Electronic Supplementary Information (ESI)

Synthesis of novel pyrophosphorothiolate-linked dinucleoside cap analogues in a ball mill

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General information

Reagents or solvents were purchased from commercial suppliers (Aldrich or Fluka) and used without further purification unless otherwise stated. Discussions in the text are based upon the use of *N*,*O*-bis(trimethylsilyl)acetamide (Aldrich 128910) which was 95% pure. Anhydrous chloroform and CDCl₃ was dried by storing over phosphorus pentoxide and subsequently filtered under gravity through activated basic alumina immediately prior to use. Otherwise, acid-free chloroform was obtained following filtration through activated basic alumina immediately prior to use. Dichloromethane was distilled from calcium hydride and stored in the absence of light over activated 3Å molecular sieves.

Davisil silica gel 60 Å was used for flash chromatography. TLC was performed using Merck Kieselgel 60 F254 plates and materials visualized UV (254nm), 0.1% (w/v) Ellman's reagent (for thiols), gaseous HCl (for tritylated compounds) and 3% (w/v) phenol in 95:5 ethanol:conc. H_2SO_4 (for nucleosides). Where appropriate, the plates were subsequently heated at high temperature (*ca*.100 – 200 °C).

All ball mill reactions were performed using a Retsch Mixer Mill MM 400¹ and a 15mm zirconia ball (10.70 g) in a zirconia-lined vessel (25mL internal volume) according to the conditions described below.

HPLC

HPLC was performed on a ThermoFinnigan SpectraSYSTEM modular HPLC system consisting of a P2000 binary gradient pump and UV1000 sample detector. Samples were injected manually via a Rheodyne injection valve. The HPLC was interfaced via an SN4000 controller (Thermo Scientific) to a Windows PC running ChromQuest 5.0 data acquisition software (Thermo Scientific).

Buffers were prepared using H_2O purified to 18.2 M Ω by reverse osmosis (Barnstead NANOpure Diamond water purification system), acetonitrile (Aldrich 34851) triethylamine (Aldrich 471283), acetic acid (Aldrich 320099), tetrabutylammonium hydrogen sulfate (Fluka 86853) and CO_2 generated by sublimation of the solid compound.

Analytical HPLC was performed using a Phenomenex Clarity 5µm Oligo-RP (150 x 4.60 mm) column eluting at 1 mL min⁻¹, monitoring at 260 nm using gradients 1 or 2.

Preparative HPLC was performed using a Phenomenex Clarity 5μ m Oligo-RP – (250 x 21.2 mm) column eluting at 8mL min⁻¹, monitoring at 280 nm using gradients 3, 4 or 5.

Triethylammonium acetate (TEAA) buffers were prepared from solutions of acetic acid in H₂O containing acetonitrile (typically 8-30% v/v) following neutralisation with triethylamine to pH 6.5 and suitable dilution to give a final concentration of 100 mM TEAA in 5% (v/v) MeCN (Buffer A) or in 65% (v/v) MeCN (Buffer B).

Ion pair (IP) buffers were prepared from solutions containing a mixture of tetrabutylammonium hydrogen sulfate (final concentration 6 mM) and acetic acid (final concentration 30 mM) in H₂O following

neutralisation with triethylamine to pH 6.3 and suitable dilution with water (Buffer A) or to give 50% (v/v) acetonitrile (Buffer B).

Desalting (TEAB) buffers were prepared from 1 M stock solutions of triethylammonium bicarbonate in H₂O. These were prepared by bubbling CO₂ through a sintered frit into a mixture of triethylamine and H₂O at 0°C to give homogenous solutions. Stock solutions were stored at 4°C until required (up to 2 days) and then further diluted as required to give: 100 mM TEAB (aq.), pH 7.8 (Buffer A); or 100 mM TEAB in 65:35 (v/v) MeCN:H₂O, pH 8.2 (Buffer B).

<u>Gradient G1</u> (analytical - TEAA buffers): 0-5 min, 0% Buffer B; 5-25 min, 0-20% Buffer B; 25-30 min, 20-100% Buffer B; 30-32 min, 100% Buffer B; 32-40 min.

<u>Gradient G2</u> (analytical -IP buffers): 0-5 min, 20% Buffer B; 5-25 min, 20-60% Buffer B; 25-30 min, 60% Buffer B; 30-35 min, 60-20% Buffer B; 35-45 min, 20% Buffer B.

