 7.36-7.29 (m, 4H, H-4), 5.879 (dd, 1H,  $J = 6.0$  Hz,  $J = 4.2$  Hz, H-1'), 5.56-5.50 (m, 1H, H-3'), 4.59-4.39 (m, 3H, H-4',5'), 2.49-2.42 (m, 1H, H-2' $\alpha$ ), 2.375, 2.370 (2s, 6H, H-6), 2.37-2.30 (m, 1H, H-2' $\beta$ ).

<sup>13</sup>C{<sup>1</sup>H} NMR (125.7 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  165.485, 165.319 (2C, C1); 144.062, 143.846 (2C, C5); 129.430, 129.316, 129.299, 129.247 (8C, C3,4); 126.705, 126.435 (2C, C2); 91.671 (C1'); 82.056 (C4'); 74.590 (C3'); 63.974 (C5'); 37.832 (C2'); 21.188, 21.166 (2C, C6).

HRMS (ESI +): calcd. for C<sub>21</sub>H<sub>21</sub>N<sub>3</sub>NaO<sub>5</sub><sup>+</sup> [M+Na]<sup>+</sup> 418.1379; found 418.1374.



**2-Deoxy-3,5-bis[O-(4-methylbenzoyl)]- $\alpha,\beta$ -D-erythro-pentofuranosyl 2-chloroacetamide (**16**)** was obtained by two-step one-pot procedure starting from 2-deoxyribofuranosyl azide **15**.

To a solution of azide **15** (19.8 g, 50.0 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (250 mL), Pd/C (2.5 g, 10% Pd) was added and the mixture was vigorously stirred 3 h at room temperature under H<sub>2</sub>. After a reduction of azide is complete (control by TLC in CH<sub>2</sub>Cl<sub>2</sub>) the mixture was filtered, cooled to 0 °C, and Et<sub>3</sub>N (7.65 mL, 55 mmol) followed by chloroacetyl chloride (4.38 mL, 55 mmol) were added, and the reaction mixture was stirred on a melting ice-bath overnight. After filtering the organic layer was washed with H<sub>2</sub>O (300 mL), satd. NaHCO<sub>3</sub> (300 mL), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated *in vacuo*. Purification was performed by column chromatography on silica gel (20-45  $\mu$ m particles size, 40  $\times$  150 mm column) eluting with a gradient of acetone (0 $\rightarrow$ 10%) in CH<sub>2</sub>Cl<sub>2</sub> over 30 min at a flow rate of 100 mL/min. Fractions containing product were evaporated *in vacuo* and resulting solid was dissolved in AcOEt (50 mL) upon heating and precipitated with hexane (250 mL) overnight at -20 °C. Solids were filtered, washed with hexane/AcOEt (8:2, v/v) and dried *in vacuo* to result in chloroacetamide **16** (8.56 g, 38%) with  $\beta/\alpha$  ratio about 1:1.

$R_f$  0.53 ( $\beta$ -**16**), 0.50 ( $\alpha$ -**16**) (AcOEt/hexane, 4:6, v/v).

**NB** Pure  $\beta$ -**16** can be obtained by crystallisation of anomeric mixture from toluene.

**$\beta$ -16:**

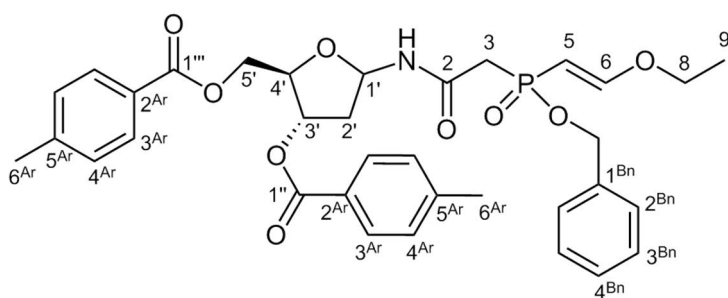
<sup>1</sup>H NMR (500 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  9.01 (d, 1H,  $^3J_{NH,1'} = 8.9$  Hz), 7.89 (m, 4H, H-3), 7.33 (m, 4H, H-4), 5.84-5.77 (m, 1H, H-1'), 5.50 (m, 1H, H-3'), 4.40 (m, 2H, H-5'), 4.31 (m, 1H, H-4'), 4.11 (s, 2H, CH<sub>2</sub>Cl); 2.384, 2.378 (2s, 6H, H-6); 2.36 (m, 2H, H-2').

<sup>13</sup>C{<sup>1</sup>H} NMR (125.8 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  166.4 (CONH), 165.5, 165.3 (2C, C1), 144.0, 143.8 (2C, C5), 129.4, 129.35, 129.3 (8C, C3,4), 126.7, 126.6 (2C, C2), 80.3 (C4'), 80.1 (C1'), 75.0 (C3'), 64.5 (C5'), 42.6 (CH<sub>2</sub>Cl), 35.9 (C2'), 21.18, 21.17 (2C, C6).

**$\alpha$ -16 (NMR extracted from 75/25 mixture of  $\alpha/\beta$ -16):**

<sup>1</sup>H NMR (400 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  8.77 (d, 1H,  $^3J_{NH,1'} = 7.9$  Hz), 7.89 (m, 4H, H-3), 7.33 (m, 4H, H-4), 5.83-5.75 (m, 1H, H-1'), 5.41 (m, 1H, H-3'), 4.50 (m, 1H, H-4'), 4.41 (m, 2H, H-5'), 4.11 (d, 2H,  $J = 1.4$  Hz, CH<sub>2</sub>Cl); 2.76 (m, 1H, H-2'), 2.39, 2.38 (2s, 6H, H-6); 2.14 (dt, 1H,  $J = 14.2$  Hz,  $J = 3.8$  Hz, H-2').

HRMS (ESI +): calcd. for C<sub>23</sub>H<sub>24</sub><sup>35</sup>ClNNaO<sub>6</sub><sup>+</sup> [M+Na]<sup>+</sup> 468.1190; found 468.1188.



***N*-{2-Deoxy-3,5-bis[*O*-(4-methylbenzoyl)]- $\alpha,\beta$ -D-erythro-pentofuranosyl}-2-[(benzyloxy)(*E*)-2-ethoxyvinyl]phosphoryl]acetamide (**17**).**

To a solution of chloroacetamide **16** (18.2 g, 40.9 mmol) in dichloroethane (80 mL), phosphinate **10** (13.9 g, 61.3 mmol) followed by HMDS (34.0 mL, 0.16 mol) were added and the reaction mixture was stirred 24 h at 90 °C (oil bath). The hot mixture was carefully quenched with MeOH (15 mL, *caution*: exothermic reaction), evaporated *in vacuo* and purified by column chromatography on silica gel eluting with a stepwise gradient of acetone (0→40%) in toluene. Fractions containing both anomers of **17** were combined, evaporated and dried *in vacuo* resulting in linear **17** (8.36 g, 32%) as a mixture of two anomers.

Analytical sample of **17** (0.50 g) was subjected to the separation of anomers by column chromatography on silica gel (20-45  $\mu$ m particles size, 25  $\times$  150 mm column) eluting with a gradient of acetone (0→30%) in CH<sub>2</sub>Cl<sub>2</sub> over 20 min at a flow rate of 40 mL/min. Fractions containing pure anomers were combined, evaporated and dried *in vacuo*.

**$\alpha$ -17:**

R<sub>f</sub> 0.42 (acetone/CH<sub>2</sub>Cl<sub>2</sub>, 15:85, v/v).

<sup>1</sup>H NMR (700 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  8.630, 8.627 (2d, 1H, <sup>3</sup>J<sub>NH,1'</sub> = 8.3 Hz, NH), 7.94, 7.86 (2m, 4H, H-3<sup>Ar</sup>), 7.40-7.24 (m, 9H, H-2<sup>Bn</sup>, 3<sup>Bn</sup>, 4<sup>Bn</sup>, 4<sup>Ar</sup>), 7.09 (m, 1H, H-6), 5.78 (m, 1H, H-1'), 5.39 (m, 1H, H-3'), 5.02-4.88 (m, 3H, H-5, CH<sub>2</sub>Ph), 4.47-4.36 (m, 3H, H-4', 5'), 3.86 (m, 2H, H-8), 3.06-2.90 (m, 2H, H-3), 2.75 (m, 1H, H-2' $\beta$ ), 2.38, 2.374, 2.370 (3s, 6H, H-6<sup>Ar</sup>), 2.03, 1.98 (2dt, 1H, J = 14.3 Hz, J = 3.9 Hz, H-2' $\alpha$ ), 1.19 (m, 3H, H-9).

<sup>13</sup>C{<sup>1</sup>H} NMR (176.1 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  165.01, 164.98 (C2); 162.55, 162.51 (2d, <sup>2</sup>J<sub>C<sub>6</sub>,P</sub> = 17.9 Hz, C6); 165.52, 165.50, 165.49 (2C, C1'', 1'''); 143.89, 143.87, 143.81 (2C, C5<sup>Ar</sup>); 137.04 (m, CH<sub>2</sub>Ph); 129.60, 129.59, 129.33, 129.25, 129.18, 129.17, 128.27, 128.25, 127.81, 127.79, 127.37, 127.33 (13C, C3<sup>Ar</sup>, 4<sup>Ar</sup>, 2<sup>Bn</sup>, 3<sup>Bn</sup>, 4<sup>Bn</sup>); 126.65, 126.57 (2C, C2<sup>Ar</sup>); 91.39, 91.35 (2d, <sup>1</sup>J<sub>C<sub>5</sub>,P</sub> = 140.0 Hz, C5); 80.13, 80.11 (C4'); 80.07, 80.04 (C1'); 75.01, 74.97 (C3'); 65.83 (C8); 64.77 (d, <sup>2</sup>J<sub>C,P</sub> = 5.6 Hz, CH<sub>2</sub>Ph); 64.35 (C5'); 39.13, 39.11 (2d, <sup>1</sup>J<sub>C<sub>3</sub>,P</sub> = 93.5 Hz, C3); 36.89, 36.87 (C2'), 21.18, 21.16 (2C, C6<sup>Ar</sup>); 14.149, 14.140 (C9).

<sup>31</sup>P{<sup>1</sup>H} NMR (202.5 MHz, *d*<sub>6</sub>-DMSO, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  39.44, 38.32 (diastereomers).

HRMS (ESI +): calcd. for C<sub>34</sub>H<sub>38</sub>NNaO<sub>9</sub>P<sup>+</sup> [M+Na]<sup>+</sup> 658.2182; found 658.2176.

**$\beta$ -17:**

R<sub>f</sub> 0.30 (acetone/CH<sub>2</sub>Cl<sub>2</sub>, 15:85, v/v).

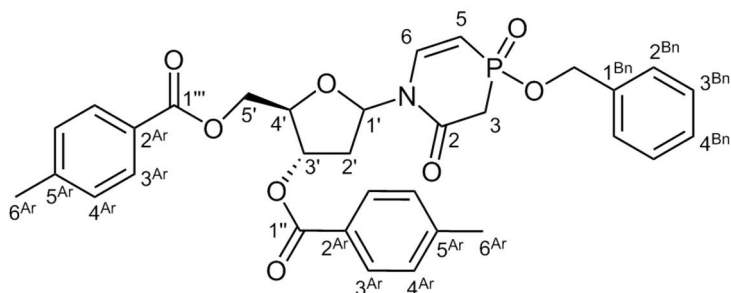
<sup>1</sup>H NMR (700 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  8.84-8.78 (m, 1H, NH), 7.91-7.87 (m, 4H, H-3<sup>Ar</sup>), 7.40-7.26 (m, 9H, H-4<sup>Ar</sup>, 2<sup>Bn</sup>, 3<sup>Bn</sup>, 4<sup>Bn</sup>); 7.10, 7.09 (2dd, 1H, J = 13.7 Hz, J = 11.1 Hz, H-6); 5.82-5.76 (m, 1H, H-1'), 5.51-5.45 (m, 1H, H-3'), 5.01-4.90 (m, 3H, H-5, CH<sub>2</sub>Ph), 4.43-4.33 (m, 2H, H-5'), 4.31-4.26 (m, 1H, H-4'), 3.92-3.84 (m, 2H, H-8), 3.02-2.88 (m, 2H, H-3); 2.385, 2.372, 2.368 (3s, 6H, H-6<sup>Ar</sup>); 2.35-2.17 (m, 2H, H-2'), 1.215, 1.206 (2t, 3H, J = 7.0 Hz, H-9).

<sup>13</sup>C{<sup>1</sup>H} NMR (176.1 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  165.53 (br, C1'''); 165.28-165.18 (2C, C2, 1''); 162.54 (d, <sup>2</sup>J<sub>C,P</sub> = 17.8 Hz), 162.45 (d, <sup>2</sup>J<sub>C,P</sub> = 17.5 Hz) (C6); 143.955, 143.946, 143.78, 143.76 (2C, C5<sup>Ar</sup>); 137.08 (d, <sup>3</sup>J<sub>C,P</sub> = 7.1 Hz), 137.03 (d, <sup>3</sup>J<sub>C,P</sub> = 6.8 Hz) (C1<sup>Bn</sup>); 129.39, 129.34, 129.30 (br), 129.28, 129.26, 128.27, 128.26, 127.81, 127.80, 127.42, 127.34 (13C, C3<sup>Ar</sup>, 4<sup>Ar</sup>, 2<sup>Bn</sup>, 3<sup>Bn</sup>, 4<sup>Bn</sup>); 126.68, 126.59 (2C, C2<sup>Ar</sup>); 91.41, 91.29 (2d, <sup>1</sup>J<sub>C,P</sub> = 141.3 Hz, C5); 80.07 (br, C4'); 79.80, 79.77 (C1'); 75.08, 75.04 (C3'); 65.78 (br, C8); 64.79 (d, <sup>2</sup>J<sub>C,P</sub> = 5.6 Hz, CH<sub>2</sub>Ph); 64.59, 64.58 (C5'); 39.39, 39.34 (2d, <sup>1</sup>J<sub>C,P</sub> = 94 Hz, C3, from DEPT); 36.1 (C2'); 21.18, 21.16 (2C, C6<sup>Ar</sup>); 14.16, 14.15 (C9).



$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  39.1, 38.9 (diastereomers).  
HRMS (ESI +): calcd. for  $\text{C}_{34}\text{H}_{38}\text{NNaO}_9\text{P}^+$   $[\text{M}+\text{Na}]^+$  658.2182; found 658.2177.

**NB** Anomerically pure **17** can be obtained starting from anomerically pure **16**, no isomerisation happens during the reaction.



**4-(Benzyloxy)-1-{2-deoxy-3,5-bis[O-(4-methylbenzoyl)]- $\alpha,\beta$ -D-erythro-pentofuranosyl}-3-hydro-1,4-azaphosphinin-2(1H)-one-4-oxide (**14**).** Linear nucleoside **17** (11.4 g, 17.5 mmol) was co-evaporated with  $\text{CH}_3\text{CN}$  (100 mL), dissolved in  $\text{CH}_3\text{CN}$  (110 mL) and TMSOTf (634  $\mu\text{L}$ , 3.51 mmol) was added. The mixture was stirred 2.5 h at 40  $^\circ\text{C}$  (oil bath), quenched with  $\text{Et}_3\text{N}$  (1.20 mL), evaporated to dryness *in vacuo* and purified by column chromatography on silica gel (40  $\times$  150 mm column) eluting with a gradient of acetone (0 $\rightarrow$ 15%) in  $\text{CH}_2\text{Cl}_2$  over 20 min at a flow rate of 100 mL/min. Fractions containing cyclic **14** were combined and evaporated *in vacuo* resulting in 6.65 g (64 %) of yellowish foam. The  $\alpha/\beta$  ratio determined from  $^{31}\text{P}$  NMR is about 3:2.

