Supporting information

Table of Contents

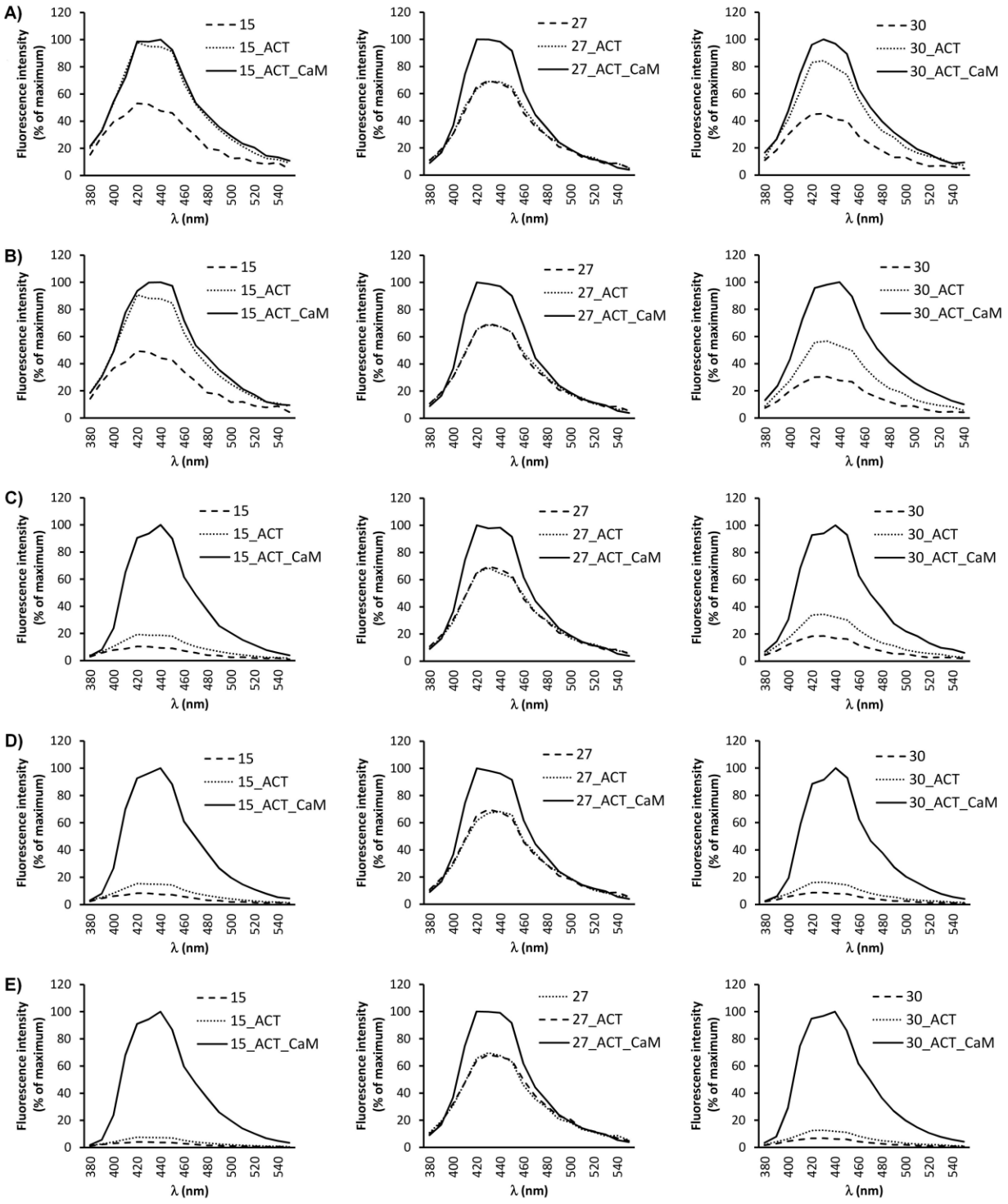

Figure S1. Direct fluorescence of compounds **15**, **27** and **30**. 100 nM of compound, 2.4 µM ACT, and 2.4 µM CaM were added to well in sequence. The excitation wavelength was 350 nm, and emission was scanned from 380 to 550 nm in 30 s intervals (A-E).

Superimposed recordings of a representative experiment are shown. Similar data were obtained in three independent experiments.

Experimental Section

Starting compounds and other chemicals were purchase from commercial suppliers or prepared according to the published procedures. Solvents were dried by standard procedures. Solvents were evaporated at 40 °C/2 kPa. Analytical TLC was performed on plates of Kieselgel 60 F_{254} (Merck). NMR spectra were recorded on Bruker Avance 500 (^{1}H at 500 MHz, ^{13}C at 125.8 MHz) and Bruker Avance 400 (^{1}H at 400 MHz, ^{13}C at 100.6 MHz) spectrometers with TMS or dioxane (3.75 ppm for ^{1}H, 67.19 ppm for ^{13}C NMR) as internal standard or referenced to the residual solvent signal. Mass spectra were measured on UPLC-MS (Waters SQD-2). HR MS were taken on a LTQ Orbitrap XL spectrometer. The microwave-assisted reactions were carried out in CEM Discover (Explorer) microwave apparatus. Preparative HPLC purification of triphosphate analogues was performed on a column packed with POROS® HQ 50 mm (50mL) with use of a gradient of TEAB in water (0.05-0.5 M). The purity of the tested compounds was determined by HPLC (H_2O-CH_3CN, linear gradient) and was higher than 95%.

General procedures

General procedure 1 (GP1). Reaction of a hydroxy derivative with 5-chloroisatoic anhydride: A hydroxy derivative (1 mmol) in dry DMF (8 mL) was treated with NaH (44 mg, 1.1 mmol) under Ar at RT and the resulting mixture was stirred at room temperature for 30 min. 5-Chloroisatoic anhydride (0.4 g, 2 mmol) was added and the mixture was stirred at 80 °C for 3 h. The reacting mixture was cooled to room temperature and

poured to EtOAc (50 mL) and extracted twice with saturated $NaHCO_3$ (50 mL) and brine (50 mL) and dried over $MgSO_4$.