<u>Gradient G3</u> (preparative -TEAA buffers): 0-10 min, 0% Buffer B; 10-60 min; 0-14% Buffer B; 60-70 min, 14% Buffer B; 70-90 min, 14-100% Buffer B; 90-100 min, 100% Buffer B; 100-120 min, 100-0% Buffer B; 120-140 min, 0% Buffer B.

<u>Gradient G4</u> (preparative IP buffers): 0-13 min, 20% Buffer B; 13-63 min, 20-60% Buffer B; 63-75 min, 60% Buffer B; 75-83 min, 60-20% Buffer B; 83-113 min 20% Buffer B.

<u>Gradient G5</u> (preparative TEAB buffers): 0-10 min, 0% Buffer B; 10-60 min, 0-30% Buffer B; 60-80 min, 30-100% Buffer B; 80-90 min, 100% Buffer B; 90-110 min, 100-0% Buffer B; 110-120 min, 100% Buffer A.

¹H, ¹³C or ³¹P NMR NMR spectra were recorded on a Bruker III-400 MHz at 300K.

Mass spectra were recorded using a VG Quattro II Triple Quadrupole Mass Spectrometer (Electrospray). Mass spectrometry was performed by Analytical Services and Environmental Projects (ASEP) at Queen's University Belfast.

Experimental procedures and material characterisation

3'-deoxy-3'-(benzothiazoyl-2-disulfanyl)thymidine (1a) - dTSSBt.



A suspension of 2,2'-dithiobis(benzothiazole) (4.53g, 13.6 mmol, 5 eq.) in argon-purged, acid-free chloroform (300 mL) was warmed gently to effect (essentially complete) dissolution. The saturated solution was allowed to cool to ambient temperature and stirred during dropwise addition of 5'-O-(mono-*p*-methoxytriphenylmethyl)-3'-deoxy-3'-thiothymidine² (1.44g, 2.72 mmol) in

argon-purged, acid-free chloroform (50 mL) over 30 minutes at ambient temperature in the absence of oxygen. Stirring was continued for a further 30 minutes after which time the solvent was removed *in vacuo* and the crude, tritylated product isolated following short column chromatography on silica (neutralised by pre-elution with 0.2% (v/v) triethylamine in DCM) eluting with 0 and 0.5% (v/v) methanol in DCM. The crude product was dissolved in glacial acetic acid (21.5 mL) at room temperature and stirred during addition of water (3.5 mL). These conditions were maintained for 5 hours after which time absolute ethanol (25 mL) was added and the solvent removed *in vacuo*. The residue was triturated with ether / hexane (1/1 - 20 mL) and purified following silica gel column chromatography eluting with a gradient of 0-2% (v/v) methanol in chloroform. Appropriate fractions were pooled and evaporated *in vacuo* to yield pure **1a** (0.72g 1.7 mmol, 63%) as a cream solid.

¹H NMR (400 MHz, D₆-DMSO) $\delta_{\rm H} = 11.27$ (1H, s, NH), 8.08 (1H, d, ${}^{3}J_{HH}$ =8.2Hz, ArH), 7.88 (1H, d, ${}^{3}J_{HH}$ =8.2Hz, ArH), 7.75 (1H, s, H6), 7.51 (1H, ψ t, J_{HH} =7.5Hz, ArH), 7.45-7.39 (1H, ψ t, J_{HH} =8.0Hz, ArH), 6.13 (1H, ψ t, ${}^{3}J_{HH}$ =5.9Hz, H1'), 5.24 (1H, ψ t, ${}^{3}J_{HH}$ =5.3Hz, OH), 4.05-3.90 (2H, m, H3', H4'), 3.90-3.60 (2H, m, H5', H5''), 2.60-2.50 (¶2H, m, H2', H2''), 1.75 (3H, s, CH₃).

¹³C NMR (101MHz, D₆-DMSO) $\delta_{\rm C} = 171.17$, 163.71, 154.39, 150.31, 136.14, 135.29, 126.65, 125.00, 122.07, 121.83, 109.17, 84.52, 83.50, 60.42, 46.81, 37.04, 12.21.