$R_f$  0.56-0.46 (acetone/ $\text{CH}_2\text{Cl}_2$ , 15:85, v/v). Visually consists of three separate spots close to each other; UV-active, stains with  $\text{KMnO}_4$ .

$^1\text{H}$  and  $^{13}\text{C}$  NMR ( $d_6$ -DMSO) are not very informative because compound is present as a mixture of two anomers,  $\alpha$  and  $\beta$ , both having two diastereomers on phosphorus atom.

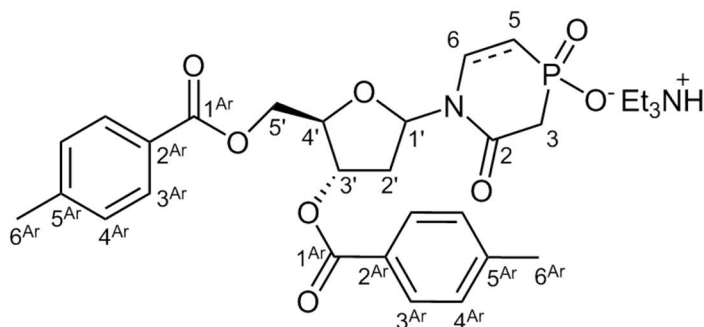
$\delta$  7.93-7.81 (m, 4H, H-3 $^{\text{Ar}}$ ), 7.52-7.20 (m, 10H, H-4 $^{\text{Ar}}$ , 2 $^{\text{Bn}}$ , 3 $^{\text{Bn}}$ , 4 $^{\text{Bn}}$ , 6), 6.40-6.16 (m, 1H, H-1'), 5.57-5.46 (m, 1H, H-5).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  31.47, 31.24 ( $\alpha$ -anomer), 30.71, 30.39 ( $\beta$ -anomer).

$^1\text{H}$ - $^1\text{H}$  COSY clearly shows four cross-spots of H-5 and H-6.

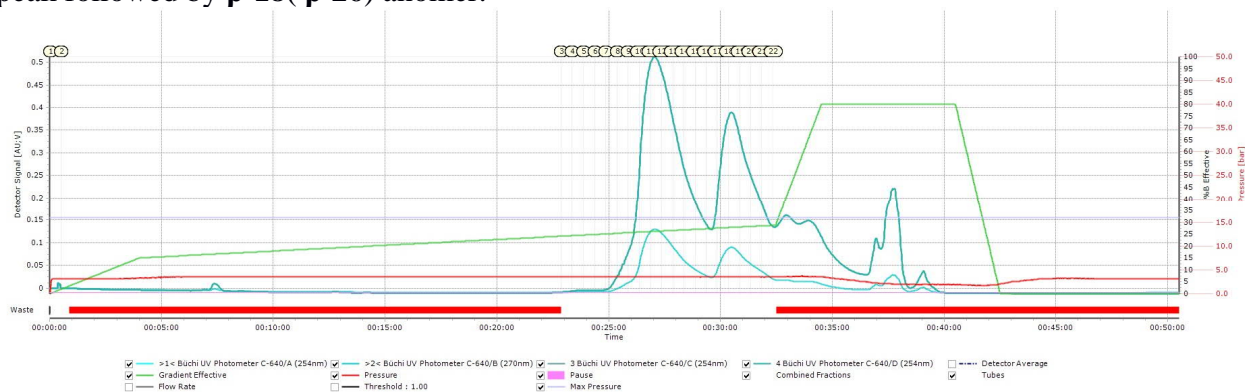
HRMS (ESI +): calcd. for  $\text{C}_{32}\text{H}_{32}\text{NNaO}_8\text{P}^+$   $[\text{M}+\text{Na}]^+$  612.1763; found 612.1763.

**NB** The cyclisation of pure  $\alpha$ -**17** and  $\beta$ -**17** leads to **14** with the same  $\alpha/\beta$  ratio  $\sim$ 3:2.

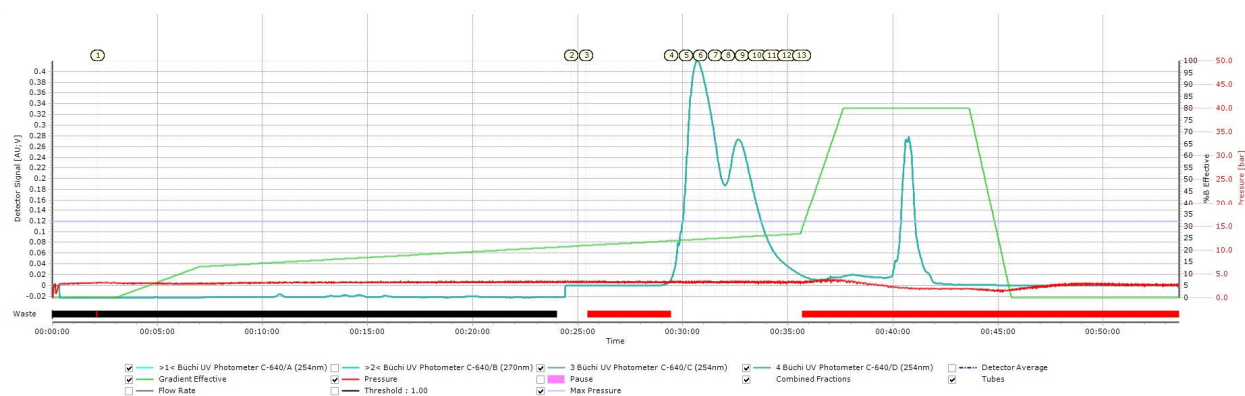


**General procedure for preparation of 1-{2-deoxy-3,5-bis[O-(4-methylbenzoyl)]- $\alpha,\beta$ -D-erythro-pentofuranosyl}-4-hydroxy-3-hydro-1,4-azaphosphinin-2(1H),4-dione (**18**) and 1-{2-deoxy-3,5-bis[O-(4-methylbenzoyl)]- $\alpha,\beta$ -D-erythro-pentofuranosyl}-4-hydroxy-1,4-azaphosphinan-2,4-dione (**26**), triethylammonium salts.** To a solution of benzyl-protected nucleoside **14** (1.00 g, 1.70 mmol) in  $\text{CH}_2\text{Cl}_2$  (17 mL),  $\text{Et}_3\text{N}$  (0.47 mL, 3.38 mmol) followed by the catalyst (0.50 g of 5%Pd/ $\text{CaCO}_3$ /3%Pb for the synthesis of **18** or 0.50 g of 10%Pd/C for the synthesis of **26**) were added and the reaction mixture was stirred 1.5 h (for **18**) or overnight (for **26**) under  $\text{H}_2$  at room temperature.

The catalyst was filtered off, the filtrate was evaporated, dried *in vacuo* and stock solution in H<sub>2</sub>O (10 mL) was prepared with concentration of **18(26)** ~100 mg/mL. Individual anomers ( $\alpha$ - and  $\beta$ -) were separated by column chromatography on 21 × 129 mm column packed in-lab with Jupiter C18 HPLC bulk media, 15  $\mu$ m. A 3 mL portion of stock solution of **18(26)** was diluted to 15 mL with H<sub>2</sub>O, loaded on the column and eluted with the following gradient of CH<sub>3</sub>CN in H<sub>2</sub>O at flow rate of 10 mL/min: 0→13% over 4 min, then 13→28% over 31 min.  $\alpha$ -**18**( $\alpha$ -**26**) Elutes as a first significant peak followed by  $\beta$ -**18**( $\beta$ -**26**) anomer.



**Figure S1.** Chromatogram showing separation of compound **18**.



**Figure S2.** Chromatogram showing separation of compound **26**.

Fractions containing pure  $\alpha$ - and  $\beta$ -anomers were combined separately, all volatile solvents were evaporated *in vacuo* and the residue was freeze-dried from H<sub>2</sub>O.

**$\alpha$ -18, triethylammonium salt:**

Yield: 346 mg (34%)

$R_f$  0.47 (MeOH/Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub>, 10:5:85, v/v/v)

<sup>1</sup>H NMR (700 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  7.89 (m, 4H, H-3<sup>Ar</sup>), 7.34 (m, 4H, H-4<sup>Ar</sup>), 6.96 (dd, 1H, <sup>3</sup> $J_{H6,P}$  = 34.6 Hz, <sup>3</sup> $J_{H5,H6}$  = 10.9 Hz, H-6), 6.33 (dd, 1H,  $J$  = 7.6 Hz,  $J$  = 4.0 Hz, H-1'), 5.495 (dt, 1H,  $J$  = 7.0 Hz,  $J$  = 2.3 Hz, H-3'), 5.44 (dd, 1H, <sup>3</sup> $J_{H5,H6}$  = 10.9 Hz, <sup>2</sup> $J_{H5,P}$  = 7.3 Hz, H-5), 4.74 (dt, 1H,  $J$  = 4.8 Hz,  $J$  = 2.1 Hz, H-4'), 4.42 (dd, 1H,  $J$  = 11.7 Hz,  $J$  = 5.3 Hz, H-5'), 4.385 (dd, 1H,  $J$  = 11.7 Hz,  $J$  = 4.6 Hz, H-5'), 2.97 (q, 6H,  $J$  = 7.3 Hz, NCH<sub>2</sub>CH<sub>3</sub>), 2.87 (m, 1H, H-2' $\beta$ ), 2.81-2.62 (m, 2H, H-3), 2.38 (s, 6H, H-6<sup>Ar</sup>), 2.09 (m, 1H, H-2' $\alpha$ ), 1.14 (t, 9H,  $J$  = 7.3 Hz, NCH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C{<sup>1</sup>H} NMR (176.1 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  168.3 (C2, from HMBC); 165.46, 165.19 (2C, C1<sup>Ar</sup>); 144.02, 143.89 (2C, C5<sup>Ar</sup>); 133.30 (br, C6); 129.46, 129.39, 129.35, 129.28 (8C, C3<sup>Ar</sup>, 4<sup>Ar</sup>); 126.58, 126.40 (2C, C2<sup>Ar</sup>); 109.5 (C5, from HSQC), 84.38 (C1'), 82.26 (C4'), 74.79 (C3'), 64.29 (C5'), 45.0 (3C, NCH<sub>2</sub>CH<sub>3</sub>), 41.3 (C3, from HSQC), 36.77 (C2'); 21.19, 21.18 (2C, C6<sup>Ar</sup>), 8.4 (3C, NCH<sub>2</sub>CH<sub>3</sub>).

<sup>31</sup>P{<sup>1</sup>H} NMR (202.5 MHz, *d*<sub>6</sub>-DMSO, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  7.17 (br).

HRMS (ESI -): calcd. for C<sub>25</sub>H<sub>25</sub>NO<sub>8</sub>P<sup>-</sup> [M-Et<sub>3</sub>NH]<sup>-</sup> 498.1323; found 498.1358.

**$\beta$ -18, triethylammonium salt:**

Yield: 210 mg (21%)

$R_f$  0.47 (MeOH/Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub>, 10:5:85, v/v/v)

$^1\text{H}$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  7.90 (m, 4H, H-3<sup>Ar</sup>), 7.35 (m, 4H, H-4<sup>Ar</sup>), 6.65 (dd, 1H,  $^3J_{\text{H}6,\text{P}} = 33.0$  Hz,  $^3J_{\text{H}5,\text{H}6} = 10.8$  Hz, H-6), 6.37 (dd, 1H,  $J = 8.4$  Hz,  $J = 6.4$  Hz, H-1'), 5.51 (m, 1H, H-3'), 5.26 (dd, 1H,  $^3J_{\text{H}5,\text{H}6} = 10.8$  Hz,  $^2J_{\text{H}5,\text{P}} = 7.1$  Hz, H-5), 4.58 (dd, 1H,  $J = 11.8$  Hz,  $J = 3.9$  Hz, H-5'), 4.50 (dd, 1H,  $J = 11.8$  Hz,  $J = 4.6$  Hz, H-5'), 4.355 (m, 1H, H-4'), 2.9 (m, 6H, NCH<sub>2</sub>CH<sub>3</sub>), 2.68-2.51 (m, 2H, H-3), 2.39 (s, 6H, H-6<sup>Ar</sup>), 2.35-2.26 (m, 2H, H-2'), 1.12 (t, 9H,  $J = 7.3$  Hz, NCH<sub>2</sub>CH<sub>3</sub>).

$^{13}\text{C}\{^1\text{H}\}$  NMR (176.1 MHz,  $d_6$ -DMSO)  $\delta$  169.21 (d,  $^2J_{\text{C}2,\text{P}} = 6.7$  Hz, C2); 165.49, 165.28 (2C, C1<sup>Ar</sup>); 144.02, 143.93 (2C, C5<sup>Ar</sup>); 131.13 (br, C6); 129.43, 129.40, 129.33, 129.28 (8C, C3<sup>Ar</sup>, 4<sup>Ar</sup>); 126.57, 126.50 (2C, C2<sup>Ar</sup>); 112.54 (d,  $^1J_{\text{C}5,\text{P}} = 121.3$  Hz, C5), 82.72 (C1'), 80.395 (C4'), 74.70 (C3'), 64.12 (C5'), 44.8 (3C, NCH<sub>2</sub>CH<sub>3</sub>), 42.11 (d,  $^1J_{\text{C}3,\text{P}} = 77.2$  Hz, C3); 35.20 (C2'), 21.20, 21.18 (2C, C6<sup>Ar</sup>), 8.5 (3C, NCH<sub>2</sub>CH<sub>3</sub>).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  7.50 (br).

HRMS (ESI -): calcd. for C<sub>25</sub>H<sub>25</sub>NO<sub>8</sub>P<sup>-</sup> [M-Et<sub>3</sub>NH]<sup>-</sup> 498.1323; found 498.1356.

#### $\alpha$ -26, triethylammonium salt:

Yield: 235 mg (23%)

$R_f$  0.38 (MeOH/Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub>, 10:5:85, v/v/v)

$^1\text{H}$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  7.88 (m, 4H, H-3<sup>Ar</sup>), 7.35 (m, 4H, H-4<sup>Ar</sup>), 6.37 (dd, 1H,  $J = 7.8$  Hz,  $J = 5.5$  Hz, H-1'), 5.46 (m, 1H, H-3'), 4.59 (m, 1H, H-4'), 4.39 (dd, 1H,  $J = 11.7$  Hz,  $J = 5.2$  Hz, H-5'), 4.35 (dd, 1H,  $J = 11.7$  Hz,  $J = 4.5$  Hz, H-5'), 3.53 (m, 2H, H-6), 3.37 (q, 6H,  $J = 7.2$  Hz, NCH<sub>2</sub>CH<sub>3</sub>), 2.75 (m, 1H, H-2'), 2.52-2.30 (m, 2H, H-3), 2.39, 2.38 (2s, 6H, H-6<sup>Ar</sup>), 1.97 (m, 1H, H-2'), 1.46 (m, 2H, H-5), 1.23 (t, 9H,  $J = 7.2$  Hz, NCH<sub>2</sub>CH<sub>3</sub>).

HRMS (ESI -): calcd. for C<sub>25</sub>H<sub>27</sub>NO<sub>8</sub>P<sup>-</sup> [M-Et<sub>3</sub>NH]<sup>-</sup> 500.1480; found 500.1511.