General procedure 2 (GP2). Conversion of hydroxy group to amino group: The hydroxy derivative (1 mmol) was co-evaporated with dry pyridine (1 x 10 mL), dissolved in dry pyridine (10 mL) and treated with MsCl (0.15 mL, 2 mmol) at 0 °C and the reaction mixture was stirred at room temperature for 2 h. MeOH (5 mL) was added at 0 °C and the volatiles were evaporated. The crude product was dissolved in EtOAC (20 mL) and washed with $NaHCO_3$ (20 mL), brine (20 mL) and dried over $MgSO_4$. The obtained crude mesylate was without further purification dissolved in DMF/HMPA mixture (1:1, 6 mL) and treated with NaN_3 (325 mg, 5 mmol) at 100 °C overnight, cooled and poured into EtOAc (50 mL), washed with brine (5 x 30 mL) and dried over $MgSO_4$. The azido derivative was purified by flash chromatography ($CHCl_3$/MeOH 0-5%). The azide (3.9 mmol) in MeOH (45 mL) was treated with H_2 over Pd/C (10 wt. % loading, 350 mg) under atmospheric pressure for 24 h. The reaction mixture was filtered, evaporated and purified by flash chromatography ($CHCl_3$/MeOH 0-5%).

General procedure 3 (GP3). Reaction of amino derivative with 5-chloroisatoic anhydride: The amino derivative (0.3 mmol) in DMF/THF mixture (1:5, 3 mL) was treated with DMAP (3.7 mg, 0.03 mmol) and 5-chloroisatoic anhydride (0.12 g, 0.6 mmol) at room temperature overnight and solvents were evaporated.

General procedure 4 (GP4). Preparation of the bisamidate prodrugs: Phosphonate diester (1 mmol) was dissolved in dry pyridine (10 mL), and TMSBr (1 mL) was added. The reaction mixture was stirred at room temperature overnight. After evaporation of the volatiles, the flask was purged with Ar (without any contact with air) and amino acid ester hydrochloride (4 mmol, dried in vacuo at 30 °C and 0.1 mbar for 1 day), dry trimethylamine (2 mL) and dry pyridine (8 mL) were added, and the mixture was heated

at 70 °C to obtain a homogenous solution. Then, a solution of 2,2'-dipyridyldisulfide (6 mmol) and triphenylphosphine (6 mmol) in dry pyridine (10 mL) was added under Ar. The resulting mixture was heated at 70 °C for 72 h. After cooling to room temperature, the solvent was removed and the residue was purified by flash chromatography (gradient of MeOH (2-30%) in a mixture of hexane/EtOAc, 60:40) to remove impurities, followed by reversed-phase chromatography on C_{18} silica gel (aqueous MeOH 1-100%). The products were freeze dried from 1,4-dioxane.

General procedure 5 (GP5). Synthesis of phosphonate diphosphates: Diisopropyl ester of phosphonic acid (0.1 mmol) in pyridine (2 mL) was treated with TMSBr (0.1 mL) at room temperature overnight. The volatiles were removed under reduced pressure, the residue was co-evaporated with water and suspended in water and the free phosphonic acid was filtered off, dissolved in *t*BuOH and H_2O (1:1, 2 mL) and morpholine (35 μL) was added. Solution of DCC (82 mg) in *t*BuOH (2 mL) was added dropwise at 80 °C and the resulting mixture was heated at 80 °C until complete conversion of the reaction. The mixture was poured into H_2O/Et_2O mixture and the aqueous phase was washed with Et_2O (3 x 30 mL), finally Et_2O was washed with water. Collected aqueous phase was evaporated and the residue was co-evaporated with EtOH and dry toluene. The dried residue was treated with tri-*n*-butylammonium pyrophosphate (0.5 M solution in DMSO) at room tempertaure for 48 h. The reaction mixture was diluted with Et_2O (10 mL), Et_2O layer was poured off and the precipitate was dissolved in 0.05 M TEAB (4 mL) and applied onto a column of POROS® HQ, and eluted with a linear gradient of TEAB (0.05-0.5 M). The fractions containing product were concentrated at 27 °C and the residue was applied onto DOWEX 50 × 8 (Na^+ form), eluted with water and freeze dried.

9-[3-*O*-(4,4'-dimethoxytrityl)-2-hydroxypropyl]adenine (2). 9-(2,3-dihydroxypropyl)adenine (4.18 g, 20 mmol) in dry pyridine (200 mL) was treated with DMAP (35 mg, 0.29 mmol), TEA (2.8 mL, 20 mmol) and DMTrCl (7.53 g, 22 mmol) at

room temperature overnight. MeOH (10 mL) was added and the solvent was evaporated, the residue in EtOAc (100 mL) was washed with sat. $NaHCO_3$ and brine and dried over $MgSO_4$. The crude product was purified by flash chromatography ($CHCl_3$ with 0.5% TEA/MeOH 0-5%) to give an off-white foam (6.1 g, 88%): ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.07 (dd, *J*=9.6 and 6.3 Hz, 1H, H-3'a), 3.28 (dd, *J*=9.6 and 5.5 Hz, 1H, H-3'b), 3.80 (s, 6H, OCH_3), 4.20 (br s, 1H, OH), 4.34-4.26 (m, 1H, H-1'a), 4.42 (dd, *J*=14.3 and 2.4 Hz, H-1'b), 4.87 (m, 1H, H-2'), 5.89 (br s, 2H, NH_2), 7.40-7.42 (m, 2H) and 7.27-7.31 (m, 7H) and 6.81-6.83 (m, 4H, OTr), 7.73 (s, 1H, H-8), 8.27 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ=48.68 (C-1'), 55.56 (OCH_3), 64.76 (C-3'), 69.87 (C-2'), 86.76 (C-Tr), 113.51 (Ar), 119.67 (C-5), 127.25, 128.23, 128.28 and 130.24 (Ar), 135.90 and 135.99 (Ar), 141.87 (C-8), 144.87 (Ar), 150.35 (C-4), 152.88 (C-2), 155.79 (C-6) ppm; MS-ESI *m*/*z* (%): 513.24 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{29}H_{30}O_4N_5$: 512.22923, found: 512.22937.