HRMS (ESI, postive ion). Calculated m/z for C₁₇H₁₈N₃O₄S₃ [M+H]⁺: 424.0459; found 424.0476

<u>3'-deoxy-3'-(5-nitropyridyl-2-disulfanyl)thymidine (1b) – dTSSNPy.</u>



To a stirred solution of 5'-O-(mono-*p*-methoxytriphenylmethyl)-3'-deoxy-3'-(5nitropyridyl-2-disulfanyl)thymidine^{3, 4} (0.640g, 0.93 mmol) in glacial acetic acid (10 mL) at ambient temperature was added water (2.5 mL) and these conditions were maintained for 1 hour. Ethanol (12.5 mL) was added and the solution reduced *in vacuo* and coevaporated with ethanol until no further acetic acid odour was detected. The residue was adsorbed on silica gel and purified by

column chromatography on the same support eluting with a gradient of 2.5-5% MeOH in DCM. Fractions containing pure product were pooled and reduced *in vacuo* to yield pure **1b** (0.194g, 0.47 mmol, 50%)

¹H NMR (400 MHz, CDCl₃) $\delta_{\rm H} = 9.30$ (1H, d, ^{*4*} J_{HH} =2.7Hz, ArH), 8.43 (1H, dd, ³ J_{HH} =2.7, 8.8Hz, ArH), 7.81 (1H, d, ³ J_{HH} =8.8Hz, ArH), 7.51 (1H, s, H6), 6.08 (1H, ψ t, ³ J_{HH} =5.9Hz, H1'), 4.08-4.03 (2H, m, H4', H5'), 4.00-3.90 (1H, m, H5''), 3.90-3.80 (1H, m, H3') 2.60-2.45 (1H, m, H2', H2''), 1.87 (3H, s, CH₃). ¹³C NMR (101MHz, D₆-DMSO) $\delta_{\rm C} = 166.84$, 163.82, 150.33, 145.28, 142.28, 136.55, 131.86, 119.93, 110.97, 85.87, 84.88, 61.35, 46.06, 38.07, 12.46.

HRMS (ESI, positive ion). Calculated m/z for $C_{15}H_{17}N_4O_6S_2$ [M+H]⁺: 413.0590; found 413.0582

5'-deoxy-5'-(5-nitropyridyl-2-disulfanyl)adenosine (2) – NPySSdA.



To a mixture of 5'-deoxy-5'-(4-methoxybenzylthio)adenosine (0.81 g, 2.0 mmol) and 2,2'-dithiobis(5-nitropyridine) (1.24 g, 4.0 mmol, 2 eq.) was added trifluoroacetic acid / thioanisole (39/1 - 20 mL) and the solution stirred under argon at room temperature for 2 hours. The

reaction mixture was concentrated under vacuum, diluted with chloroform and purified by silica gel chromatography eluting with 5 - 10% methanol in chloroform. Appropriate fractions were reduced *in vacuo* to yield pure **2** (660 mg, 1.5 mmol, 75%).

¹H NMR (400 MHz, D₆-DMSO) $\delta_{\rm H} = 9.20$ (1H, d, ⁴ J_{HH} =2.7Hz, ArH), 8.51 (1H, dd, ³ J_{HH} =2.7, 9.0Hz, ArH), 8.46 (1H, s, H2), 8.21 (1H, s, H8), 8.01 (1H, d, ³ J_{HH} =8.8Hz, ArH), 7.87 (2H, brs, -NH₂), 5.89 (1H, d, ³ J_{HH} =5.9Hz, H1'), 5.52 (2H, brs, 2x -OH) 4.79 (1H, ψ t, ³ J_{HH} =5.5Hz, H2'), 4.19 (1H, m, H3'), 4.09 (1H, m, H4'), 3.40-3.30 (2H, m, H5', H5'').

¹³C NMR (101MHz, D₆-DMSO) $\delta_{\rm C}$ = 167.31, 154.94, 151.27, 149.12, 144.66, 142.10, 140.61, 132.36, 119.52, 119.12, 87.70, 82.43, 72.55, 72.45, 41.49.

HRMS (ESI, positive ion). Calculated m/z for $C_{15}H_{16}N_7O_5S_2[M+H]^+$: 438.0654; found 438.0658.