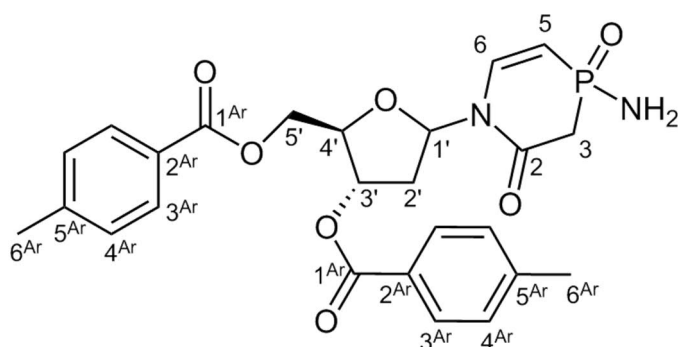
#### $\beta$ -26, triethylammonium salt:

Yield: 197 mg (19%)

$R_f$  0.38 (MeOH/Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub>, 10:5:85, v/v/v)

$^1\text{H}$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  7.89 (m, 4H, H-3<sup>Ar</sup>), 7.35 (m, 4H, H-4<sup>Ar</sup>), 6.33 (dd, 1H,  $J = 9.2$  Hz,  $J = 5.8$  Hz, H-1'), 5.47 (m, 1H, H-3'), 4.57 (dd, 1H,  $J = 11.8$  Hz,  $J = 4.0$  Hz, H-5'), 4.44 (dd, 1H,  $J = 11.8$  Hz,  $J = 4.5$  Hz, H-5'), 4.26 (m, 1H, H-4'), 3.37 (q, 6H,  $J = 7.2$  Hz, NCH<sub>2</sub>CH<sub>3</sub>), 3.31 (m, 2H, H-6), 2.43, 2.34 (2m, 2H, H-3), 2.24 (m, 2H, H-2'), 2.39 (s, 6H, H-6<sup>Ar</sup>), 1.30 (m, 2H, H-5), 1.23 (t, 9H,  $J = 7.2$  Hz, NCH<sub>2</sub>CH<sub>3</sub>).

HRMS (ESI -): calcd. for C<sub>25</sub>H<sub>27</sub>NO<sub>8</sub>P<sup>-</sup> [M-Et<sub>3</sub>NH]<sup>-</sup> 500.1480; found 500.1512.



**4-Amino-1-{2-deoxy-3,5-bis[O-(4-methylbenzoyl)]- $\alpha,\beta$ -D-erythro-pentofuranosyl}-3-hydroxy-1,4-azaphosphinin-2(1H),4-dione (19)** was prepared similar to the previously described protocol.<sup>8</sup> To a solution of phosphinic salt **18** (mixture of anomers, 0.71 g, 1.18 mmol) in CHCl<sub>3</sub> (24 mL), oxalyl chloride (0.20 mL, 2.37 mmol) was added and the mixture was stirred 15 min at room temperature (gas evolution). Satd. NH<sub>3</sub> solution in CHCl<sub>3</sub> (5 mL) was added in one portion and the mixture was stirred 10 min, evaporated and purified by column chromatography (25  $\times$  150 mm column) on silica gel eluting with a gradient of MeOH (0 $\rightarrow$ 8%) in CH<sub>2</sub>Cl<sub>2</sub> over 15 min at a flow rate of 40 mL/min. Fractions containing the product were evaporated *in vacuo* resulting in **19** as a yellowish solid (0.27 g, 46%).

$R_f$  0.35 (MeOH/CH<sub>2</sub>Cl<sub>2</sub>, 7:93, v/v)

$^1\text{H}$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  7.93-7.87 (m, 4H, H-3<sup>Ar</sup>); 7.37-7.32 (m, 4H, H-4<sup>Ar</sup>); 7.24 (t,  $J = 11.0$ ), 7.16 (t,  $J = 11.1$ ), 7.08 (dd,  $J = 10.9$ ,  $J = 5.2$ ), 7.01 (dd,  $J = 11.0$ ,  $J = 5.3$ ) (1H, H-6); 6.34 (m),

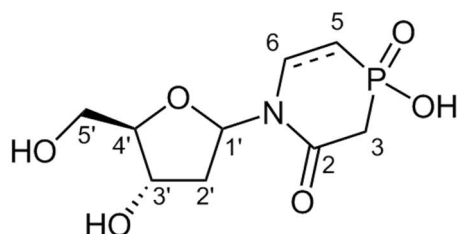
6.27 (m) (1H, H-1'); 5.57-5.49 (m, 1H, H-3'); 5.48-5.40 (m), 5.39-5.33 (m) (1H, H-5); 4.86-4.78 (m), 4.41 (m) (1H, H-4'); 4.46-4.39 (m, 2H, H-5'); 3.16-2.93 (m, 2H, H-3); 2.93-2.86 (m), 2.42-2.34 (m), 2.39 (s, 6H, H-6<sup>Ar</sup>); 2.23-2.12 (m) (2H, H-2').

<sup>13</sup>C{<sup>1</sup>H} NMR (125.8 MHz, *d*<sub>6</sub>-DMSO)  $\delta$  166.63, 166.58 (C2); 165.49, 165.48, 165.45, 165.25, 165.21, 165.17 (2C, C1<sup>Ar</sup>); 144.04, 144.02, 143.99, 143.96, 143.92, 143.91 (2C, C5<sup>Ar</sup>); 136.87, 136.71, 136.49, 136.45 (C6); 129.65-129.20 (8C, C3<sup>Ar</sup>,4<sup>Ar</sup>); 126.67, 126.57, 126.55, 126.53, 126.52, 126.46, 126.42, 126.33 (2C, C2<sup>Ar</sup>); 104.49 (d, <sup>1</sup>J<sub>C5,P</sub> = 115.9 Hz), 104.38 (d, <sup>1</sup>J<sub>C5,P</sub> = 116.3 Hz), 103.68 (d, <sup>1</sup>J<sub>C5,P</sub> = 116.5 Hz), 103.43 (d, <sup>1</sup>J<sub>C5,P</sub> = 116.2 Hz) (C5); 85.52, 84.77, 83.42, 82.95 (C1'); 83.04, 82.57, 80.76, 80.55 (C4'); 74.76, 74.65, 74.58, 74.41 (C3'); 64.24, 64.19, 64.17, 64.07 (C5'); 40.75 (d, <sup>1</sup>J<sub>C3,P</sub> = 74.4 Hz), 40.63 (d, <sup>1</sup>J<sub>C3,P</sub> = 74.8 Hz), 40.50 (d, <sup>1</sup>J<sub>C3,P</sub> = 74.0 Hz), 40.42 (d, <sup>1</sup>J<sub>C3,P</sub> = 74.2 Hz) (C3, from DEPT); 37.45, 36.97, 35.53, 35.40 (C2'); 21.20, 21.18 (2C, C6<sup>Ar</sup>).

<sup>31</sup>P{<sup>1</sup>H} NMR (202.5 MHz, *d*<sub>6</sub>-DMSO, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  16.3, 15.9.

HRMS (ESI +): calcd. for C<sub>25</sub>H<sub>27</sub>N<sub>2</sub>NaO<sub>7</sub>P<sup>+</sup> [M+Na]<sup>+</sup> 521.1453; found 521.1452.

**NB** Starting from isomerically pure  **$\alpha$ -18**, only  **$\alpha$ -19** was obtained indicating that no significant anomerisation happened during the reaction.



**General procedure for preparation of 1-(2-deoxy- $\alpha,\beta$ -D-erythro-pentofuranosyl)-4-hydroxy-3-hydroxy-1,4-azaphosphinin-2(1H),4-dione (20) or 1-(2-deoxy- $\alpha,\beta$ -D-erythro-pentofuranosyl)-4-hydroxy-1,4-azaphosphinan-2,4-dione (27).**

Toluoyl-protected nucleoside **18** or **26** in a form of triethylammonium salt (0.60 g, 1.00 mmol) was dissolved in 28% aq. NH<sub>3</sub> (50 mL) and stirred overnight at room temperature. Silica gel (2 g) was added to the mixture, all volatiles were removed *in vacuo*, and nucleoside pre-absorbed on silica was dried *in vacuo*. The nucleoside was dry-loaded on a column with silica gel (12 × 150 mm) and purified eluting with a gradient of H<sub>2</sub>O (0→50%) in CH<sub>3</sub>CN over 25 min at a flow rate of 10 mL/min. Fractions containing a nucleoside were evaporated *in vacuo* to remove CH<sub>3</sub>CN and then freeze-dried from H<sub>2</sub>O.

Compound  **$\alpha$ -20** was prepared starting from compound  **$\alpha$ -18**:

Yield: 206 mg (78%)

*R*<sub>f</sub> 0.15 (H<sub>2</sub>O/CH<sub>3</sub>CN, 20:80, v/v), UV-active

<sup>1</sup>H NMR (700 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, intn. ref. acetone)  $\delta$  7.14 (dd, 1H, <sup>3</sup>J<sub>H6,P</sub> = 36.3 Hz, <sup>3</sup>J<sub>H5,H6</sub> = 11.1 Hz, H-6), 6.24 (dd, 1H, *J* = 7.5 Hz, *J* = 5.3 Hz, H-1'), 5.67 (dd, 1H, <sup>3</sup>J<sub>H5,H6</sub> = 11.1 Hz, <sup>2</sup>J<sub>H5,P</sub> = 7.6 Hz, H-5), 4.38 (m, 1H, H-3'), 4.20 (m, 1H, H-4'), 3.70 (dd, 1H, *J* = 12.4 Hz, *J* = 3.5 Hz, H-5'), 3.60 (dd, 1H, *J* = 12.4 Hz, *J* = 5.6 Hz, H-5'), 3.05-2.87 (m, 2H, H-3), 2.65 (m, 1H, H-2' $\beta$ ), 2.00 (dt, 1H, *J* = 14.4 Hz, *J* = 4.7 Hz, H-2' $\alpha$ ).

<sup>13</sup>C{<sup>1</sup>H} NMR (176.0 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, intn. ref. acetone)  $\delta$  170.79 (d, <sup>2</sup>J<sub>C2,P</sub> = 5.1 Hz, C2), 136.39 (C6), 107.86 (d, <sup>1</sup>J<sub>C5,P</sub> = 124.2 Hz, C5), 87.60 (C4'), 85.52 (C1'), 71.07 (C3'), 62.00 (C5'), 40.13 (d, <sup>1</sup>J<sub>C3,P</sub> = 81.5 Hz, C3), 39.16 (C2').

<sup>31</sup>P{<sup>1</sup>H} NMR (202.5 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  18.04.

HRMS (ESI -): calcd. for C<sub>9</sub>H<sub>13</sub>NO<sub>6</sub>P<sup>-</sup> [M-H]<sup>-</sup> 262.0486; found 262.0502.

$\epsilon_{258}$  4230 L · mol<sup>-1</sup> · cm<sup>-1</sup>

Compound  **$\beta$ -20** was prepared starting from compound  **$\beta$ -18**:

Yield: 243 mg (92%)

*R*<sub>f</sub> 0.15 (H<sub>2</sub>O/CH<sub>3</sub>CN, 20:80, v/v), UV-active



$^1\text{H}$  NMR (700 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, intn. ref. acetone)  $\delta$  6.97 (dd, 1H,  $^3J_{\text{H}_6,\text{P}} = 36.0$  Hz,  $^3J_{\text{H}_5,\text{H}_6} = 11.0$  Hz, H-6), 6.37 (t, 1H,  $J = 7.1$  Hz, H-1'), 5.68 (dd, 1H,  $^3J_{\text{H}_5,\text{H}_6} = 11.0$  Hz,  $^2J_{\text{H}_5,\text{P}} = 7.6$  Hz, H-5), 4.38 (m, 1H, H-3'), 3.92 (m, 1H, H-4'), 3.78 (dd, 1H,  $J = 12.3$  Hz,  $J = 3.7$  Hz, H-5'), 3.70 (dd, 1H,  $J = 12.3$  Hz,  $J = 5.5$  Hz, H-5'), 3.06-2.89 (m, 2H, H-3), 2.26-2.18 (m, 2H, H-2').

$^{13}\text{C}\{^1\text{H}\}$  NMR (176.0 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, intn. ref. acetone)  $\delta$  170.86 (d,  $^2J_{\text{C}_2,\text{P}} = 5.2$  Hz, C2), 135.93 (C6), 108.55 (d,  $^1J_{\text{C}_5,\text{P}} = 123.9$  Hz, C5), 86.36 (C4'), 84.21 (C1'), 71.33 (C3'), 62.07 (C5'), 40.21 (d,  $^1J_{\text{C}_3,\text{P}} = 81.3$  Hz, C3), 38.01 (C2').

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  17.65.

HRMS (ESI -): calcd. for C<sub>9</sub>H<sub>13</sub>NO<sub>6</sub>P<sup>-</sup> [M-H]<sup>-</sup> 262.0486; found 262.0499.

Compound **27** as an anomeric mixture was prepared starting from compound **26**:

Yield: 241 mg (39%)

$R_f$  0.13 (H<sub>2</sub>O/CH<sub>3</sub>CN, 25:75, v/v), non-UV-active, stains with 2% aq. H<sub>2</sub>SO<sub>4</sub>/heat.

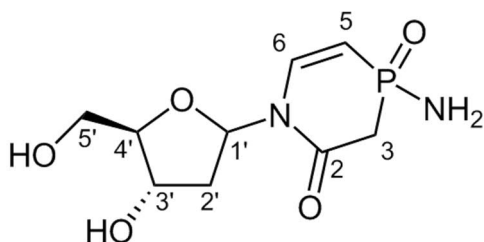
The nucleoside exists as a pair of  $\alpha$  and  $\beta$  anomers, therefore two sets of signals are present in NMR spectra. Designations " $\alpha$ " and " $\beta$ " are used to distinguish between the isomers. Integral intensities of the signals are reported as fractional numbers that reflect the ratio of isomers.

$^1\text{H}$  NMR (700 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, internal. ref. *d*<sub>4</sub>-TMSP)  $\delta$  6.33 (dd, 0.4H,  $J = 8.2$  Hz,  $J = 6.5$  Hz, H-1'- $\beta$ ), 6.21 (t, 0.6H,  $J = 6.7$  Hz, H-1'- $\alpha$ ), 4.37 (m, 1H, H-3'- $\alpha,\beta$ ), 4.13 (m, 0.6H, H-4'- $\alpha$ ), 3.89 (m, 0.4H, H-4'- $\beta$ ), 3.82-3.58 (m, 4H, H-6,5'- $\alpha,\beta$ ), 2.94-2.78 (m, 2H, H-3- $\alpha,\beta$ ), 2.59 (m, 0.6H, H-2'- $\alpha$ ), 2.18 (ddd, 0.4H,  $J = 14.1$  Hz,  $J = 8.3$  Hz,  $J = 6.8$  Hz, H-2'- $\beta$ ), 2.09 (ddd, 0.4H,  $J = 14.1$  Hz,  $J = 6.4$  Hz,  $J = 3.3$  Hz, H-2'- $\beta$ ), 1.95-1.85 (m, 2.6H, H-2'- $\alpha,3-\alpha,\beta$ ).