9-[2-*O*-(5-Chloroanthraniloyl)-3-hydroxypropyl)]adenine (3) and **9-[3-*O*-(5-chloroanthraniloyl)-2-hydroxypropyl)]adenine (4).** Prepared from **2** (708 mg, 1.38 mmol) according to GP1. The crude product was treated with AcOH (80%, 20 mL) at room temperature for 2h. Acetic acid was evaporated and the resulting crude product was purified by flash chromatography ($CHCl_3$/MeOH 0-5%) and freeze dried from 1,4-dioxane to give inseparable mixture of compounds **3** and **4** (5:1, 396 mg, 79% overall yield). Major product **3**: ^{1}H NMR (400 MHz, [D_6]DMSO): δ=4.10-4.35 (m, 5H, H-1', H-2', H-3'), 5.66 (br s, 1H, OH), 6.79 (br s, 2H, NH_2), 6.81 (d, *J*=8.9 Hz, 1H, H-3''), 7.21 (brs, 2H, NH_2), 7.29 (dd, *J*=8.9 and 2.6 Hz, H-4''), 7.79 (d, *J*=2.6 Hz, 1H, H-6''), 8.10 (s, 1H, H-8), 8.13 (s, 1H, H-2) ppm; MS-ESI *m*/*z* (%): 362.9 (100) $[M+H]^+$. Minor product **4**: ^{1}H NMR (400 MHz, [D_6]DMSO): δ=4.10-4.35 (m, 3H, H-2', H-3'), 4.54 (m, 2H, H-1'), 5.25 (br s, 1H, OH), 6.65 (br s, 2H, NH_2), 6.74 (d, *J*=8.9 Hz, 1H, H-3''), 7.19 (brs, 2H, NH_2), 7.24 (dd, *J*=8.9 and 2.6 Hz, H-4''), 7.67 (d, *J*=2.6 Hz, 1H, H-6''), 8.12 (s, 1H, H-8), 8.14 (s, 1H, H-2) ppm; MS-ESI *m*/*z* (%): 363.1 (100) $[M+H]^+$.

9-[2-Amino-3-*O*-(4,4'-dimethoxytrityl)propyl]adenine (5). Prepared from **2** by GP2. White foam, overall yield (two steps) 50%: ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.16 (d, *J*=5.6 Hz, 2H, H-1'), 3.42-3.51 (m, 1H, H-2'), 3.82 (s, 6H, OCH_3), 4.17 (dd, *J*=14.0 and 7.4 Hz, 1H, H-3'a), 4.37 (dd, *J*=14.0 and 4.7 Hz, 1H, H-3'b), 5.85 (br s, 2H, NH_2), 6.84-6.87 (m, 4H), 7.25-7.27 (m, 1H), 7.29-7.35 (m, 6H), 7.44-7.46 (m, 2H, OTr), 7.74 (s, 1H, H-8), 8.34 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ=48.01 (C-1'), 51.57 (C-2'), 55.37 (OCH_3), 65.63 (C-3'), 86.41 (C-Tr), 113.34 (OTr), 119.63 (C-5), 127.07, 128.05,128.18, 130.11, 135.85 and 135.87 (OTr), 141.44 (C-8), 144.75 and 150.54 (arom.), 153.06 (C-2), 155.56 (C-4), 158.70 (C-6) ppm; MS-ESI *m*/*z* (%): 511.41 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{29}H_{31}O_3N_6$: 511.24522, found: 511.24539. Also, intermediate 9-[2'-azido-3'-O-(4,4'-dimethoxytrityl)propyl]adenine was isolated as white foam, yield 85%: ^{1}H NMR (400 MHz, [D_6]DMSO): δ=3.06 (dd, *J*=10.1 and 6.5 Hz, 1H, H-3'a), 3.28 (dd, *J*=10.1 and 3.3 Hz, 1H, H-3'b), 3.64 (s, 6H, OCH_3), 4.23-4.29 (m, 3H, H-2', H-1'), 6.85-6.89 (m, 4H, arom.), 7.22-7.24 (m, 5H, arom.), 7.31 (m, 2H, arom.), 7.39 (m, 2H, arom.), 8.04 and 8.12 (H-2, H-8) ppm; MS-ESI *m*/*z* (%): 537.58 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{29}H_{29}O_3N_8$: 537.23571, found: 537.23580.

9-[2-*N*-(5-Chloroanthraniloyl)-3-hydroxypropyl)]adenine (6) and **2-(3-(1-(6-amino-9H-purin-9-yl)-3-hydroxypropan-2-yl)ureido)-5-chlorobenzoic acid (7)**. Prepared according to GP3. The crude product was treated with 80% AcOH (10 mL) at room temperature for 3 h. Acetic acid was evaporated and the residue was purified by flash chromatography ($CHCl_3$/MeOH 0-5%) and freeze dried to give **6** (167 mg, 84%) as a white foam. ^{1}H NMR (400 MHz, [D_6]DMSO): δ=3.44-3.55 (m, 2H, H-3'), 4.29 (dd, *J*=13.1 Hz, *J*=8.3 Hz, 1H, H-1'b), 4.35-4.39 (m, 1H, H-2'), 4.44 (dd, *J*=13.1 Hz, *J*=3.5 Hz, 1H, H-1'a), 5.09 (t, *J*=5.7 Hz, 1H, OH), 6.36 (br s, 2H, NH_2), 6.67 (d, *J*=8.8 Hz, 1H, H-3''), 7.14 (dd, *J*=8.8 Hz, *J*=2.5 Hz, H-4''),7.20 (br s, 2H, NH_2), 7.50 (d, *J*=2.5 Hz, 1H, H-6''), 8.04 (s, 1H, H-8), 8.15 (s, 1H, H-2), 8.23 (d, *J*=8.2 Hz, 1H, NH) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=44.24 (C-1'), 51.48 (C-2'), 61.37 (C-3'), 115.87 (C-1''), 118.08 (C-5''), 118.31 (C-3''), 119.07 (C-5), 127.95 (C-6''), 131.87 (C-4''), 141.68 (C-8), 148.83 (C-4), 150.26 (C-2''), 152.75 (C-2), 156.40 (C-6), 167.92 (COO) ppm; MS-ESI m/z (%): 362.2