<u>3'-thiothymidine 3'-pyrophosphate (P' \rightarrow 5') adenosine (5) - dTSppA - from 1a.</u>



A suspension of 3'-deoxy-3'-(benzothiazoyl-2-disulfanyl)thymidine (1a) (97.0 mg, 0.228 mmol) in 4:1 anhydrous chloroform:BTMSA (3 mL) was stirred at ambient temperature under argon for 30 min after which time a clear solution was formed. To this stirred solution was added (TMSO)₃P (84 μ L, 0.251 mmol , 1.1 eq) in 4:1 anhydrous chloroform:BTMSA (1 mL; total BTMSA 3.11 mmol, 13.6 eq.) and these conditions maintained for a further 30 minutes. ³¹P NMR indicated complete reaction had occured. After 60 minutes, half of the reaction mixture by mass (3.05g) was directly transferred into a zirconia-lined vessel and into a second such vessel were rinsed the residues from both the reaction flask and NMR tube

with anhydrous chloroform (2 x 1mL). Both vessels were stored in a desiccator under vacuum until volatiles had been removed and the residue had the consistency of a paste. The residues were subsequently treated as follows:

a) Pyrophosphate coupling in the presence of 3.0 eq. of AMP-morpholidate (4)

The jar was then charged sequentially with adenosine-5'-phosphoromorpholidate 4-morpholine-N.N'dicyclohexylcarboxamidine salt (4) (243 mg, 0.342 mmol, 3.0 eq.), tetrazole (17 mg, 0.24 mmol, 2.1 eq.), MgCl₂•(H₂O)₆ (35 mg, 0.17 mmol, 1.5 eq.), H₂O (25.0 μL, 1.37 mmol, 12 eq.) and a 15 mm zirconia ball. The vessel was vibrated at 30 Hz for 90 minutes and allowed to cool to room temperature. The crude reaction mixture was extracted from the vessel using both physical fracturing of the solid residues (with a polypropylene automatic pipette tip) and successive rinsing with H₂O (1 mL), MeCN (1 mL), H₂O (0.5 mL), MeCN (0.5 mL), MeOH (1.5 mL), H₂O (1 mL), MeOH (1 mL), and finally MeCN (1 mL) into eppendorf tubes. The aqueous suspensions were sonicated, filtered in 1 mL aliquots through a Spin-X cellulose acetate centrifuge filter (0.45 µm) at 12,000 rpm and the residual solids washed with H₂O (0.5 mL). Organic solutions (1.5 mL aliquots) were reduced in a vacuum centrifuge to approximately 0.1 mL, diluted with H₂O (1 mL) and the suspensions then sonicated and centrifuge-filtered as above. The remaining solids were rinsed with H₂O (0.5 mL) and the combined extracts analysed by ³¹P NMR. The reaction mixture was purified by C18 RP-HPLC initially using TEAA buffers (gradient G3) from which a single peak which contained both dTSppA (5) and Ap(M)pA (6) was collected and reduced in vacuo. The concentrated solution was then repurified using IP buffers (G4) from which a single peak corresponding to pure 4 was collected. Combined pure fractions were concentrated in vacuo and subject to desalting following HPLC isolation using TEAB buffers (G5) and coevaporation with deionised water.

Isolated yield of pure dTSppA•~2.0(Et₃NH): 1008 OD^{260nm} units (0.043 mmol, 38 %).

b) Pyrophosphate coupling in the presence of 1.5 eq. of AMP-morpholidate (4)

The jar was then charged sequentially with adenosine-5'-phosphoromorpholidate 4-morpholine-N,N'-dicyclohexylcarboxamidine salt (4) (121 mg, 0.171 mmol, 1.5 eq.), tetrazole (17 mg, 0.24 mmol, 2.1 eq.), MgCl₂•(H₂O)₆ (35 mg, 0.17 mmol, 1.5 eq.), H₂O (25.0 µL, 1.37 mmol, 12 eq.) and a 15 mm zirconia ball. The vessel was vibrated at 30 Hz for 90 minutes and allowed to cool to room temperature. The crude reaction mixture was extracted from the vessel using both physical fracturing of the solid residues (with a polypropylene automatic pipette tip) and successive rinsing with H₂O (1 mL), MeCN (1 mL), H₂O (0.5 mL) and finally MeOH (1 mL) into eppendorf tubes. The aqueous suspensions were sonicated, filtered in 1 mL aliquots through a Spin-X cellulose acetate centrifuge filter (0.45 µm) at 12,000 rpm and the residual solids washed with H₂O (0.5 mL). Organic solutions were seperately reduced in a vacuum centrifuge to approximately 0.25 mL, diluted with H₂O (1 mL) and the suspensions then sonicated and centrifuge-filtered as above. The remaining solids were rinsed with H₂O (0.5 mL) and the combined extracts analysed by ³¹P NMR. The reaction mixture was purified by C18 RP-HPLC initially using TEAA buffers (gradient G3) from which a single peak which contained both dTSppA (**5**) and Ap(M)pA (**6**) was collected and reduced *in vacuo*. The concentrated solution was then repurified using IP buffers (G4) from which a single peak corresponding to pure dTSppA was collected. Combined pure fractions were concentrated *in vacuo* and

subject to desalting following HPLC isolation using TEAB buffers (G5) and coevaporation with deionised water.