$^{13}\text{C}\{^1\text{H}\}$  NMR (176.0 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, intn. ref. *d*<sub>4</sub>-TMSP)  $\delta$  174.48 (d,  $^2J_{\text{C}_2,\text{P}} = 4.6$  Hz, C2 $\beta$ ), 174.24 (d,  $^2J_{\text{C}_2,\text{P}} = 4.8$  Hz, C2 $\alpha$ ), 88.71 (C4' $\alpha$ ), 88.09 (C4' $\beta$ ), 86.30 (C1' $\alpha$ ), 85.81 (C1' $\beta$ ), 73.87 (C3' $\beta$ ), 73.17 (C3' $\alpha$ ), 64.55 (C5' $\beta$ ), 64.16 (C5' $\alpha$ ), 42.31 (d,  $^1J_{\text{C}_3,\text{P}} = 72.8$  Hz, C3 $\beta$ ), 42.26 (d,  $^1J_{\text{C}_3,\text{P}} = 72.9$  Hz, C3 $\alpha$ ), 40.88 (d,  $^2J_{\text{C}_6,\text{P}} = 5.7$  Hz, C6 $\alpha$ ), 40.54 (d,  $^2J_{\text{C}_6,\text{P}} = 5.7$  Hz, C6 $\beta$ ), 39.96 (C2' $\alpha$ ), 38.71 (C2' $\beta$ ), 31.27 (br d,  $^1J_{\text{C}_5,\text{P}} = 96.1$  Hz, C5 $\alpha,\beta$ ).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  36.51 ( $\alpha$ ), 36.20 ( $\beta$ ).

HRMS (ESI -): calcd. for C<sub>9</sub>H<sub>13</sub>NO<sub>6</sub>P<sup>-</sup> [M-H]<sup>-</sup> 264.0642; found 264.0658.



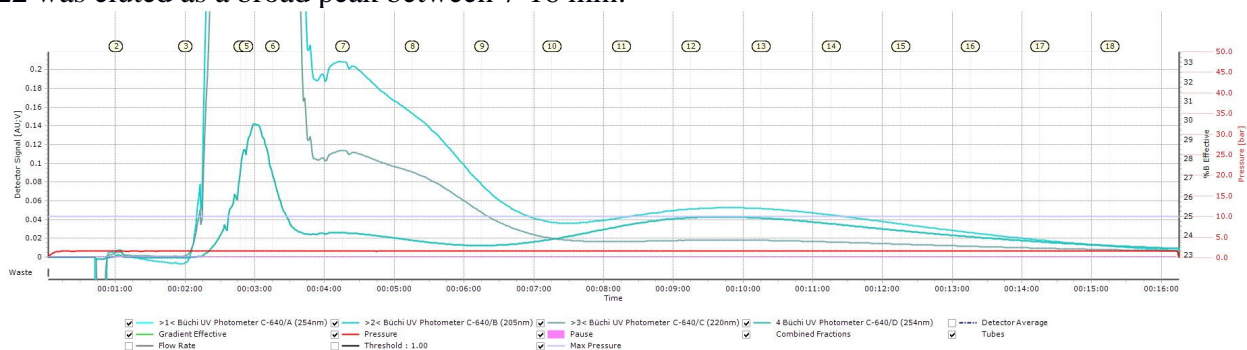
**1-(2-Deoxy- $\alpha,\beta$ -D-erythro-pentofuranosyl)-4-amino-3-hydro-1,4-azaphosphinin-2(1H),4-dione (22).**

To a heterogeneous mixture of nucleoside **20** (0.85 mmol) in CHCl<sub>3</sub> (50 mL), TBDPSCI (1.33 mL, 5.11 mmol) followed by Et<sub>3</sub>N (1.18 mL, 8.48 mmol) were added and the mixture was refluxed for 2 h and evaporated *in vacuo* to dryness. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and oxalyl chloride (0.25 mL, 3.95 mmol) was added (slow gas evolution). The mixture was stirred 30 min at room temperature and quenched with satd. NH<sub>3</sub> solution in CHCl<sub>3</sub> (10 mL), evaporated *in vacuo* to dryness and purified by column chromatography (25 × 150 mm column) on silica gel eluting with a gradient of MeOH (0→10%) in CH<sub>2</sub>Cl<sub>2</sub> over 20 min at a flow rate of 40 mL/min. Fractions containing UV-active product with  $R_f$  0.16 (acetone/CH<sub>2</sub>Cl<sub>2</sub>, 1:1, v/v) were combined and evaporated to result in TBDPS-protected phosphinamide **21** (86 mg, 14%).



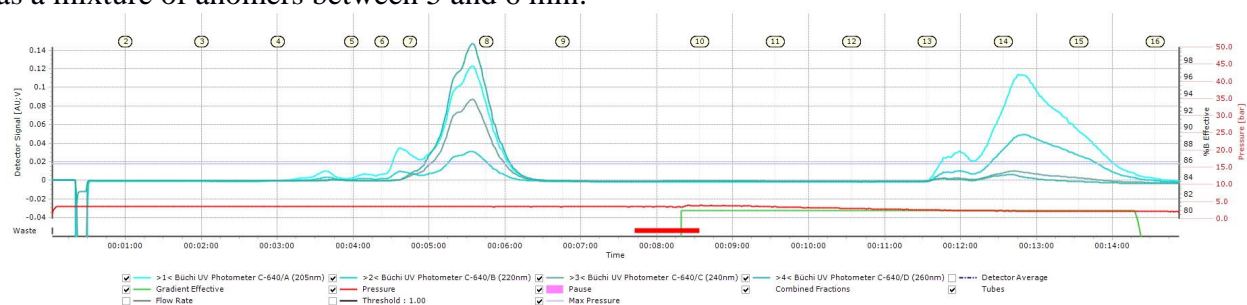
HRMS (ESI +): calcd. for  $C_{41}H_{51}N_2NaO_5PSi_2^+ [M+Na]^+$  761.2972; found 761.2968.

To a solution of compound **21** (68 mg, 92  $\mu$ mol) in THF (5 mL), solution of  $Bu_4NF \cdot 3H_2O$  (72 mg, 0.23 mmol) in THF (1 mL) was added and the mixture was stirred 1 h at room temperature. All volatiles were evaporated *in vacuo* and the residue was purified by column chromatography (12  $\times$  150 mm column) on silica gel eluting with *i*PrOH over 20 min at a flow rate of 5 mL/min. Nucleoside **22** was eluted as a broad peak between 7-16 min:



**Figure S3.** Chromatogram showing separation of compound **22** on a silica gel column.

Fractions containing compound **22** were evaporated *in vacuo* to dryness and the residue was purified by column chromatography on 21  $\times$  129 mm column packed in-lab with Jupiter C18 HPLC bulk media, 15  $\mu$ m, and eluting with  $H_2O$  at a flow rate of 10 mL/min. Phosphoramidate nucleoside **22** elutes as a mixture of anomers between 5 and 6 min:



**Figure S4.** Chromatogram showing separation of compound **22** on a reverse-phase column.

Fractions containing the product were freeze-dried to result in 7.5 mg (31%) of **22** with  $\alpha/\beta$  ratio  $\sim$ 2:1.  $R_f \sim$ 0.5 (EtOH), broad spot, UV-active.

The nucleoside exists as a pair of  $\alpha$  and  $\beta$  anomers, therefore two sets of signals are present in NMR spectra. Designations “ $\alpha$ ” and “ $\beta$ ” are used to distinguish between the isomers. Integral intensities of the signals are reported mostly as fractional numbers that reflect the ratio of isomers.

$^1H$  NMR (700 MHz, 20 mM Na-phosphate, pH 7.0, 10% v  $D_2O$ , intn. ref. acetone)  $\delta$  7.44 (dd,  $J = 38.5$  Hz,  $J = 10.9$  Hz), 7.42 (dd,  $J = 38.7$  Hz,  $J = 10.9$  Hz) (0.7H, H-6- $\alpha$ ); 7.29 (dd,  $J = 38.4$  Hz,  $J = 11.2$  Hz), 7.26 (dd,  $J = 38.6$  Hz,  $J = 11.0$  Hz) (0.3H, H-6- $\beta$ ); 6.36, 6.35 (2t, 0.3H,  $J = 7.2$  Hz, H-1'- $\beta$ ); 6.23 (dd,  $J = 7.7$  Hz,  $J = 4.2$  Hz), 6.22 (dd,  $J = 8.2$  Hz,  $J = 4.2$  Hz) (0.7H, H-1'- $\alpha$ ); 5.73-5.62 (m, 1H, H-5- $\alpha,\beta$ ); 4.39 (m, 1H, H-3'- $\alpha,\beta$ ); 4.26 (m, 0.7H, H-4'- $\alpha$ ); 3.95 (m, 0.3H, H-4'- $\beta$ ); 3.82-3.77, 3.74-3.69 (2m, 0.6H, H-5'- $\beta$ ); 3.69 (dd,  $J = 12.6$  Hz,  $J = 3.9$  Hz), 3.60 (dd,  $J = 12.6$  Hz,  $J = 5.5$  Hz) (1.7H, H-5'- $\alpha$ ); 3.40-3.24 (m, 2H, H-3- $\alpha,\beta$ ); 2.67 (m, 0.7H, H-2'- $\alpha$ ); 2.28-2.16 (m, 0.6H, H-2'- $\beta$ ); 2.02, 2.00 (2t, 0.7H,  $J = 4.0$  Hz, H-2'- $\alpha$ ).

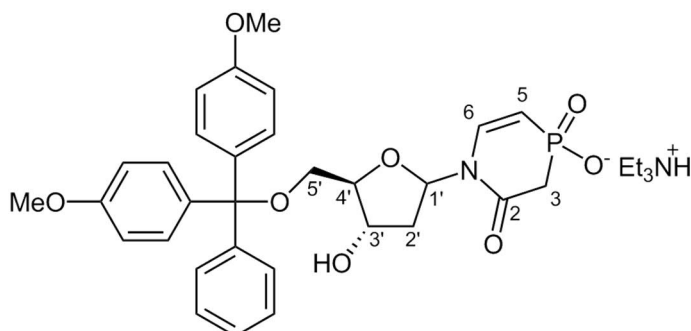
$^{13}C\{^1H\}$  NMR (176.0 MHz, 20 mM Na-phosphate, pH 7.0, 10% v  $D_2O$ , intn. ref. acetone)  $\delta$  168.46, 168.44 (C2- $\beta$ ); 168.37, 168.34 (C2- $\alpha$ ); 141.16, 141.10 (C6- $\alpha$ ); 140.6 (C6- $\beta$ ); 101.28, 100.94 (2d,  $^1J_{C5,P} = 120.0$  Hz, C5- $\beta$ ); 100.25, 100.02 (2d,  $^1J_{C5,P} = 120.0$  Hz, C5- $\alpha$ ); 88.35, 88.31 (C4'- $\alpha$ ); 86.68, 86.66 (C4'- $\beta$ ); 86.49, 86.31 (C1'- $\alpha$ ); 84.71, 84.66 (C1'- $\beta$ ); 71.32, 71.29 (C3'- $\beta$ ); 71.09 (C3'- $\alpha$ ); 62.08,

62.03, 62.00 (C5'- $\alpha,\beta$ ); 39.67, 39.54 (C2'- $\alpha$ ); 39.62 (d,  $^1J_{C3,P} = 76.9$  Hz), 39.26 (d,  $^1J_{C3,P} = 77.3$  Hz) (C3- $\beta$ ); 39.56 (d,  $^1J_{C3,P} = 76.9$  Hz), 39.21 (d,  $^1J_{C3,P} = 77.7$  Hz) (C3- $\alpha$ ); 38.43, 38.17 (C2'- $\beta$ ).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz, 20 mM Na-phosphate, pH 7.0, 10% v D<sub>2</sub>O, extn. ref. 85% H<sub>3</sub>PO<sub>4</sub>)  $\delta$  26.34 ( $\alpha$ ), 25.89 ( $\beta$ ).

HRMS (ESI +): calcd. for C<sub>9</sub>H<sub>15</sub>N<sub>2</sub>NaO<sub>5</sub>P<sup>+</sup> [M+Na]<sup>+</sup> 285.0616; found 285.0613.

UV-vis:  $\epsilon_{262}$  4730 L·mol<sup>-1</sup> cm<sup>-1</sup>

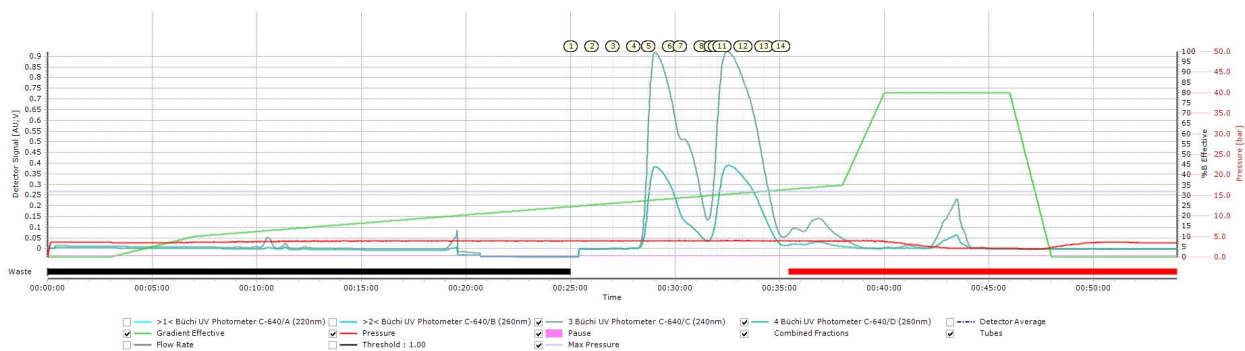


**1-[2-Deoxy-5-O-(4,4'-dimethoxytrityl)- $\alpha,\beta$ -D-erythro-pentofuranosyl]-4-hydroxy-3-hydro-1,4-azaphosphinin-2(1H),4-dione, triethylammonium salt (**23**).**

To a solution of benzyl-protected nucleoside **14** (2.95 g, 5.00 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL), Et<sub>3</sub>N (1.39 mL, 10.0 mmol) followed by 5%Pd/CaCO<sub>3</sub>/3%Pb (1.48 g) were added and the reaction mixture was stirred 1.5 h under H<sub>2</sub> at room temperature. The catalyst was filtered off, the filtrate was evaporated *in vacuo* and the residue was dissolved in 28% aq. NH<sub>3</sub> (150 mL) and stirred overnight at room temperature. The mixture was concentrated to the volume ~50 mL and cooled at +4 °C for 1 h, crystals were filtered off and washed with a cold water. Combined water layer (200 mL) was extracted with AcOEt (200 mL), and water layer was evaporated *in vacuo* to dryness, co-evaporated with MeOH, CH<sub>3</sub>CN and dried *in vacuo* resulting in compound **20**.

Dry pyridine (200 mL) was added to compound **20**, heating was required to dissolve major part of nucleoside **20**. After the mixture was cooled down to room temperature, DMTCI (3.05 g, 9.00 mmol) was added in one portion and the reaction mixture was stirred for 24 h at room temperature. The residue obtained after evaporation of solvents *in vacuo* and co-evaporation with toluene was purified by column chromatography (25 × 150 mm column) on silica gel eluting with a gradient of solvent B (0→30%) in solvent A over 20 min at a flowrate 50 mL/min (solvent A: Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub> 5:95 v/v, solvent B: Et<sub>3</sub>N/MeOH 5:95 v/v). Fractions containing the product with *R<sub>f</sub>* 0.4 (MeOH/Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub>, 10:5:85, v/v/v) were combined and evaporated *in vacuo* resulting in 3.20 g (96%) of **23** as a mixture of anomers.

The stock solution of compound **23** in H<sub>2</sub>O (32 mL) was prepared with concentration of **23** ~100 mg/mL. Anomers  $\alpha$ - and  $\beta$ - were separated by column chromatography on 21 × 129 mm column packed in-lab with Jupiter C18 HPLC bulk media, 15  $\mu$ m. A 3-10 mL portions of stock solution of **23** were diluted to 15 mL with H<sub>2</sub>O, loaded on the column and eluted with the following gradient of CH<sub>3</sub>CN in H<sub>2</sub>O at a flow rate of 10 mL/min: 0→10% over 4 min, then 10→45% over 31 min.  $\beta$ -**23** Elutes as a first significant peak followed by  $\alpha$ -**23** anomer:



**Figure S5.** Chromatogram showing separation of  $\alpha$ - and  $\beta$ -anomers of compound **23**.

Fractions containing pure  $\alpha$ - and  $\beta$ -anomers were combined separately,  $\text{Et}_3\text{N}$  (1 mL) was added to each compound and all volatile solvents were evaporated *in vacuo*, the residue was co-evaporated with  $\text{CH}_3\text{CN}$  ( $2 \times 200$  mL) and  $\text{CH}_2\text{Cl}_2$  ( $2 \times 200$  mL).