(21), 364.2 (8) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{15}H_{17}O_2N_7Cl$: 362.11268, found: 362.11286. As a second product compound **7** (10 mg, 8%) was isolated as a white foam. ^{1}H NMR (400 MHz, [D_6]DMSO): δ=3.45 (m, 2H, H-3‘), 4.10 (m, 1H, H-2‘), 4.25 (dd, *J*=13.9 Hz, *J*=8.6 Hz, 1H, H-1’b), 4.40 (dd, *J*=13.9 Hz, *J*=4.7 Hz, 1H, H-1’a), 5.44 (br s, 1H, OH), 7.15 (dd, *J*=8.9 Hz, *J*=2.8 Hz, 1H, H-4‘‘), 7.19 (br s, 2H, NH_2), 7.71 (br s, 1H, NH), 7.87 (d, *J*=2.8 Hz, H-6‘‘), 8.07 (s, 1H, H-8), 8.13 (d, *J*=8.9 Hz, 1H, H-3‘‘), 8.16 (s, 1H, H-2), 12.60 (br s, 1H, 2‘‘-NH) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=44.54 (C-1‘), 51.83 (C-2‘), 62.08 (C-3‘), 119.01 (C-5), 120.00 (C-3‘‘), 122.98 (C-1‘‘), 125.90 (C-5‘‘),129.55 (C-4‘‘), 130.79 (C-6‘‘), 141.54 (C-2‘‘), 141.84 (C-8), 150.12 (C-4), 152.77 (C-2), 155.37 (NH-COO), 156.35 (C-6), 169.32 (COOH) ppm; MS-ESI *m*/*z* (%): 406.1 (100), 408.2 (67) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{16}H_{15}O_4N_7Cl$: 404.08795, found: 404.08782.

9-{3-[(Diethoxyphosphoryl)methoxy]-2-hydroxypropyl}adenine (9). Compound **8** (800 mg, 2.1 mmol) was treated with ammonia in EtOH (15 mL) and heated under microwave irradiation (50 W) at 100 °C for 1 h. The solvent was evaporated and the residue was purified by flash chromatography ($CHCl_3$/MeOH 0-5%) to give **9** as a colorless oil (550 mg, 72%): ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.36 (t, *J*=7.1 Hz, 6H CH_3), 3.50 (br s, 1H, H-2’), 3.63 (m, 2H, PCH_2), 3.85 (m, 2H, H-3’), 4.18 (m, 4H, $POCH_2$), 4.27 (m, 1H, H-1’a), 4.43 (m, 1H, H-1’b), 5.36 (brs, 1H, OH), 5.99 (brs, 2H, NH_2), 7.92 (s, 1H, H-8), 8.31 (s, 1H, H-2) ppm; MS-ESI *m*/*z* (%): 359.97 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{13}H_{23}O_5N_5P$: 360.14313, found: 360.14321.

9-{3-[(Diethoxyphosphoryl)methoxy]-2-*O*-(5-chloroanthraniloyl)propyl}adenine (10). Prepared from **9** (360 mg, 1 mmol) by GP1 at room temperature, purified by flash chromatography ($CHCl_3$/MeOH 0-5%) to give yellowish oil (462 mg, 90%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.19 (2 x t, *J*=7.1 Hz, 6H, CH_3), 3.81-3.85 (m, 2H, H-3‘), 3.92 (m, 2H, PCH_2), 4.00-4.06 (m, 4H, P-OCH_2), 4.51-4.57 (m, 2H, H-1‘), 5.51 (m, 1H, H-2‘), 6.68 (br s, 2H, NH_2), 6.75 (d, *J*=8.9, 1H, H-3‘‘), 7.20 (br s, 2H, NH_2), 7.25 (dd, *J*=8.9 Hz,

J=2.7 Hz, 1H, H-4‘‘), 7.64 (d, *J*=2.7 Hz, 1H, H-6‘‘), 8.13 (s, 1H, H-8), 8.14 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=16.69 (d, *J*=5.5 Hz, CH_3), 43.53 (C-1‘), 62.30 (d, *J*=6.3 Hz, P-OCH_2), 64.88 (d, *J*=161.6 Hz, PCH_2), 71.14 (C-2‘), 71.66 (d, *J*=10.7 Hz, C-3‘), 109.19 (C-1‘‘), 118.09 (C-5‘‘), 118.81 (C-3‘‘), 118.92 (C-5), 129.99 (C-6‘‘), 134.53 (C-4‘‘), 141.50 (C-8), 150.27 (C-2‘‘), 150.78 (C-4), 153.00 (C-2), 156.38 (C-6), 165.78 (COO) ppm; MS-ESI *m*/*z* (%): 513.13 (100) [*M*+H]$^+$.

Bis(L-phenylalanine isopropyl ester) prodrug of ((3-(6-amino-9H-purin-9yl)-2-*O*-(5-chloroanthraniloyl)propoxy)methyl)phosphonic acid (11). Prepared from **10** (110 mg, 0.2 mmol) by GP4 as a white foam (95 mg, 56%): ^{1}H NMR (500 MHz, [D_6]DMSO): δ=1.00-1.16 (m, 12H, CH_3ipr.), 2.76-2.92 (m, 4H, Ph-$\underline{CH_2}$), 3.23-3.39 (m, 2H, PCH_2), 3.52-3.61 (m, 2H, H-3‘), 3.88-4.02 (m, 2H, P-NH-$\underline{CH}$), 4.27 (m, 1H, NH), 4.45-4.56 (m, 2H, H-1‘), 4.60 (m, 1H, NH), 4.74-4.85 (m, 2H, CHipr.), 5.40 (m, H-2‘), 6.69-6.75 (m, 3H, H-3‘‘, 2‘‘-NH_2), 7.12-7.25 (m, 13H, 6-NH_2, H-4‘‘, Ph-H-2, Ph-H-3, Ph-H-4), 7.65 and 7.62 (d, *J*=2.7 Hz, 1H, H-6‘‘), 8.10-8.14 (m, 2H, H-2, H-8) ppm; ^{13}C NMR (125 MHz, [D_6]DMSO): δ=21.48-21.67 (m, CH_3ipr.), 40.00 ($\underline{CH_2}$-Ph), 43.20 and 43.17 (C-1‘), 54.07-54.33 (m, P-NH-$\underline{CH}$), 68.00 and 67.97 (d, *J*=134.9 Hz, PCH_2), 68.15 and 68.03 (CHipr.), 70.71 and 70.87 (m, C-2‘, C-3‘), 108.95 and 108.96 (C-1‘‘), 117.88 (C-5‘‘), 118.57 (C-3‘‘), 118.64 and 118.65 (C-5), 126.66, 126.61 (C-4-Ph), 128.27 and 128.24 (C-3-Ph), 129.55-129.78 (m, C-2-Ph, C-6‘‘), 134.30 (C-4‘‘), 137.31, 137.29 and 137.23 (C-1-Ph), 141.34 and 141.32 (C-8), 149.99 and 149.98 (C-2‘‘), 150.57 and 150.56 (C-4), 152.81 and 152.79 (C-2), 156.15 (C-6), 165.60 and 165.58 (1‘‘-COO), 172.35-172.53 (m, COOipr.) ppm; MS-ESI *m*/*z* (%): 849.5 (56) [*M*+H]$^+$; HRMS-ESI *m*/*z* [*M*+H]$^+$ calcd for $C_{41}H_{51}O_8N_8ClP$: 849.32505, found: 849.32538.