Isolated yield of pure dTSppA: 608 OD^{260nm} units (0.026 mmol, 23 %).

<u>3'-thiothymidine 3'-pyrophosphate (P' \rightarrow 5') adenosine (5) - dTSppA - from 1b.</u>

A suspension of 3'-deoxy-3'-(5-nitropyridyl-2-disulfanyl)thymidine (1b) (50.0 mg, 0.114 mmol) in 4:1 anhydrous chloroform:BTMSA (1.5 mL) was stirred at ambient temperature under argon for 30 min after which time a clear solution had formed. To this stirred solution was added (TMSO)₃P (42.0μ L, 0.125 mmol, 1.1 eq) in 4:1 anhydrous chloroform: BTMSA (0.5 mL - total BTMSA 1.55 mmol, 13.6 eq.) and these conditions maintained for 20 minutes. ³¹P NMR was performed and after a total of 60 minutes, the reaction mixture was transferred into a zirconia-lined vessel and residues rinsed from both the reaction flask and NMR tube with anhydrous chloroform (2 x 1mL). The vessel was stored in a desiccator under vacuum until volatiles had been removed and the residue had the consistency of a paste. The jar was then charged sequentially with adenosine-5'-phosphoromorpholidate 4-morpholine-N,N'-dicyclohexylcarboxamidine salt (4) (121 mg, 0.171 mmol, 1.5 eq.), tetrazole (17 mg, 0.24 mmol, 2.1 eq.), MgCl₂•(H₂O)₆ (35 mg, 0.17 mmol, 1.5 eq.), H₂O (25.0 µL, 1.37 mmol, 12 eq.) and a 15 mm zirconia ball. The vessel was vibrated at 30 Hz for 90 minutes and allowed to cool to room temperature. The crude reaction mixture was extracted from the vessel by rinsing with H₂O (2 mL), into eppendorf tubes. The aqueous suspensions were sonicated, filtered in 1 mL aliquots through a Spin-X cellulose acetate centrifuge filter (0.45 µm) at 12,000 rpm and the filter rinsed with H₂O (0.5 mL). The combined extracts were analysed by ³¹P NMR. The reaction mixture was purified by C18 RP-HPLC initially using TEAA buffers (gradient G3) from which a single peak which contained both dTSppA (5) and Ap(M)pA (6) was collected and reduced *in vacuo*. The concentrated solution was then repurified using IP buffers (G4) from which a single peak corresponding to pure dTSppA was collected. Combined pure fractions were concentrated in vacuo and subject to desalting following HPLC isolation using TEAB buffers (G5) and coevaporation with deionised water. Isolated yield of pure dTSppA: 658 OD^{260nm} units (0.028 mmol, 25 %).

HPLC retention time (gradient G1): 22.1 min

¹H NMR (400 MHz, D₂O) $\delta_{\rm H} = 8.39$ (1H, s, H2), 8.10 (1H, s, H8), 7.47 (1H, s, H6-dT), 5.99 (1H, d, ${}^{3}J_{HH}$ =5.7Hz, H1'-rA), 5.96 (1H, ψ t, ${}^{3}J_{HH}$ =6.1Hz, H1'-dT), 4.60 (1H, ψ t, ${}^{3}J_{HH}$ =5.3Hz, H2'-rA), 4.42 (1H, ψ t, ${}^{3}J_{HH}$ =4.5Hz, H3'-rA), 4.29 (1H, m, H4'-rA), 4.25-4.10 (2H, m, H5',H5''-rA), 3.90-3.82 (1H, m, H4'-dT), 3.82-3.68 (2H, m, H5',H5''-dT), 3.65-3.52 (1H, m, H3'-dT), 2.51-2.45 (2H, m, H2',H2''-dT), 1.66 (3H, s, CH₃).