### $\alpha$ -**23**, triethylammonium salt:

0.82 g (25%)

$R_f$  0.4 (MeOH/ $\text{Et}_3\text{N}$ / $\text{CH}_2\text{Cl}_2$ , 10:5:85, v/v/v)

Contains  $\text{Et}_3\text{NCH}_2\text{Cl}^+ \text{Cl}^-$  as an impurity with a molar ratio  $\alpha$ -**23**/impurity 1:0.6.

$^1\text{H}$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  5.35 (s, 2H,  $\text{Et}_3\text{NCH}_2\text{Cl}$ ), 3.37 (q, 6H,  $J = 7.2$  Hz,  $\text{CH}_3\text{CH}_2\text{N}$ ), 1.23 (t, 9H,  $J = 7.2$  Hz,  $\text{CH}_3\text{CH}_2\text{N}$ ).

$^{13}\text{C}\{^1\text{H}\}$  NMR (176.1 MHz,  $d_6$ -DMSO)  $\delta$  62.62 ( $\text{Et}_3\text{NCH}_2\text{Cl}$ ), 52.00 (3C,  $\text{CH}_3\text{CH}_2\text{N}$ ), 7.20 (3C,  $\text{CH}_3\text{CH}_2\text{N}$ ).

$^1\text{H}$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  7.38 (m, 2H, H-2''), 7.31 (m, 2H, H-3''), 7.25 (m, 4H, H-2'''), 7.21 (m, 1H, H-4''), 6.89 (m, 4H, H-3'''), 6.86 (dd, 1H,  $^3J_{\text{H}6,\text{P}} = 32.2$  Hz,  $J_{\text{H}5,\text{H}6} = 10.8$  Hz, H-6), 6.33 (dd, 1H,  $J = 7.7$  Hz,  $J = 5.7$  Hz, H-1'), 5.38 (dd, 1H,  $J_{\text{H}5,\text{H}6} = 10.8$  Hz,  $^2J_{\text{H}5,\text{P}} = 7.3$  Hz, H-5), 4.08 (m, 1H, H-3'), 4.05 (m, 1H, H-4'), 3.73 (s, 6H,  $\text{OCH}_3$ ), 3.02 (dd, 1H,  $J = 10.1$  Hz,  $J = 3.3$  Hz, H-5'), 2.90 (dd, 1H,  $J = 10.1$  Hz,  $J = 5.2$  Hz, H-5'), 2.54-2.38 (m, 9H, H-3,2',  $\text{CH}_3\text{CH}_2\text{N}$ ), 1.69 (dt, 1H,  $J = 13.5$  Hz,  $J = 4.9$  Hz, H-2'), 0.93 (t, 9H,  $J = 7.1$  Hz,  $\text{CH}_3\text{CH}_2\text{N}$ ).

$^{13}\text{C}\{^1\text{H}\}$  NMR (176.1 MHz,  $d_6$ -DMSO)  $\delta$  169.66 (d,  $^2J_{\text{C}6,\text{P}} = 5.4$  Hz, C2), 158.03 (2C, C4'''), 144.87 (C1''); 135.63, 135.56 (2C, C1'''); 131.26 (C6), 129.66, 129.65 (4C, C2'''), 127.85 (2C, C3'''), 127.67 (2C, C2''), 126.62 (C4''), 113.72 (d,  $^1J_{\text{C}3,\text{P}} = 117.1$  Hz, C5), 113.21 (4C, C3'''), 85.49 ( $\text{CAr}_3$ ), 85.19 (C4'), 82.95 (C1'), 70.98 (C3'), 64.30 (C5'), 55.02 (2C,  $\text{OCH}_3$ ), 42.76 (d,  $^1J_{\text{C}5,\text{P}} = 74.6$  Hz, C3), 39.19 (C2', from DEPT).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  5.66.

HRMS (ESI  $-$ ): calcd. for  $\text{C}_{30}\text{H}_{31}\text{NO}_8\text{P}^-$  [ $\text{M-Et}_3\text{NH}$ ] $^-$  564.1793; found 564.1829.

### $\beta$ -**23**, triethylammonium salt:

0.66 g (20%)

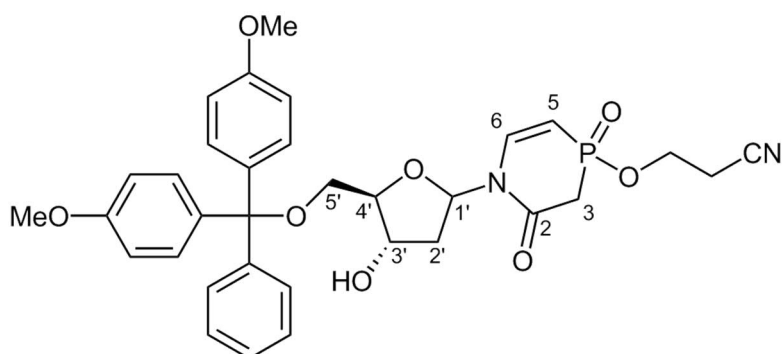
$R_f$  0.4 (MeOH/ $\text{Et}_3\text{N}$ / $\text{CH}_2\text{Cl}_2$ , 10:5:85, v/v/v)

$^1\text{H}$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  7.39 (m, 2H, H-2''), 7.31 (m, 2H, H-3''), 7.26 (m, 4H, H-2'''), 7.21 (m, 1H, H-4''), 6.89 (m, 4H, H-3'''), 6.62 (dd, 1H,  $^3J_{\text{H}6,\text{P}} = 32.0$  Hz,  $J_{\text{H}5,\text{H}6} = 10.8$  Hz, H-6), 6.26 (t, 1H,  $J = 6.9$  Hz, H-1'), 5.20 (dd, 1H,  $J_{\text{H}5,\text{H}6} = 10.8$  Hz,  $^2J_{\text{H}5,\text{P}} = 7.3$  Hz, H-5), 4.16 (m, 1H, H-3'), 3.77 (m, 1H, H-4'), 3.73 (s, 6H,  $\text{OCH}_3$ ), 3.12 (m, 2H, H-5'), 2.53, 2.39 (2m, 2H, H-3), 1.91 (m, 2H, H-2').

$^{13}\text{C}\{^1\text{H}\}$  NMR (176.1 MHz,  $d_6$ -DMSO)  $\delta$  169.54 (d,  $^2J_{\text{C}6,\text{P}} = 5.5$  Hz, C2), 158.07 (2C, C4'''), 144.87 (C1''); 135.54, 135.45 (2C, C1'''); 130.30 (C6), 129.76, 129.74 (4C, C2'''), 127.85 (2C, C3'''), 127.71 (2C, C2''), 126.67 (C4''), 113.89 (d,  $^1J_{\text{C}3,\text{P}} = 117.0$  Hz, C5); 113.23, 113.21 (4C, C3'''), 85.62 ( $\text{CAr}_3$ ), 84.41 (C4'), 82.29 (C1'), 70.45 (C3'), 63.79 (C5'), 55.05 (2C,  $\text{OCH}_3$ ), 42.61 (d,  $^1J_{\text{C}3,\text{P}} = 75.0$  Hz, C3), 38.82 (C2').

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  5.59.

HRMS (ESI  $-$ ): calcd. for  $\text{C}_{30}\text{H}_{31}\text{NO}_8\text{P}^-$  [ $\text{M-Et}_3\text{NH}$ ] $^-$  564.1793; found 564.1828.



**General procedure for preparation of 1-[2-Deoxy-5-O-(4,4'-dimethoxytrityl)- $\alpha,\beta$ -D-erythro-pentofuranosyl]-4-(2-cyanoethoxy)-3-hydro-1,4-azaphosphinin-2(1H),4-dione (**24**).**

To a solution of pure  $\alpha$ - or  $\beta$ -anomer of salt **23** (0.67 g, 1.00 mmol) in  $\text{CH}_2\text{Cl}_2$  (20 mL), 3-hydroxypropionitrile (0.34 mL, 5.00 mmol),  $\text{Et}_3\text{N}$  (0.42 mL, 3.00 mmol), and TBTU (0.64 g, 2.00 mmol) were added, and the mixture was refluxed for 1 h. Reaction mixture was diluted with  $\text{CH}_2\text{Cl}_2$  to 100 mL and washed with  $\text{H}_2\text{O}$  (100 mL). The emulsion formed was centrifuged for 5 min at 5000 rpm and organic layer was separated and evaporated *in vacuo* to dryness. The residue was purified by column chromatography (21  $\times$  129 mm column) on silica gel. Column was washed with  $\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2$  (1:99, v/v, 200 mL) and  $\text{CH}_2\text{Cl}_2$  (200 mL), sample was loaded on the column and it was eluted with a gradient of *i*PrOH (0 $\rightarrow$ 15%) in  $\text{CH}_2\text{Cl}_2$  over 20 min at a flow rate of 25 mL/min. The product was eluted as two separable diastereomers on phosphorus center, that were combined and evaporated *in vacuo* to dryness to result in  $\alpha$ -**24** or  $\beta$ -**24** (both compounds appear as a yellowish foam).

**$\alpha$ -24:**

Yield: 165 mg (27%)

$R_f$  0.33, 0.30 (*i*PrOH/ $\text{CH}_2\text{Cl}_2$ , 7:93, v/v)

$^1\text{H}$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  7.60 (dd,  $J = 41.7$  Hz,  $J = 11.3$  Hz), 7.55 (dd,  $J = 41.1$  Hz,  $J = 11.2$  Hz), (1H, H-6); 7.37 (m, 2H, H-2''), 7.32 (m, 2H, H-3''), 7.27-7.20 (m, 5H, H-2''',4''), 6.90 (m, 4H, H-3'''); 6.30 (dd,  $J = 7.9$  Hz,  $J = 3.9$  Hz), 6.27 (dd,  $J = 7.9$  Hz,  $J = 4.0$  Hz) (1H, H-1'); 5.66-5.57 (m, 1H, H-5), 4.23-4.17 (m, 1H, H-4'), 4.17-4.06 (m, 3H, H-3',  $\text{CH}_2\text{CH}_2\text{CN}$ ), 3.74 (s, 6H,  $\text{OCH}_3$ ); 3.62-3.22 (m, 2H, H-3), 3.08-3.00, 2.97-2.91 (2m, 2H, H-5'); 2.91-2.86 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CN}$ ), 2.62-2.50, 1.79-1.69 (2m, 2H, H-2').

$^{13}\text{C}\{^1\text{H}\}$  NMR (125.7 MHz,  $d_6$ -DMSO)  $\delta$  165.66 (d,  $^2J_{\text{C}_2,\text{P}} = 5.3$  Hz, C2), 158.07 (2C, C4'''); 144.77, 144.76 (C1''); 142.22, 142.13 (C6); 135.52, 135.51, 135.43, 135.40 (2C, C1'''); 129.65, 129.64 (4C, C2'''); 127.87 (2C, C3'''), 127.63 (2C, C2''), 126.69 (C4''); 118.34, 118.31 (CN); 113.23 (4C, C3'''); 97.50 (d,  $^1J_{\text{C}_5,\text{P}} = 124.8$  Hz), 97.25 (d,  $^1J_{\text{C}_5,\text{P}} = 125.2$  Hz) (C5); 86.68, 86.67 (C4'); 85.66, 85.63 ( $\text{C}_{\text{Ar}_3}$ ); 84.63, 84.60 (C1'); 70.80, 70.78 (C3'); 64.05 (C5'); 59.48, 59.42 (d,  $^2J = 5.4$  Hz,  $\text{CH}_2\text{CH}_2\text{CN}$ ); 55.03 (2C,  $\text{OCH}_3$ ); 39.98, 39.72 (C2'); 36.92 (d,  $^1J_{\text{C}_3,\text{P}} = 83.5$  Hz, C3), 19.42 (d,  $^3J = 6.3$  Hz,  $\text{CH}_2\text{CH}_2\text{CN}$ ).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  32.9, 32.4.

HRMS (ESI +): calcd. For  $\text{C}_{33}\text{H}_{35}\text{N}_2\text{NaO}_8\text{P}^+$  [ $\text{M}+\text{Na}$ ] $^+$  641.2029; found 641.2025.

**$\beta$ -24:**

Yield: 450 mg (73%)

$R_f$  0.72, 0.69 (*i*PrOH/ $\text{CH}_2\text{Cl}_2$ , 10:90, v/v)

$^1\text{H}$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  7.38 (m, 2H, H-2''), 7.36-7.28 (m, 3H, H-6,3''), 7.28-7.21 (m, 5H, H-4'',2'''), 6.94-6.88 (m, 4H, H-3'''), 6.21 (m, 1H, H-1'), 5.34-5.25 (m, 2H, H-5,OH), 4.28-4.19 (m, 1H, H-3'), 4.17-4.05 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CN}$ ), 3.84 (m, 1H, H-4'), 3.76-3.73 (m, 6H,  $\text{OCH}_3$ ), 3.50-3.29 (m, 2H, H-3), 3.25-3.12 (m, 2H, H-5'), 2.91-2.85 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CN}$ ), 2.11-1.95 (m, 2H, H-2').

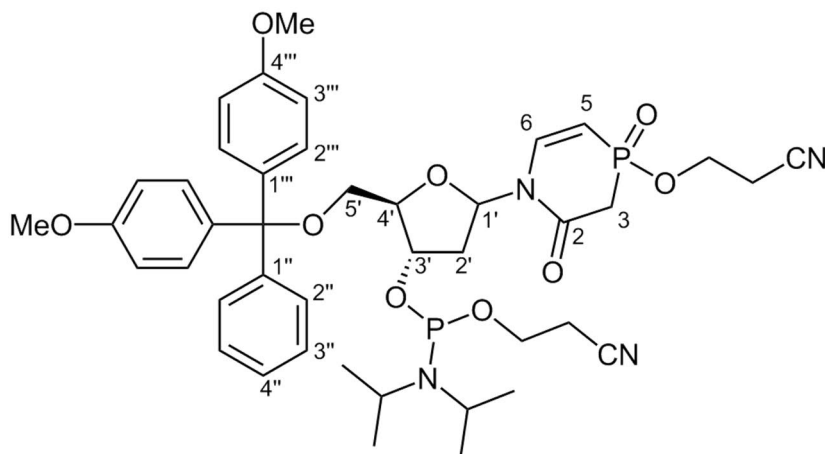
$^{13}\text{C}\{^1\text{H}\}$  NMR (125.7 MHz,  $d_6$ -DMSO)  $\delta$  165.56 (d,  $^2J_{\text{C}_2,\text{P}} = 5.7$  Hz), 165.49 (d,  $^2J_{\text{C}_6,\text{P}} = 4.9$  Hz) (C2); 158.13, 158.12, 158.11 (2C, C4'''); 144.72, 144.69 (C1''); 141.16, 140.97 (C6); 135.47, 135.41, 135.26, 135.24 (2C, C1'''); 129.75, 129.73 (4C, C2'''); 127.91 (2C, C3'''), 127.69 (2C, C2''), 126.75 (C4''); 118.30, 118.26 (CN); 113.28, 113.25, 113.23 (4C, C3'''); 98.13 (d,  $^1J_{\text{C}_5,\text{P}} = 124.5$  Hz), 97.47



(d,  $^1J_{C5,P} = 125.6$  Hz) (C5); 85.78, 85.76 ( $C_{Ar3}$ ); 85.08 (C4'); 83.35, 83.25 (C1'); 70.27, 70.09 (C3'); 63.53, 63.41 (C5'); 59.61, 59.43 (d,  $^2J = 5.5$  Hz,  $CH_2CH_2CN$ ); 55.06, 55.05 (2C,  $OCH_3$ ); 39.45, 38.96 (C2'); 37.18 (d,  $^1J_{C3,P} = 81.9$  Hz), 36.86 (d,  $^1J_{C3,P} = 83.7$  Hz) (C3); 19.44, 19.38 (d,  $^3J = 6.4$  Hz,  $CH_2CH_2CN$ ).