9-{-2-Amino-3-[(diethoxyphosphoryl)methoxy]propyl}adenine (12). The hydroxy derivative **9** (550 mg, 1.5 mmol) in dry pyridine (10 mL) was treated with MsCl (0.22 mL, 3 mmol) at 0 °C a allowed to stir at room temperature for 2 h. MeOH (5 mL) was added at 0 °C and the volatiles were evaporated. The crude product was dissolved in EtOAC

(20 mL) and washed with $NaHCO_3$ (20 mL), brine (20 mL) and dried over $MgSO_4$. The mesylate was without further purification dissolved in DMF/HMPA mixture (1:1, 5 mL) and treated with NaN_3 (487 mg, 7.5 mmol) and stirred at room temperature for 3 days, further NaN_3 (487 mg, 7.5 mmol) was added and the mixture was stirred for further 3 days and the resulting mixture was poured into EtOAc (50 mL) and washed with brine (5 x 30 mL) and dried over $MgSO_4$ and purified by flash chromatography ($CHCl_3$/MeOH 0-5%) to give 9-{-2-azido-3-[(diethoxyphosphoryl)methoxy]propyl}adenine (398 mg, 59%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.24 (t, *J*=7.0 Hz, 6H, CH_3), 3.64 (dd, *J*=10.5 and 6.4 Hz, 1H, H-3'a), 3.79 (dd, *J*=10.5 and 3.4 Hz, 1H, H-3'b), 3.91 (m, 2H, PCH_2), 4.05 (m, 4H, $POCH_2$), 4.21-4.33 (m, 3H, H-1', H-2'), 7.25 (brs, 2H, NH_2), 8.13 (s, 1H, H-8), 8.15 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=16.75 (d, *J*=5.5 Hz, CH_3), 43.75 (C-1'), 60.29 (C-2'), 62.29 (d, *J*=6.3 Hz, P-OCH_2), 64.79 (d, *J*=162.1 Hz, PCH_2), 72.87 (d, *J*=10.9 Hz, C-3'), 119.00 (C-5), 141.51 (C-8), 150.17 (C-4), 153.04 (C-2), 156.46 (C-6) ppm; MS-ESI *m*/*z* (%): 385.1 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{13}H_{22}O_4N_8P$: 385.14961, found: 385.14977. The azide (190 mg, 0.49 mmol) in MeOH (10 mL) was treated with Pd/C (10 wt. % loading, 15 mg) and H_2 under atmospheric pressure for 24 h. The reaction mixture was filtered, evaporated and purified by flash chromatography ($CHCl_3$/MeOH 0-30%) to give colorless oil (120 mg, 66%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.23 (t, *J*=7.0, 6H, CH_3), 3.25 (m, 1H, H-2'), 3.39 (m, 2H, H-3'), 3.83 (d, *J*=7.8 Hz, 2H, PCH_2), 3.96 (dd, *J*=13.8 and 7.7 Hz, 1H, H-1'a), 4.05 (dq, *J*=8.1 and 7.0 Hz, 4H, $POCH_2$), 4.19 (dd, *J*=13.8 and 5.0, 1H, H-1'b), 7.18 (brs, 2H, NH_2), 8.09 (s, 1H, H-8), 8.12 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=16.35 (d, *J*=5.5 Hz, CH_3), 46.50 (C-1'), 50.32 (C-2'), 61.76 (d, *J*=7.2 Hz, O$\underline{CH_2}CH_3$), 64.33 (d, *J*=162.0 Hz, PCH_2), 75.68 (d, *J*=10.0 Hz, C-3'), 118.60 (C-5), 141.58 (C-8), 149.77 (C-4), 152.29 (C-2), 155.92 (C-6) ppm; MS-ESI *m*/*z* (%): 359.22(100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{13}H_{24}O_4N_6P$: 369.15912, found: 359.15920.

9-{3-[(Diethoxyphosphoryl)methoxy]-2-*N*-(5-chloroanthraniloyl)propyl}adenine (13). Prepared from **12** (115 mg, 0.32 mmol) by GP3 to give **13** as a colorless oil (130 mg, 79%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.21 (2 x t, *J*=7.1 Hz, 6H, CH_3), 3.64 (m,

2H, H-3‘), 3.88 (d, *J*=8.0 Hz, 2H, PCH_2), 4.04 (dq, *J*=8.0 Hz, *J*=7.1 Hz, 4H, $O\underline{CH_2}CH_3$), 4.29 (dd, *J*=14.1 Hz, *J*=8.5 Hz, 1H, H-1’b), 4.40 (dd, *J*=14.1 Hz, H=4.2 Hz, H-1’a), 4.55 (m, 1H, H-2‘), 6.38 (br s, 2H, NH_2), 6.67 (d, *J*=8.8 Hz, 1H, H-3‘‘), 7.14 (dd, *J*=8.8 Hz, *J*=2.5 Hz, 1H, H-4‘‘), 7.21 (br s, 2H, NH_2), 7.46 (d, *J*=2.5 Hz, 1H, H-6‘‘), 8.04 (s, 1H, H-8), 8.14 (s, 1H, H-2), 8.33 (d, *J*=8.5 Hz, NHCO) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=16.31 (d, J=5.6 Hz, CH_3), 43.72 (C-1‘), 48.51 (C-2‘), 61.89 (d, *J*=7.2 Hz, $O\underline{CH_2}CH_3$), 64.21 (d, *J*=161.8 Hz, PCH_2), 71.85 (d, *J*=10.7 Hz, C-3‘), 115.06 (C-1‘‘), 117.61 (C-5‘‘), 117.93 (C-3‘‘), 118.60 (C-5), 127.41 (C-6‘‘), 131.56 (C-4‘‘), 141.18 (C-8), 148.47 (C-4), 149.81 (C-2‘‘), 152.39 (C-2), 155.96 (C-6), 167.53 (CON) ppm; MS-ESI *m*/*z* (%): 512.4 (88) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{20}H_{28}O_5N_7ClP$: 512.15726, found: 512.15708.