¹³C NMR (101MHz, D₂O) δ_{C} = 166.08, 155.39, 152.78, 151.33, 148.88, 139.73, 137.34, 118.43, 110.92, 87.08, 86.00 (d, ${}^{3}J_{PC}$ =9.1Hz), 84.52, 83.73 (d, ${}^{3}J_{PC}$ =9.6Hz), 74.56, 70.27, 65.24 (d, ${}^{2}J_{PC}$ =5.5Hz), 60.05, 39.96 (d, ${}^{2}J_{PC}$ =3.7Hz), 39.66 (d, ${}^{3}J_{PC}$ =3.7Hz), 11.44.

³¹P NMR (162 MHz, D₂O) $\delta_P = 5.69$ (d, ² $J_{PP}=28.5$ Hz, P_{β}), -11.94 (d, ² $J_{PP}=28.4$ Hz, P_{α}) HRMS (ESI, negative ion). Calculated m/z for C₂₀H₂₆N₇O₁₃P₂S [M+H]⁻: 666.0785; found 666.0793.

fast-adenosine 5'-pyrophosphoromorpholidate ($P \rightarrow 5'$) adenosine (*fast*-6) – *fast*-Ap(M)pA.



HPLC retention time (gradient G1): 22.1 min ¹H NMR (400 MHz, D₂O) $\delta_{\rm H}$ = 8.09 (1H, s, H2), 7.97 (1H, s), 7.94 (1H, s), 7.92 (1H, s), 5.89 (1H, d, ³J_{HH}=5.3Hz, H1'-Ap(M)), 5.86 (1H, d, ³J_{HH}=4.5Hz, H1'pA), 4.55 (1H, t, ³J_{HH}=5.3Hz, H2'-Ap(M)), 4.49 (1H, ψ t,

 ${}^{3}J_{HH}$ =5.0Hz, H2'-pA), 4.38 (1H, ψ t, ${}^{3}J_{HH}$ =4.8Hz, H3'-Ap(M)), 4.36 (1H, ψ t, ${}^{3}J_{HH}$ =5.0Hz, H3'-pA) 4.33-4.30 (1H, m, H5'-pA) 4.30-4.25 (2H, m, H5''-pA, H4'-Ap(M)), 4.25-4.20 (1H, m, H4'-pA), 4.20-4.12 (2H, m, H5',H5''-Ap(M)), 3.58 (4H, ψ t, ${}^{3}J_{HH}$ =4.5Hz, O(CH₂)₂), 3.15-3.10 (¶4H. m, N(CH₂)₂) 31 P NMR (162 MHz, D₂O) δ = 2.02 (d, ${}^{2}J_{PP}$ =16.8Hz), -11.23 (d, ${}^{2}J_{PP}$ =16.5Hz)

HRMS (ESI, negative ion). Calculated m/z for C₂₄H₃₂N₁₁O₁₃P₂ [M]⁻: 744.1656; found 744.1664

5'-thioadenosine 5'-pyrophosphate (P' \rightarrow 5') adenosine (7) – dASppA.



A suspension of 5'-deoxy-5'-(5-nitropyridyl-2disulfanyl)adenosine (2) (50.0 mg, 0.114 mmol) in 4:1 anhydrous chloroform:BTMSA (1.5 mL) was stirred at ambient temperature under argon for 30 min after which time a clear solution was formed. To this stirred solution was added (TMSO)₃P (42 μ L, 0.125 mmol, 1.1 eq) in 4:1

anhydrous chloroform:BTMSA (0.5 mL – total BTMSA 1.55 mmol, 13.6 eq.) and these conditions maintained for 30 minutes. ³¹P NMR indicated complete reaction and was again employed to assess the solution after storage for a further six days under inert conditions. An aliquot (1.5 mL, 0.085 mmol) of the reaction mixture was transferred into a zirconia-lined vessel and stored in a desiccator under vacuum for 30 min. The jar was then charged sequentially with AMP-morpholidate (4) (90 mg, 0.127 mmol, 1.5 eq), tetrazole (12 mg, 0.178 mmol, 2.1 eq.), MgCl₂•(H₂O)₆ (26 mg, 0.127 mmol, 1.5 eq.), H₂O (18 μ L, 1.020 mmol, 12 eq.) and a 15 mm zirconia ball. The vessel was vibrated at 30 Hz for 90 minutes and allowed to cool to room temperature. The crude reaction mixture was extracted from the vessel by rinsing with H₂O (2 mL), into eppendorf tubes. The aqueous suspensions were sonicated, filtered in 1 mL aliquots through a Spin-X cellulose acetate centrifuge filter (0.45 μ m) at 12,000 rpm and the filter rinsed with H₂O (0.5 mL). The combined extracts were analysed by ³¹P NMR. The reaction mixture was purified by C18 RP-HPLC using TEAA buffers (gradient G3) from which a single peak corresponding to pure dASppA was collected. Combined pure fractions were concentrated *in vacuo* and subject to desalting following HPLC isolation using TEAB buffers (G5) and coevaporation with deionised water.