$^{31}P\{^1H\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $H_3PO_4$ )  $\delta$  32.2, 31.9.

HRMS (ESI +): calcd. for  $C_{33}H_{35}N_2NaO_8P^+$   $[M+Na]^+$  641.2029; found 641.2026.



**General procedure for preparation of 1-[2-deoxy-5-O-(4,4'-dimethoxytrityl)-3-O-(*N,N*-diisopropylamino-2-cyanoethoxyphosphanyl)- $\alpha,\beta$ -D-erythro-pentofuranosyl]-4-(2-cyanoethoxy)-3-hydroxy-1,4-azaphosphinin-2(1*H*),4-dione (**25**).** To a solution of pure  $\alpha$ - or  $\beta$ -anomer of **24** (155 mg, 0.25 mmol) in dry  $CH_2Cl_2$  (10 mL),  $Et_3N$  (209  $\mu$ L, 1.50 mmol) followed by *N,N*-diisopropylamino-2-cyanoethoxychlorophosphine (145 mg, 0.63 mmol) were added and the mixture was stirred for 1 h at room temperature. Reaction mixture was diluted with  $CH_2Cl_2$  to 100 mL, washed with satd.  $NaHCO_3$  (100 mL), dried over  $Na_2SO_4$ , filtered and evaporated *in vacuo* to dryness. The residue was purified by column chromatography (12  $\times$  150 mm column) on silica gel. Column was washed with  $Et_3N/CH_2Cl_2$  (10:90, v/v, 100 mL) and  $CH_2Cl_2$  (100 mL), sample was loaded on the column and it was eluted with a gradient of *i*PrOH (0 $\rightarrow$ 10%) in  $CH_2Cl_2$  over 10 min at a flow rate of 10 mL/min. Fractions containing product **25** were combined and evaporated *in vacuo* to dryness, the residue was co-evaporated with dry  $CH_2Cl_2$  (2  $\times$  100 mL) and finally freeze-dried from benzene.

#### $\alpha$ -**25**:

Yield: 91 mg (44%)

$R_f$  ~0.4 (*i*PrOH/ $CH_2Cl_2$ , 5:95, v/v), TLC plate treated with  $Et_3N$ /hexane (1:9, v/v) and dried prior to spotting

$^1H$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  7.50-7.39 (m, 1H, H-6); 7.39-7.35 (m, 2H, H-2''); 7.34-7.29 (m, 2H, H-3''); 7.27-7.20 (m, 5H, H-2''',4''); 6.92-6.86 (m, 4H, H-3'''); 6.37-6.30 (m, 1H, H-1'); 5.63-5.53 (m, 1H, H-5); 4.44-4.28 (m, 2H, H-3',4'); 4.15-4.07, 3.73-3.61 (2m, 4H,  $CH_2CH_2CN$ ); 3.737, 3.735 (2s, 6H,  $OCH_3$ ); 3.55, 3.30 (2m, 2H, H-3); 3.55-3.48 (m, 2H,  $CHCH_3$ ); 3.18-3.09, 3.02-2.95 (2m, 2H, H-5'); 2.92-2.85, 2.72-2.64 (2m, 4H,  $CH_2CH_2CN$ ); 2.77-2.62, 2.04-1.81 (2m, 2H, H-2'); 1.15-1.01 (m, 12H,  $CHCH_3$ ).

$^{13}C\{^1H\}$  NMR (176.0 MHz,  $d_6$ -DMSO)  $\delta$  165.72-165.62 (C2); 158.12 (2C, C4'''); 144.64 (C1''); 141.60, 141.51, 141.47 (C6); 135.42, 135.36, 135.28, 135.26, 135.25, 135.23 (2C, C1'''); 129.62 (4C, C2'''); 127.88 (2C, C3'''); 127.61, 127.57 (2C, C2''); 126.74 (C4''); 118.94, 118.84, 118.29, 118.21 (2C,  $CH_2CH_2CN$ ); 113.23 (4C, C3'''); 97.88, 97.75 (2d,  $^1J_{C5,P} = 125.3$  Hz, C5); 85.84, 85.81, 85.80 ( $C_{Ar3}$ ); 85.71, 85.69, 85.42, 85.39 (C4'); 84.82, 84.81, 84.78, 84.49 (C1'); 73.75, 73.65, 73.14, 73.06 (C3'); 63.7-63.6 (C5'); 59.58-59.40, 58.43, 58.39, 58.32, 58.28 (2C,  $CH_2CH_2CN$ ); 55.03 (2C,  $OCH_3$ ); 42.65, 42.57, 42.50 (2C,  $CHCH_3$ ); 39.3, 39.0 (C2', from DEPT); 36.9 (br d,  $^1J_{C3,P} = 83$  Hz, C3); 24.34, 24.30, 24.28, 24.24, 24.19, 24.16 24.12 ( $CHCH_3$ ); 19.86, 19.85, 19.82, 19.81, 19.77, 19.46, 19.45, 19.43, 19.41, 19.39 (2C,  $CH_2CH_2CN$ )



$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  149.00, 148.98, 148.52, 148.36 (P(III)); 32.50, 32.48, 32.09 (P(V)).  
HRMS (ESI +): calcd. for  $\text{C}_{42}\text{H}_{52}\text{N}_4\text{NaO}_9\text{P}_2^+$   $[\text{M}+\text{Na}]^+$  841.3107; found 841.3103.

### $\beta$ -25:

Yield: 119 mg (58%)

$R_f \sim 0.4$  ( $i\text{PrOH}/\text{CH}_2\text{Cl}_2$ , 5:95, v/v), TLC plate treated with  $\text{Et}_3\text{N}/\text{hexane}$  (1:9, v/v) and dried prior to spotting.

$^1\text{H}$  NMR (700 MHz,  $d_6$ -DMSO)  $\delta$  7.41-7.36 (m, 2H, H-2''); 7.36-7.27 (m, 3H, H-6,3''); 7.27-7.22 (m, 5H, H-4'',2''); 6.93-6.87 (m, 4H, H-3'''); 6.22 (m, 1H, H-1'); 5.34 (m, 1H, H-5); 4.47 (m, 1H, H-3'); 4.10 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CN}$ ); 4.02-3.92 (m, 1H, H-4'); 3.76-3.57 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CN}$ ); 3.74 (m, 6H,  $\text{OCH}_3$ ); 3.57-3.45 (m, 2H,  $\text{CHCH}_3$ ); 3.26-3.19 (m, 2H, H-5'); 2.90-2.86 (m, 2H), 2.75 (m, 1H), 2.64 (m, 1H) ( $\text{CH}_2\text{CH}_2\text{CN}$ ); 2.30-2.08 (m, 2H, H-2'); 1.14-0.96 (m, 12H,  $\text{CHCH}_3$ ).

$^{13}\text{C}\{^1\text{H}\}$  NMR (176.0 MHz,  $d_6$ -DMSO)  $\delta$  165.56 (m, C2); 158.16, 158.14 (2C, C4''); 144.60 (C1''); 141.24, 141.05, 140.92, 140.76 (C6); 135.34, 135.28, 135.19, 135.14, 135.10 (2C, C1''); 129.74, 129.73, 129.69 (4C, C2'''); 127.89 (2C, C3''); 127.69, 127.64 (2C, C2''); 126.78 (C4''); 118.93, 118.92, 118.75, 118.29, 118.28, 118.27, 118.22 (2C,  $\text{CH}_2\text{CH}_2\text{CN}$ ); 113.24, 113.23, 113.22 (4C, C3''); 99.0-97.2 (m, C5); 85.95, 85.92, 85.88, 85.85 ( $\text{CAr}_3$ ); 84.05, 84.03, 83.69, 83.66 (C4'); 83.40, 83.36, 83.21, 83.10 (C1'); 72.80 (d,  $^2J_{\text{C3}',\text{P}} = 17.9$  Hz), 72.58 (d,  $^2J_{\text{C3}',\text{P}} = 17.6$  Hz), 72.45 (d,  $^2J_{\text{C3}',\text{P}} = 16.4$  Hz), 72.20 (d,  $^2J_{\text{C3}',\text{P}} = 16.3$  Hz) (C3'); 63.06, 62.93, 62.90, 62.74 (C5'); 59.60, 59.59 (2d,  $^2J = 5.4$  Hz), 59.48 (d,  $^2J = 5.7$  Hz), 59.45 (d,  $^2J = 5.9$  Hz), 58.34, 58.33, 58.29 (3d,  $^2J = 18.4$  Hz) (2C,  $\text{CH}_2\text{CH}_2\text{CN}$ ); 55.06, 55.05, 55.04 (2C,  $\text{OCH}_3$ ); 42.62, 42.60, 42.55, 42.53 (2C,  $\text{CHCH}_3$ ); 38.29, 38.18, 37.80, 37.65 (C2'); 37.5-36.4 (m, C3); 24.38-24.10 (4C,  $\text{CHCH}_3$ ); 19.84-19.70, 19.47-19.31 (2C,  $\text{CH}_2\text{CH}_2\text{CN}$ ).

$^{31}\text{P}\{^1\text{H}\}$  NMR (202.5 MHz,  $d_6$ -DMSO, extn. ref. 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  148.95, 148.90, 148.64, 148.56 (P(III)); 32.07, 32.01, 31.70, 31.69 (P(V)).

HRMS (ESI +): calcd. for  $\text{C}_{42}\text{H}_{52}\text{N}_4\text{NaO}_9\text{P}_2^+$   $[\text{M}+\text{Na}]^+$  841.3107; found 841.3102.

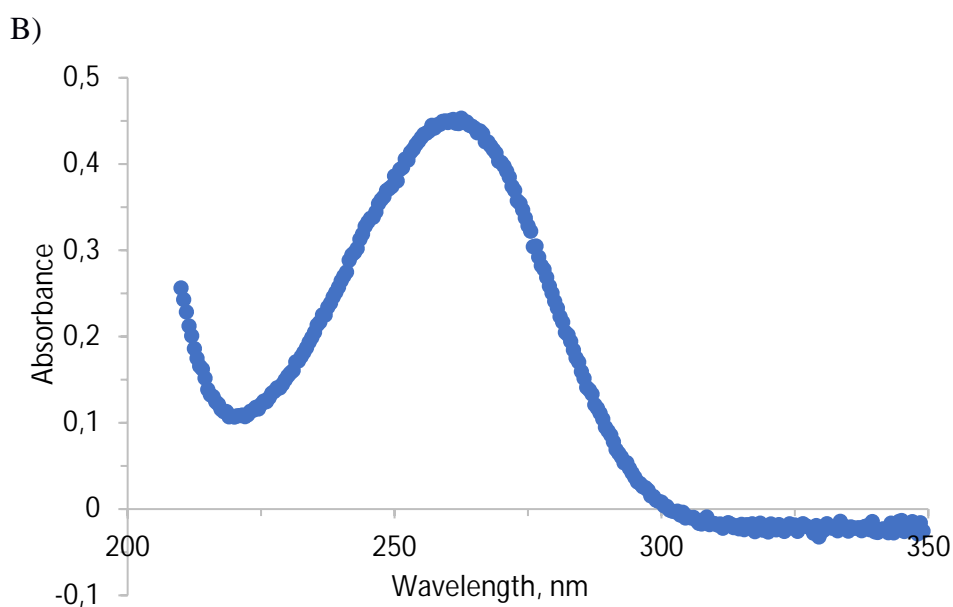
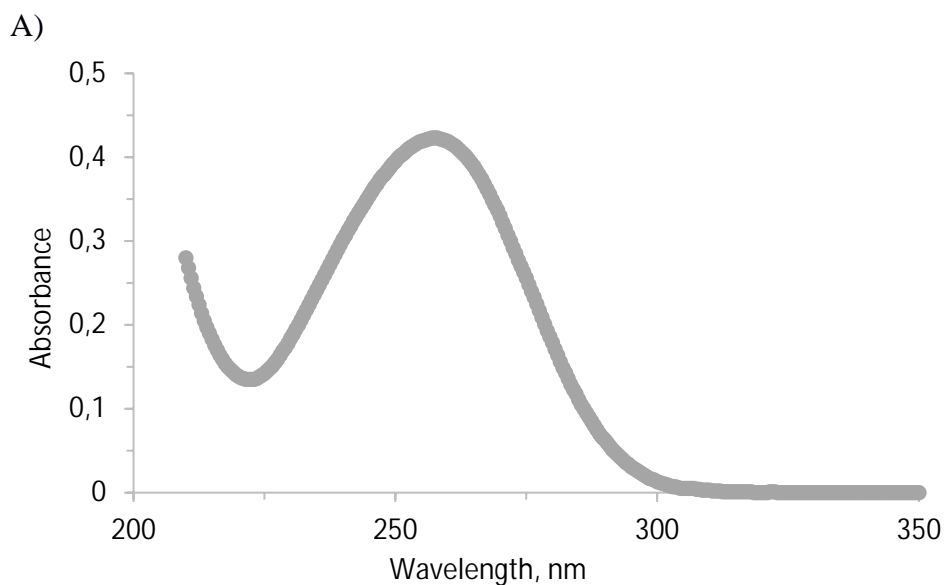
### Determination of molar extinction coefficient of nucleosides 20 and 22.

A sample of nucleoside (3-7 mg) was dissolved in a 20.0 mM phosphate buffer, containing 10 %  $\text{D}_2\text{O}$  at pH 7.0.  $^{31}\text{P}$  NMR spectrum without  $^1\text{H}$  decoupling was recorded overnight with a relaxation delay increased to 10 s. Concentration of nucleoside in the solution was determined by integration bands corresponding to phosphinate group of the nucleoside and phosphate buffer according to the equation:

$$c(\text{nucleoside}) = c(\text{phosphate}) \cdot \frac{I(\text{nucleoside})}{I(\text{phosphate})}$$

A dilution series with a factor 10 were prepared from the solution of nucleoside and UV absorption spectrum was recorded in the range 220-350 nm in 1 cm quartz cuvette. A molar extinction coefficient was determined using equation:

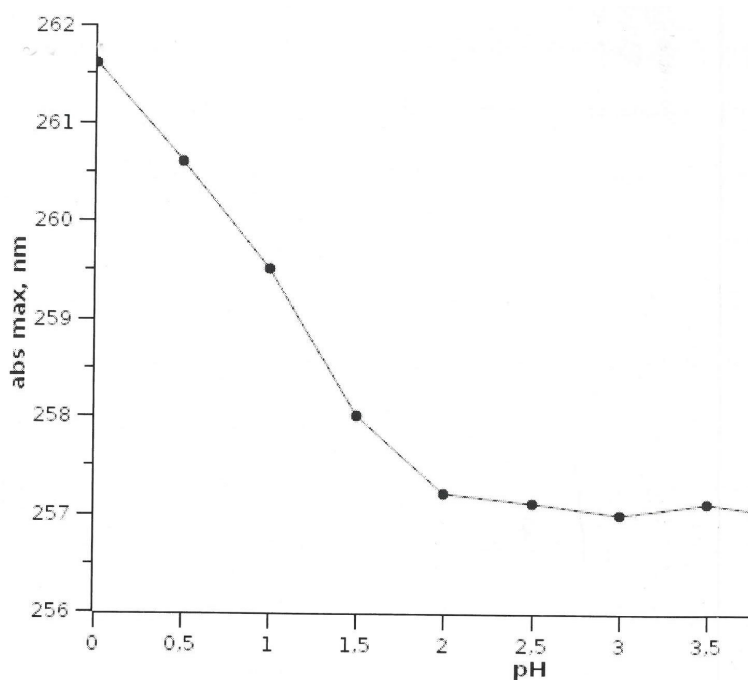
$$\epsilon = \frac{OD}{c(\text{nucleoside})}$$



**Figure S6.** UV-vis spectra of compounds **20** (A) and **22** (B) at 100  $\mu$ M in 20 mM phosphate buffer, containing 10 % D<sub>2</sub>O at pH 7.0.