Bis(L-phenylalanine isopropyl ester) prodrug of ((3-(6-amino-9H-purin-9yl)-2-*N*-(5-chloroanthraniloyl)propoxy)methyl)phosphonic acid (14). Prepared from **13** (65 mg, 0.126 mmol) by GP4, white foam (58 mg, 54%): ^{1}H NMR (500 MHz, [D_6]DMSO): δ=0.99-1.16 (m, 12H, CH_3ipr.), 2.76-2.91 (m, 4H, Ph-$\underline{CH_2}$), 3.16-3.46 (m, 4H, PCH_2, H-3‘), 3.89-4.04 (m, 2H, P-NH-$\underline{CH}$), 4.27-4.44 (m, 3H, NH-P, H-1‘), 4.48 (m, 1H, H-2‘), 4.61-4.71 (m, 1H, NH-P), 4.74-4.87 (m, 2H, CHipr.), 6.41 and 6.40 (s, 2H, NH_2-2‘‘), 6.68 and 6.67 (d, *J*=8.8, 1H, H-3‘‘), 7.12-7.26 (m, 13H, Ph-H-2, Ph-H-3, Ph-H-4, 6-NH_2, H-4‘‘), 7.49 and 7.47 (d, *J*=2.5 Hz, 1H, H-6‘‘), 8.02 and 8.00 (s, 1H, H-8), 8.14 and 8.14 (s, 1H, H-2), 8.33 and 8.29 (d, *J*=8.2 Hz, 1H, NH) ppm; ^{13}C NMR (125 MHz, [D_6]DMSO): δ=21.48-21.69 (m, CH_3ipr.), 39.9 (Ph-$\underline{CH_2}$), 43.69 and 43.64 (C-1‘), 48.83 and 48.68 (C-2‘), 54.02-54.42 (m, P-NH-$\underline{CH}$), 67.84 and 67.71 (d, *J*=134.6 Hz, *J*=135.0 Hz, PCH_2), 68.16 and 68.01 (CHipr.), 71.42-71.62 (m, C-3‘), 115.32 and 115.30 (C-1‘‘), 117.84 and 117.81 (C-5‘‘), 118.10 (C-3‘‘), 118.77 (C-5), 126.67, 126.63 and 126.61 (C-4-Ph), 127.74 and 127.73 (C-6‘‘), 128.28, 128.26 and 128.24 (C-3-Ph), 129.69, 129.65 and 129.63 (C-2-Ph), 131.75 (C-4‘‘), 137.33, 137.32, 137.29 and 137.26 (C-1-Ph), 141.46 and 141.42 (C-8), 148.70 (C-2‘‘), 150.03 and 150.01 (C-4), 152.61 (C-2), 156.18 and 156.17 (C-6), 167.73 and 167.73 (CON), 172.40-172.61 (m, COO) ppm; MS-ESI *m*/*z*

(%): 834.7 (20) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{40}H_{50}O_7N_9ClP$: 834.32539, found: 834.32569.

((3-(6-Amino-9H-purin-9yl)-2-*N*-(5-chloroanthraniloyl)propoxy)methyl)phosphonic acid diphosphate, sodium salt (15). Prepared from **13** (51.2 mg, 0.1 mmol) by GP5, white foam (12 mg, 17%): ^{1}H NMR (500 MHz, D_2O): δ=3.87-3.98 (m, 4H, H-3‘, PCH_2), 4.51 (dd, *J*=14.6, *J*=9.9, 1H, H-1’b), 4.64 (dd, *J*=14.6 Hz, *J*=4.0 Hz, 1H, H-1’a), 4.73 (m, 1H, H-2‘), 6.72 (d, *J*=8.8 Hz, H-3‘‘), 7.04 (d, *J*=2.5 Hz, 1H, H-6‘‘), 7.21 (dd, *J*=8.8 Hz, *J*=2.5 Hz, 1H, H-4‘‘), 8.19 (s, 1H, H-2), 8.22 (s, 1H, H-8) ppm; ^{13}C NMR (125 MHz, D_2O): δ=45.57 (C-1‘), 50.07 (C-2‘), 67.91 (d, *J*=162.4 Hz, PCH_2), 72.20 (d, *J*=11.3 Hz, C-3‘), 118.93 (C-5), 119.31 (C-1‘‘), 119.49 (C-3‘‘), 123.23 (C-5‘‘), 128.10 (C-6‘‘), 132.64 (C-4‘‘), 143.70 (C-8), 145.64 (C-2‘‘), 150.15 (C-4), 153.08 (C-2), 156.14 (C-6), 170.37 (CON) ppm; ^{31}P NMR (202 MHz, D_2O): δ=-21.73 (dd, *J*=19.6 and 25.6 Hz, P-β), -7.55 (m, P-α), 9.22 (d, *J*=25.6, P-γ) ppm; HRMS-ESI *m*/*z* $[M+H]^-$ calcd for $C_{16}H_{19}O_{11}N_7ClNaP_3$: 635.99471, found: 635.99464.

9-(3-Hydroxy-2-(hydroxymethyl)propyl)-*N*^{6}-benzoyladenine (17). *N*^{6}-benzoyladenine (1.84 g, 7.7 mmol) in dry DMF (25 mL) was treated with NaH (0.372 g, 9.3 mmol, 60% susp. In mineral oil) at 0 °C for 30 min. and compound **16** (2.6 g, 11.6 mmol) in dry DMF (5 mL) was added and the reaction mixture was heated at 60 °C for 24 h. The resulting mixture was cooled and diluted with EtOAc (200 mL) and washed with brine (3 x 10 mL) and dried over $MgSO_4$. The crude product was treated with 80% AcOH (25 mL) at 60 °C for 30 min. The mixture was cooled and acetic acid was evaporated and co-evaporated with water and EtOH. Flash chromatography gave **17** (1.3 g, 53%) as a white solid: ^{1}H NMR (400 MHz, [D_6]DMSO): δ=2.21 (m, 1H, H-2‘), 4.38-4.46 (m, 4H, H-3‘, H-4‘), 4.30 (d, *J*=7.1, 2H, H-1‘), 4.66 (t, 2H, *J*=5.1 Hz, OH), 7.62-7.64 (m, 1H) and 7.53-7.57 (m, 2H), 8.04-8.06 (m, 2H, arom.), 8.42 (s, 1H, H-8), 8.73 (s, 1H, H-2), 11.13 (br s, 1H, NHCO) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=42.58 (C-1‘), 43.59 (C-2‘), 59.34 (C-3‘), 125.50 (C-5), 128.47 (C-3‘‘, C-2‘‘), 132.39 (C-4‘‘), 133.48 (C-1‘‘), 145.35 (C-8),

150.02 (C-6), 151.35 (C-2), 152.68 (C-4), 165.57 (C=O) ppm; MS-ESI *m*/*z* (%): 328.24 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{16}H_{18}N_5O_3$: 328.14042, found: 328.14053.