Isolated yield of dASppA•~1.9(Et₃NH): 531 OD^{260nm} units (0.021 mmol, 25 % from MA product).

HPLC retention time (gradient G1): 18.1 min

¹H NMR (400 MHz, D₂O) $\delta_{\rm H} = 8.17$ (1H, s, H2), 7.98 (1H, s, H2), 7.92 (1H, s, H8), 7.92 (1H, s, H8), 5.85 (1H, d, ${}^{3}J_{HH}$ =2.5Hz, H1'-rA), 5.75 (1H, d, ${}^{3}J_{HH}$ =5.3Hz, H1'-dA), 4.48 (1H, ψ t, ${}^{3}J_{HH}$ =5.0Hz, H2'-dA), 4.45 (1H, ψ t, ${}^{3}J_{HH}$ =5.0Hz, H2'-rA), 4.36 (1H, t, ${}^{3}J_{HH}$ =4.7Hz, H3'-rA), 4.28-4.23 (3H, m, H3'-dA, H4'-dA, H4'-rA), 4.22-4.12 (2H, m, H5',H5''-rA), 3.25-3.08 (2H, m, H5',H5''-dA).

¹³C NMR (101MHz, D₂O) $\delta_{\rm C}$ = 154.87, 154.76, 152.41, 152.28, 148.20, 148.16, 139.47, 139.36, 118.03, 117.88, 87.25, 87.15, 83.37 (d, ³*J*_{*PC*}=9.5Hz), 83.27 (d, ³*J*_{*PC*}=6.2Hz) 74.74, 74.03, 71.61, 69.92, 65.28 (d, ²*J*_{*PC*}=4.8Hz), 32.51 (d, ²*J*_{*PC*}=3.7Hz).

³¹P NMR (162 MHz, D₂O) δ_P = 7.32 (d, ²*J*_{PP}=29.9Hz, P_β), 11.88 (d, ²*J*_{PP}=28.9Hz, P_α).

HRMS (ESI, negative ion). Calculated m/z for $C_{20}H_{25}N_{10}O_{12}P_2S$ [M+H]⁻: 691.0849, found 691.0871

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dTSSBt – 1a

HRMS (ESI, positive ion)













dTSSNPy-1b

HRMS (ESI, positive ion)







¹³C NMR 101 MHz

D₆-DMSO



NPySSdA – 2

HRMS (ESI, positive ion)











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Analytical C18 RP-HPLC of dTSppA (5) + *fast*-Ap(M)pA (6) - gradient G2 (IP buffers) (from 1a + 3.0 eq. AMP-morpholidate (4))



Analytical C18 RP-HPLC of pure dTSppA (5) - gradient G1















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dTSppA – 5

HRMS (ESI, negative ion)











HRMS (ESI, negative ion)









Analytical C18 RP-HPLC of crude dASppA (7) reaction mixture - gradient G1 (from 2 + 1.5 eq. AMP-morpholidate (4))



Analytical C18 RP-HPLC of pure dASppA (7) – gradient G1.









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HRMS (ESI, negative ion)



dTSMP after C18 RP-HPLC purification (in buffer at pH 6.5) ³¹P NMR 162 MHz D₂O (external lock)



a) dTSMP after 0.5 h







c) dTSMP after 27.5 h



d) dTSMP after 27.5 h + treatment with 1:1 100 mM DTT:100 mM NaHCO₃



e) Coinjection of hydrolysed dTSMP (d) above) with dTSH + BtSH (f) below)



f) dTSSBt (1a) after treatment with 1:1 100 mM DTT:100 mM NaHCO₃ (\rightarrow dTSH + BtSH)



g) Coinjection dTSH + BtSH (f) above) with BtSH (h) below)



h) (BtS)₂ after treatment with 1:1 100 mM DTT:100 mM NaHCO₃ (\rightarrow BtSH)