**Estimation of p*K*<sub>a</sub> of nucleoside **20**.**

A change of  $\lambda_{\text{max}}$  of nucleoside **20** as a function of pH of the solution was plotted (Figure below) and p*K*<sub>a</sub> was estimated to be  $\leq 1.5$ .



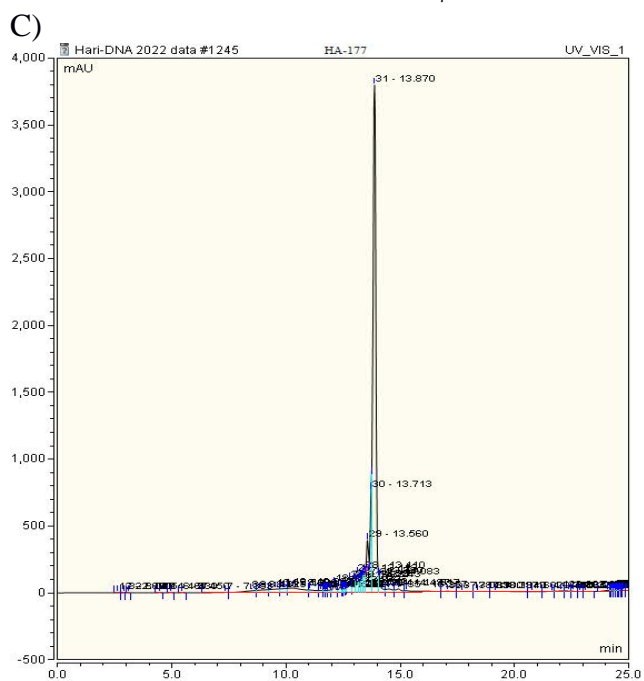
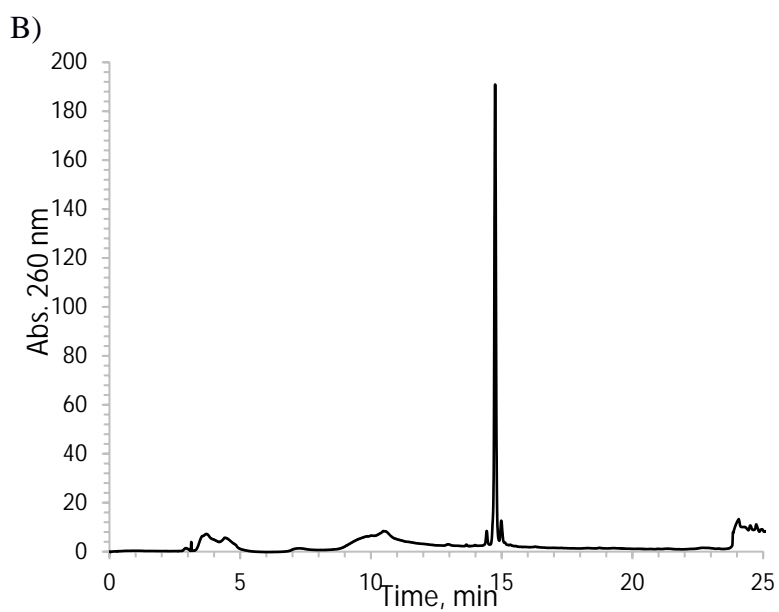
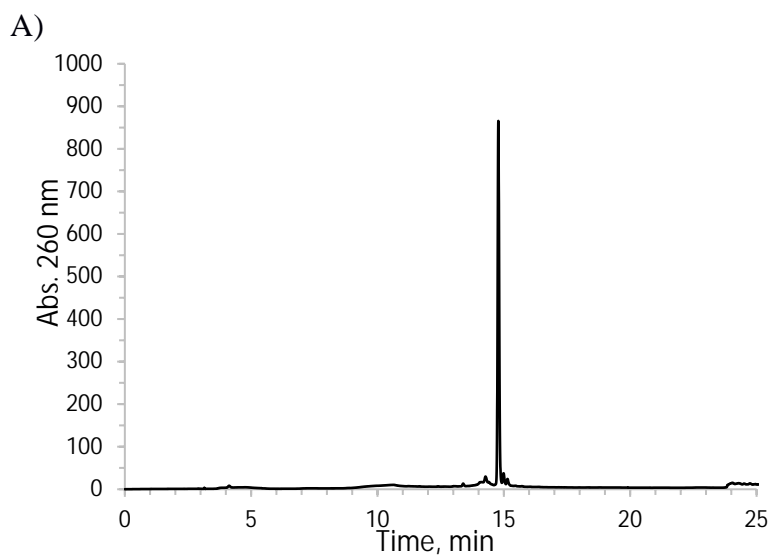
**Figure S7.** Change in  $\lambda_{\max}$  of nucleoside **20** as a function of pH.

### 3. Synthesis of modified oligos

Oligodeoxynucleotides were prepared on a MerMade-4 DNA/RNA synthesizer (BioAutomation) on a 5  $\mu$ mol scale using standard manufacturer's protocol. Phosphoramidites  **$\alpha$ -25** and  **$\beta$ -25** were used in the synthesis of modified oligonucleotide sequences. Each phosphoramidite (30 mg) was dissolved in 420  $\mu$ L of freshly distilled, dry acetonitrile. Coupling time of modified nucleosides was increased to 5 min. After DNA assembly, the final detritylation step was performed and DMT-OFF oligonucleotides were cleaved from the solid support and deprotected with conc. ammonia solution (1.0 mL) at room temperature for 24 h. After filtering, ammonia was removed on a speed-vac (1h).

ONs were purified by HPLC using IE-column (TSKgel Super Q-5PW). Buffer A (20 mM Tris-HCl, 1 mM Na<sub>2</sub>-EDTA, pH 9.0), buffer B (20 mM Tris-HCl, 1 mM Na<sub>2</sub>-EDTA, 1 M NaCl, pH 9.0). Gradients: 3.7 min 100 % A, convex curve gradient to 30 % B in 7.3 min, linear gradient to 50 % B in 18.5 min, concave gradient to 100 % B in 7.4 min, keep 100 % B for 7.4 min and then 100 % A in 7.4 min. Collected individual UV-absorbing fractions ( $\lambda=260$  nm) were desalted using NAP-25 column (GE Healthcare) against 'saltless buffer'. The composition of each fraction was confirmed by ESI-MS, and the fractions containing desired modified ONs were identified, analyzed by analytical RP-HPLC, ESI-MS (Figures S7 and S8) and concentrated by freeze-drying.

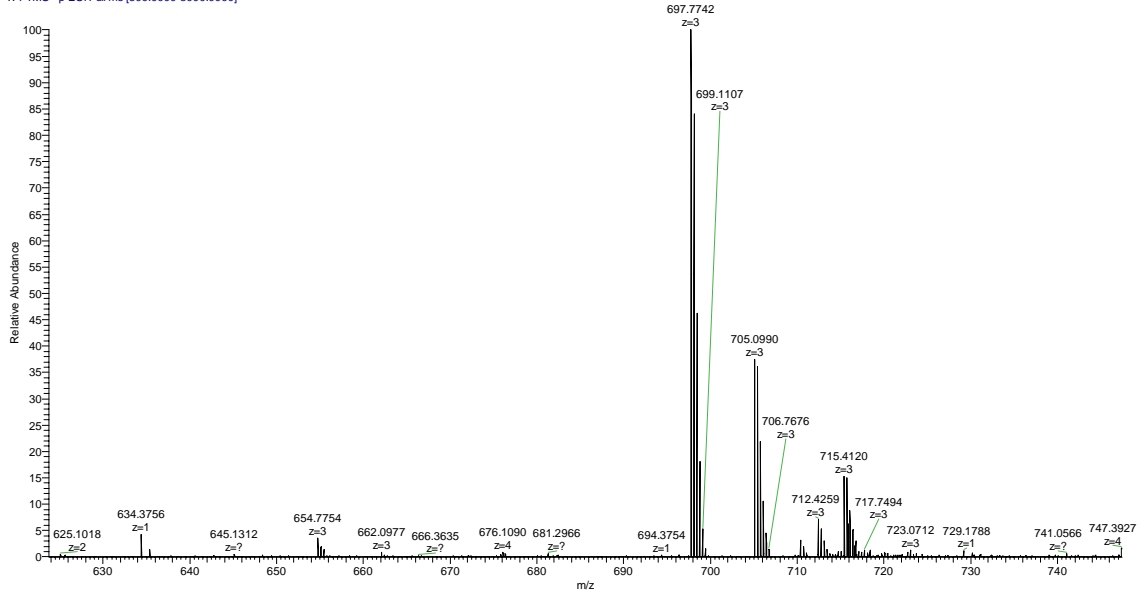
The oligonucleotides obtained were analysed by reverse-phase HPLC on 250/4.6 mm, 5  $\mu$ m, 300 Å C18 column (Thermo Fisher Scientific) in a gradient of CH<sub>3</sub>CN (0→20% for 20 min, 1.3 mL/min) in 0.1 M TEAA buffer (pH 7.0) with a detection at 260 nm ESI-MS was performed in negative mode using 10  $\mu$ M aq. oligo solution supplemented with 20% MeOH.



**Figure S8.** Analytical RP-HPLC profiles of oligos T4  $\beta$ 20 AT (A), T4  $\alpha$ 20 AT (B) and TGCGCTT  $\beta$ 20 GCGCT (C). Note that peaks at 3-5, 9-11 and 24-26 min are artefacts form the column/buffer in A and B.

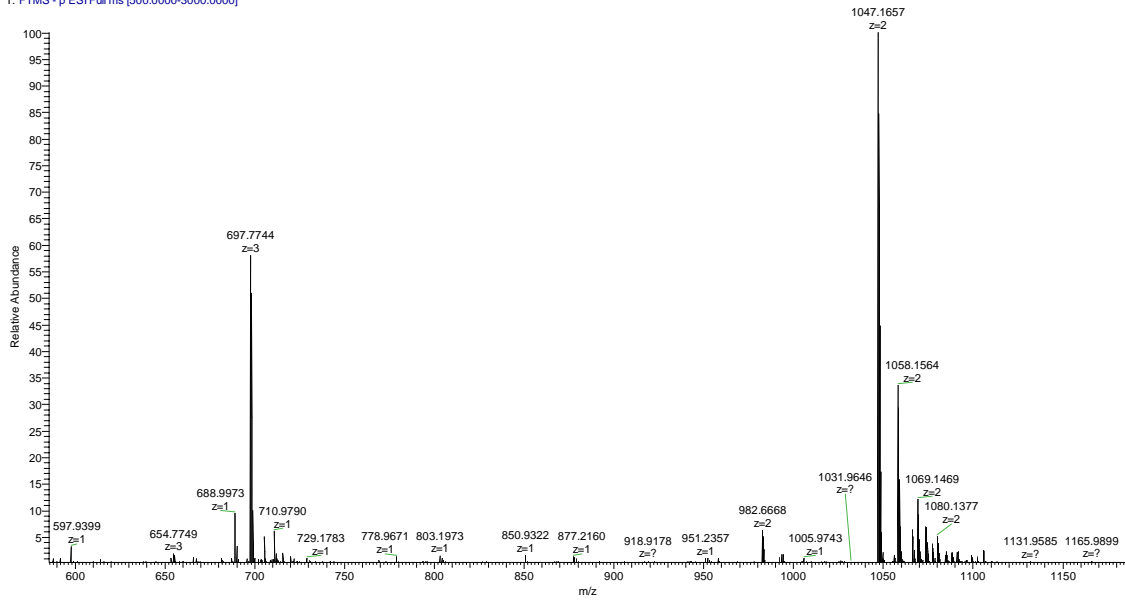
A)

426-142 #83-93 RT: 0.18-0.27 AV: 22 NL: 5.36E6  
T: FTMS - p ESI Full ms [500.0000-3000.0000]

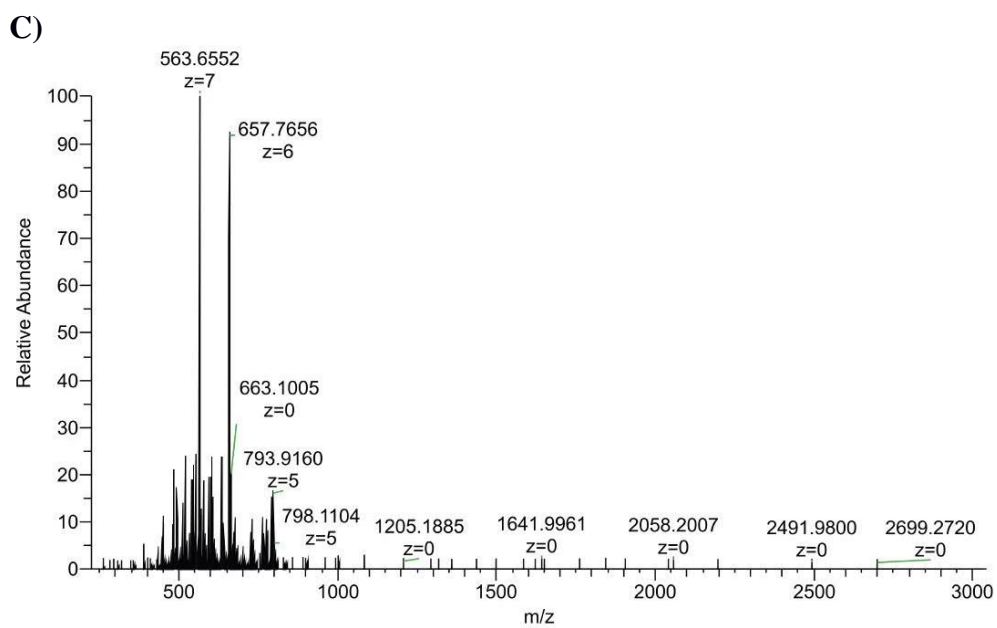


B)

426-142 #83-93 RT: 0.38-0.43 AV: 11 NL: 2.20E6  
T: FTMS - p ESI Full ms [500.0000-3000.0000]







**Figure S9.** ESI-MS profiles of ODNs T4  $\beta$ 20 AT (A), T4  $\alpha$ 20 AT (B) and T4  $\beta$ 20 GCGCT (C). Calcd for  $[T4 \beta 20 AT - 2H]^{-2} = 1047.17$  found 1047.17 for T4  $\beta$ 20 AT. Calcd for  $[T4 \beta 20 AT - 3H]^{-3} = 697.78$  found 697.77 for both  $\beta$  and  $\alpha$  ODN. Calcd for  $[T4 \beta 20 GCGCT - 7H]^{-7} = 563.5193$ , found 563.6552.