9-(3-*O*-(*t*-Butyldimethylsilyl)-2-(hydroxymethyl)propyl)-*N*^{6}-benzoyladenine (18). Dihydroxy derivative **17** (1.48 g, 4.5 mmol) in dry DMF (25 mL) was treated with imidazole (0.46 g, 6.75 mmol) and TBSCl (0.75 g, 4.95 mmol) was added in portions and the resulting mixture was stirred at room temperature for 24 h. The solvent was evaporated and the crude product was purified by flash chromatography ($CHCl_3$/MeOH 0-10%) to give **18** (1 g, 50%) as a white solid: ^{1}H NMR (400 MHz, [D_6]DMSO): δ=0.10 and 0.11 (2 x s, 2 x 3H, Si-CH_3), 0.95 (s, 9H, CH_3-*t*Bu), 2.31 (m, 1H, H-2‘), 3.42 (dd, 1H, *J*=12.0 Hz, *J*=7.5 Hz, H-4’b), 3.51 (m, 1H, H-4’a), 3.55 (dd, 1H, *J*=10.5 Hz, *J*=7.5 Hz, H-3’b), 3.68 (dd, 1H, *J*=10.5 Hz, *J*=4.8 Hz, H-3‘a), 4.16 (br s, 1H, OH), 4.59-4.43 (m, 2H, H-1‘), 8.03 (m, 2H) and 7.61-7.65 (m, 1H) and 7.52-7.56 (m, 2H, arom.), 8.06 (s, 1H, H-8), 8.79 (s, 1H, H-2), 9.18 (br s, 1H, NH) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=-5.45 (CH_3-Si), 18.21 (C-Si), 25.88 (CH_3-*t*Bu), 41.44 (C-1‘), 43.81 (C-2‘), 60.05 (C-4‘), 61.65 (C-3‘), 122.79 (C-5), 127.89 (C-3‘‘), 128.87 (C-2‘‘), 132.84 (C-4‘‘), 133.57 (C-1‘‘), 144.15 (C-8), 149.69 (C-6), 152.42 (C-2), 152.49 (C-4), 164.41 (C=O) ppm; MS-ESI *m*/*z* (%): 441.98 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{22}H_{32}N_5O_3Si$: 442.22689, found: 442.22697.

9-(3-*O*-(*t*-Butyldimethylsilyl)-2-(hydroxymethyl)propyl)adenine (19). Compound **18** (830 mg, 1.9 mmol) in MeOH (15 mL) was treated with MeONa (2 mL, 1M solution in MeOH) at room temperature overnight. The reaction mixture was neutralized with AcOH and evaporated. The crude product was purified by flash chromatography ($CHCl_3$/MeOH 0-10%) to give **19** (510 mg, 81%) as a white solid: ^{1}H NMR (400 MHz, [D_6]DMSO): δ=-0.02 and -0.02 (s, 2 x 3H, CH_3-Si), 0.89 (s, 9H, CH_3-*t*Bu), 2.28-2.13 (m, 1H, H-2‘), 3.36-3.38 (m, 2H, H-4‘), 3.54 (d, *J*=5.4 Hz, 2H, H-1‘), 4.14 (d, *J*=7.1 Hz, 2H, H-3‘), 4.71 (br, 1H, OH), 7.20 (br s, 2H, NH_2), 8.03 (s, 1H, H-8), 8.13 (s, 1H, H-2) ppm;

^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=-5.12 (CH_3-Si), 18.40 (C-Si), 26.24 (CH_3-*t*Bu), 42.47 (C-1‘), 43.89 (C-2‘), 59.33 (C-4‘), 61.39 (C-3‘), 119.15 (C-5), 141.74 (C-8), 150.16 (C-4), 152.78 (C-2), 156.38 (C-6) ppm; MS-ESI *m*/*z* (%): 337.99 (100) [*M*+H]$^+$; HRMS-ESI *m*/*z* [*M*+H]$^+$ calcd for $C_{15}H_{28}O_2N_5Si$: 338.20068, found: 338.20072.

9-(3-*O*-(5-Chloroanthraniloyl)-2-(hydroxymethyl)propyl)adenine (20). Prepared from **19** (170 mg, 0.5 mmol) by GP1. The crude product was treated with AcOH (80%, 10 mL) at 50 °C for 8 h. Acetic acid was evaporated, the residue was co-evaporated with water and EtOH and purified by flash chromatography ($CHCl_3$/MeOH 0-10%) and freeze dried from 1,4-dioxane to give 20 (93 mg, 49%) as a white foam: ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=2.54 (m, 1H, H-2‘), 3.48 (m, 2H, H-3‘), 4.18 (m, 2H, H-1‘), 4.27 (m, 2H, H-4‘), 4.95 (t, *J*=5.2 Hz, 1H, OH), 6.77 (br s, 2H, NH_2), 6.79 (d, *J*=8.9, 1H, H-3‘‘), 7.19 (br s, 2H NH_2), 7.27 (dd, *J*=8.9 Hz, *J*=2.7 Hz, 1H, H-4‘‘), 7.58 (d, *J*=2.7 Hz, 1H, H-6‘‘), 8.12 (s, 1H, H-8), 8.13 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=41.16 (C-2‘), 42.48 (C-1‘), 59.47 (C-3‘), 63.36 (C-4‘), 109.92 (C-1‘‘), 118.21 (C-5‘‘), 118.90 (C-3‘‘), 119.12 (C-5), 129.86 (C-6‘‘), 134.35 (C-4‘‘), 141.70 (C-8), 150.19 (C-2‘‘), 150.19 (C-4), 152.87 (C-2), 156.42 (C-6), 166.67 (COO) ppm; MS-ESI *m*/*z* (%): 377.1 (100) [*M*+H]$^+$; HRMS-ESI *m*/*z* [*M*+H]$^+$ calcd for $C_{16}H_{18}O_3N_6Cl$: 377.11234, found: 377.11245.