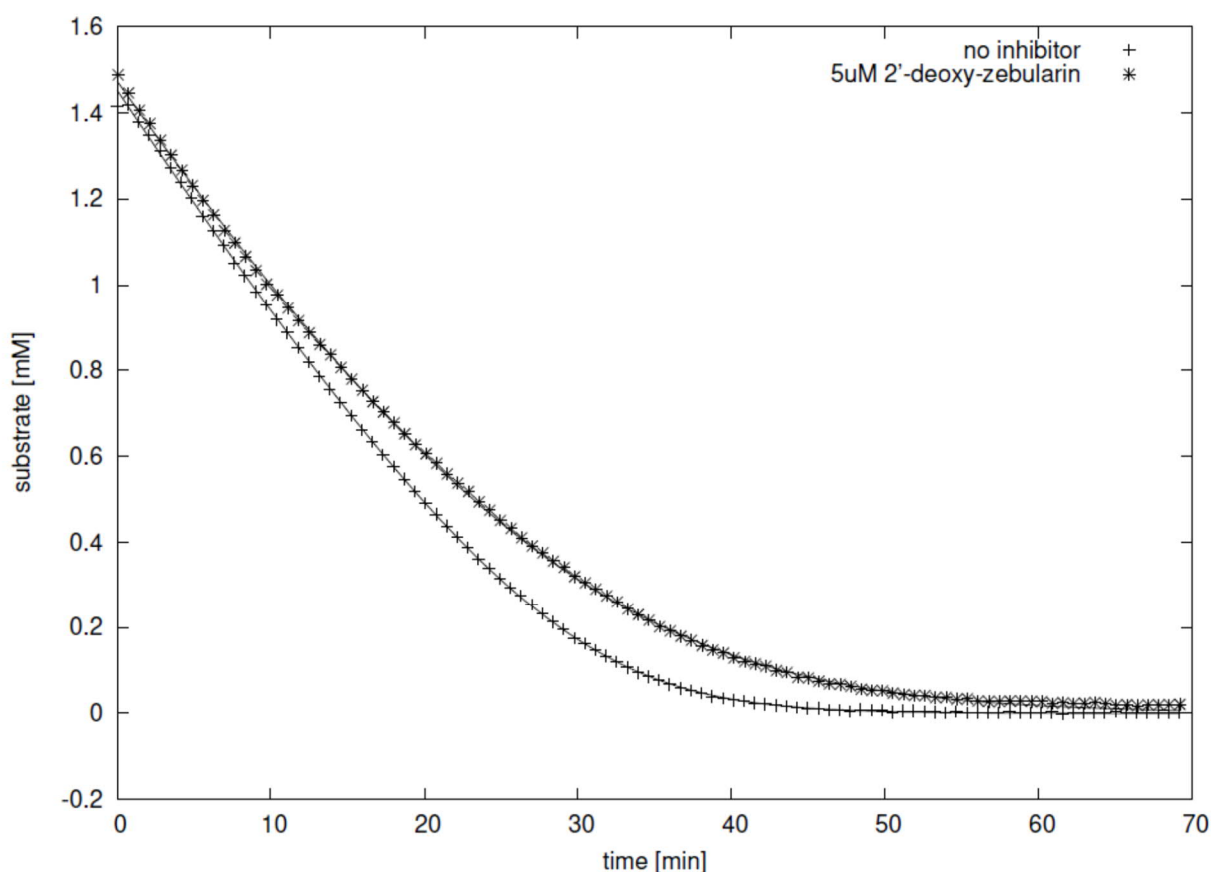
#### 4. Protein expression and purification

Recombinant human cytidine deaminase (hCDA) expressed in *E.Coli* was purchased from Sigma-Aldrich, product No SRP6372.

A3A-mimic (A3B<sub>CTD</sub>-QM- $\Delta$ L3-AL1swap) and wild-type A3A were expressed and purified as previously described.<sup>9</sup>

#### 5. UV kinetic assay of hCDA-catalysed 2'-deoxycytidine deamination

UV spectroscopy was used to monitor hCDA-catalysed deamination of 2'-deoxycytidine at 286 nm in 100 mM NaCl, 50 mM phosphate buffer at 25 °C at various pH as mentioned in the text. Cary 300 Bio UV-vis spectrometer from Varian was used. Enzymatic reactions were performed in a quartz cuvette (0.1 cm). Deamination of 2'-deoxycytidine leads to a decrease in absorbance at 286 nm (Figure S9).



**Figure S10.** hCDA (27nM) catalysed deamination of 2'-deoxycytidine (~1.5 mM) in the absence (plus) and presence of 5  $\mu$ M 2'-deoxyzebularine (asterisk) monitored by measuring absorbance at 286 nm over time in 100 mM NaCl, 1 mM TCEP, 50 mM phosphate buffer at 25 °C, at pH 7.4.

The uninhibited deamination (pluses, Figure S9) and the inhibited reaction start at about the same speed. As the reaction progresses, the inhibited reaction becomes slower, as its observed  $K_M$  is larger than in the uninhibited case. In order to be able to visualize the fitted curves, only every 5th data point is plotted. The data was then fitted using Gnuplot to the integrated Michaelis Menten equation:<sup>10</sup>

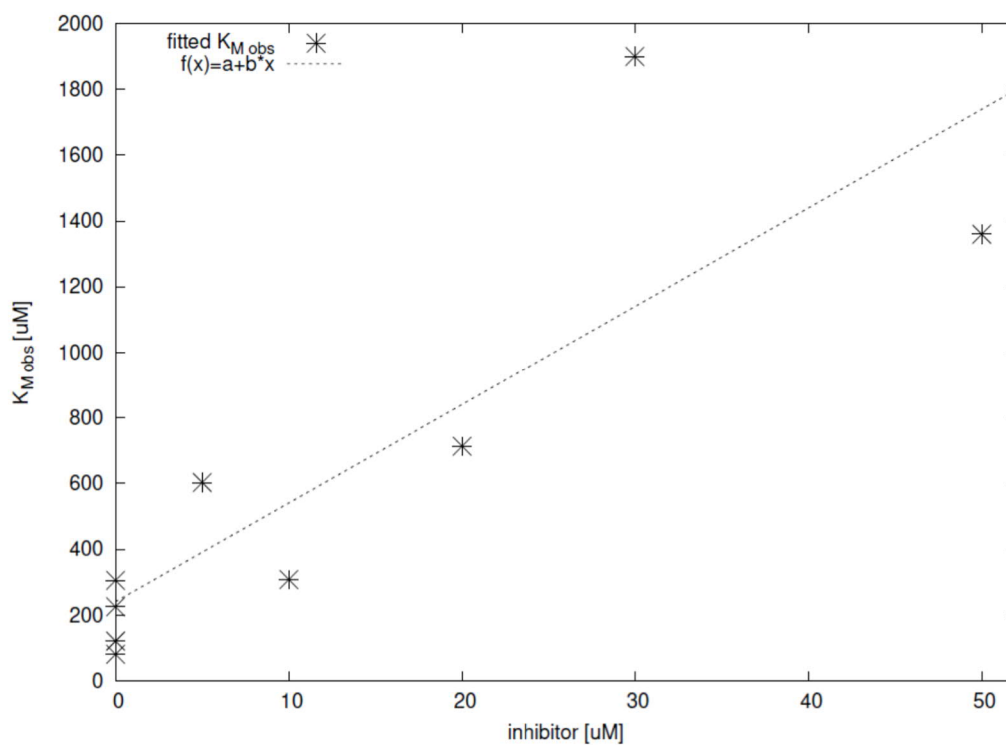
$$mm(t) = K_M * Lambert W\left(\frac{c}{K_M} * e^{\left(\frac{c}{K_M} - t * \frac{v_{max}}{K_M}\right)}\right) + d$$

where  $mm(t)$  calculates the current substrate concentration with respect to the time,  $t$ . The four parameters which are fitted are  $K_M$  and  $v_{max}$ , the Michaelis-Menten constants,  $c$  is the initial substrate concentration and  $d$  is a baseline correction parameter. A summary of the measured inhibition constants is given in Table 1 in the main text.

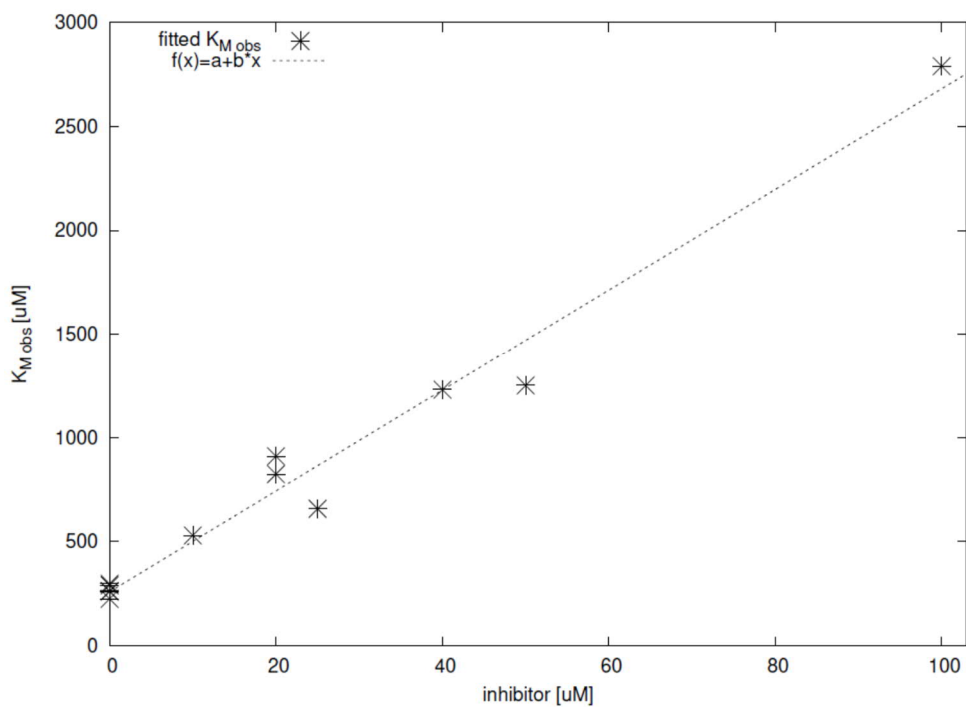
An increase in concentration of an inhibitor in enzymatic reaction leads to an increase in the observed  $K_m$  value. The plot of observed  $K_m$  values *versus* inhibitor concentration (Figures S10-S12) was then fitted with linear regression ( $y = ax + b$ ) to derive the inhibition constant ( $K_i$ ) using the following formula:

$$K_i = a/b$$

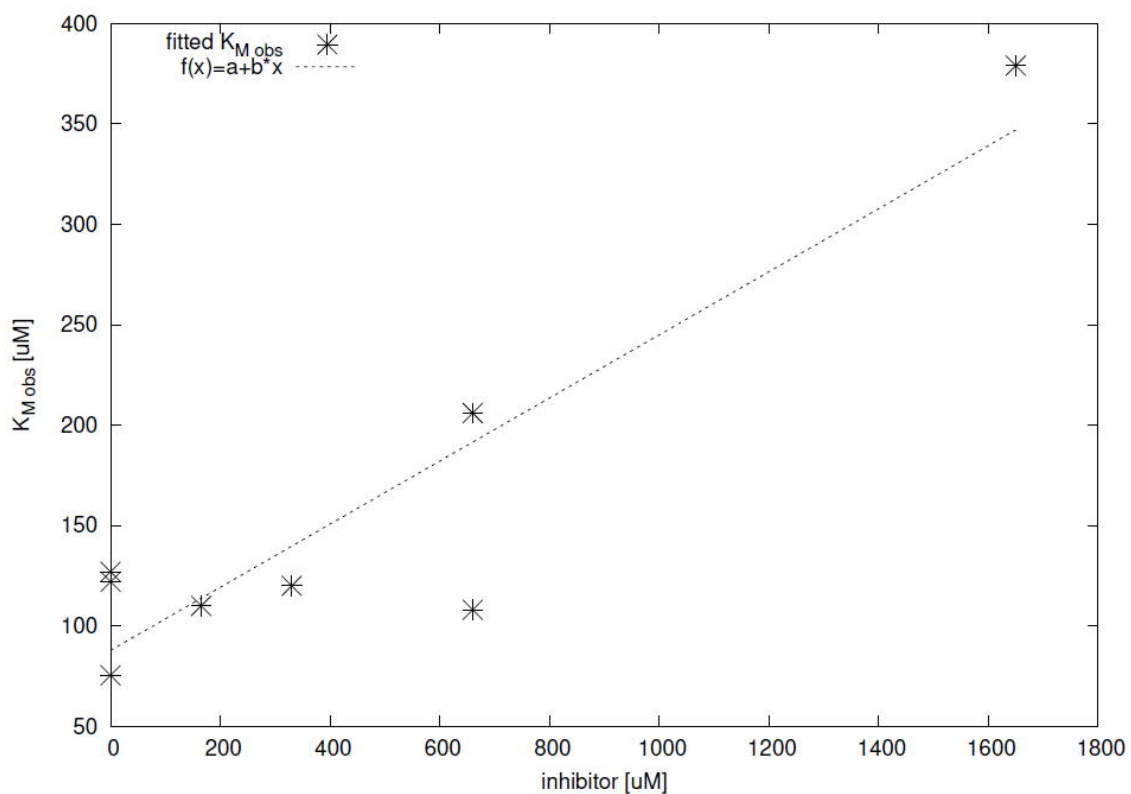
Uncertainty of  $K_i$  was calculated using error-propagation method.



**Figure S11.** A plot of observed  $K_m$  versus various concentration of **dZ** in hCDA (27 nM) catalysed deamination of 2'-deoxycytidine in 100 mM NaCl, 1 mM TCEP, 50 mM phosphate buffer at 25 °C, at pH 7.4.



**Figure S12.** A plot of observed  $K_m$  versus concentration of compound **22** in hCDA (27 nM) catalysed deamination of 2'-deoxycytidine in 100 mM NaCl, 1 mM TCEP, 50 mM phosphate buffer at 25 °C, at pH 7.4.



**Figure S13.** A plot of observed  $K_m$  versus various concentration of compound **20** in hCDA (27nM) catalysed deamination of 2'-deoxycytidine in 100 mM NaCl, 1 mM TCEP, 50 mM phosphate buffer at 25 °C, at pH 4.7.

## 6. <sup>1</sup>H-NMR kinetic assay of A3-catalysed deamination

Competitive inhibition of A3A-mimic and A3A by the synthesised oligonucleotides was performed by a similar procedure reported by us previously.<sup>11, 12</sup> We used 400 μM of a standard 7-mer oligonucleotide substrate 5'-dT<sub>4</sub>CAT, 8 or 32 μM of inhibitors, 260 nM of A3A-mimic (A3B<sub>CTD</sub>-QM-ΔL3-AL1swap) in a buffer containing 50 mM sodium phosphate (pH 6.0), 100 mM NaCl, 2.5 mM β-mercaptoethanol, 0.1 M EDTA, 50 μM TSP. The H-5 proton doublet signals of the cytosine from the substrate was baselined and integrated. A peak of TSP was used as an internal standard to determine the concentration of the substrate which appears at 5.88 ppm ( $J = 7.7$  Hz) and converted to 5.71 ppm ( $J = 8.3$  Hz) during the reaction over time. The integrated signal area was converted to substrate concentration and plotted *versus* time of the reaction. The data were then fitted with linear regression to determine the initial speed of the reaction in the absence of presence of inhibitors.

## 7. References

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