9-(2-(Aminomethyl)-3-*O*-(*t*-butyldimethylsilyl)propyl)adenine (21). Prepared from **20** (120 mg, 0.36 mmol) by the same procedure as compound **12**. Intermediate 9-(2-(azidomethyl)-3-*O*-(*t*-butyldimethylsilyl)propyl)adenine was obtained as a white solid (110 mg, 85%): ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=0.0 (s, 6H, CH_3-Si), 0.85 (s, 9H, CH_3-*t*Bu), 2.44-2.35 (m, 1H, H-2‘), 3.41 (m, 2H, H-4‘), 3.49 (dd, *J*= 10.5 Hz, *J*=5.5 Hz, 1H, H-3’b), 3.58 (dd, *J*=10.5 Hz, *J*=4.6, 1H, H-3’a), 4.15 (m, 2H, H-1‘), 7.20 (br s, 2H, NH_2), 8.06 (s, 1H, H-8), 8.13 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=-5.20 (CH_3-Si), 18.38 (C-Si), 26.21 (CH_3-*t*Bu), 41.08 (C-2‘), 42.71 (C-1‘), 50.26 (C-4‘), 61.14 (C-3‘), 119.17 (C-5), 141.55 (C-8), 150.18 (C-4), 152.91 (C-2), 156.42 (C-6) ppm; MS-ESI *m*/*z* (%): 363.0 (100) [*M*+H]$^+$; HRMS-ESI *m*/*z* [*M*+H]$^+$ calcd for $C_{15}H_{27}ON_8Si$: 363.20716,

found: 363.20722. The amino derivative (21) was obtained as a white solid (35 mg, 34%): ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=-0.01 (s, 6H, CH$_3$-Si), 0.85 (s, 9H, CH$_3$-*t*Bu), 2.07 (m, 1H, H-2‘), 2.47 (m, 2H, H-4‘), 3.53 (m, 2H, H-3‘), 4.15 (m, 2H, H-1‘), 7.17 (br s, 2H, NH$_2$), 8.03 (s, 1H, H-8), 8.12 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=-5.56 (CH$_3$-Si), 17.92 (C-Si), 25.79 (CH$_3$-*t*Bu), 40.08 (C-4‘), 42.32 (C-1‘), 43.69 (C-2‘), 61.61 (C-3‘), 118.62 (C-5), 141.19 (C-4), 152.34 (C-2), 155.90 (C-6) ppm; MS-ESI *m*/*z* (%): 337.2 (100) [*M*+H]$^+$; HRMS-ESI *m*/*z* [*M*+H]$^+$ calcd for $C_{15}H_{29}ON_6Si$: 337.21666, found: 337.21671.

9-(3-*N*-(5-Chloroanthraniloyl)-2-(hydroxymethyl)propyl)adenine (22). Prepared from **21** (35 mg, 0.1 mmol) by GP3. The crude product was treated with AcOH (80%, 5 mL) at 60 °C for 3 h. The solvent was evaporated, co-distilled with water and EtOH and the residue was purified by flash chromatography ($CHCl_3$/MeOH 0-10%) and freeze dried from 1,4-dioxane to give **22** (27 mg, 53%) as a white foam: ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=2.28 (m, 1H, H-2‘), 3.27 and 3.13 (m, 2 x 1H, H-4‘), 3.35 (m, 2H, H-3‘), 4.18 (m, 2H, H-1‘), 4.82 (t, *J*=5.3 Hz, OH), 6.49 (br s, 2H, NH$_2$), 6.72 (d, *J*=8.8 Hz, 1H, H-3‘‘), 7.17 (dd, *J*=8.8 Hz, *J*=2.5 Hz, H-4‘‘), 7.26 (br s, 2H, NH$_2$), 7.57 (d, *J*=2.5 Hz, 1H, H-6‘‘), 8.13 (s, 1H, H-8), 8.14 (s, 1H, H-2), 8.45 (t, *J*=5.7 Hz, 1H, NH-CO) ppm; ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=38.23 (C-4‘), 41.46 (C-2‘), 42.61 (C-1‘), 59.70 (C-3‘), 115.63 (C-1‘‘), 117.78 (C-5‘‘), 118.01 (C-3‘‘), 118.64 (C-5), 127.41 (C-6‘‘), 131.43 (C-4‘‘), 141.45 (C-8), 148.45 (C-4), 149.74 (C-2‘‘), 152.36 (C-2), 156.03 (C-6), 167.96 (C=O) ppm; MS-ESI *m*/*z* (%): 374.2 (100) [*M*+H]$^-$; HRMS-ESI *m*/*z* [*M*+H]$^+$ calcd for $C_{16}H_{18}O_2N_7ClNa$: 398.11036, found: 398.11036.

9-(3-*O*-(*t*-Butyldimethylsilyl)-2-(diisopropoxyphosphorylmethoxymethyl)propyl)-*N*^{6}-benzoyladenine (23). Compound **18** (0.5 g, 1.13 mmol) in DMF (10 mL) was treated with (*t*BuO)$_2$Mg (0.29 g, 1.7 mmol) and *p*TsOCH$_2$P(O)(OiPr)$_2$ (0.54 g, 1.7 mmol), and the reaction mixture was heated at 60 °C for 3 days. The mixture was cooled to room temperature, diluted with EtOAc (100 mL), washed with brine (3 x 10 mL) and dried over

$MgSO_4$. The product was purified by flash chromatography ($CHCl_3$/MeOH 0-5%), colorless oil (380 mg, 44%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=0.02 (s, 6H, CH_3-Si), 0.84 (s, 9H, CH_3-*t*Bu), 1.24 (m, 12H, CH_3ipr.), 2.49 (m, 1H, H-2‘), 3.52 (m, 2H, H-4‘), 3.59 (m, 2H, H-3‘), 3.72 (d, *J*=8.0 Hz, 2H, PCH_2), 4.38-4.23 (m, 2H, H-1‘), 4.59 (m, 2H, CHipr.), 8.02-8.07 (m, 2H) and 7.69-7.59 (m, 1H) and 7.55 (m, 2H, H-Bz), 8.41 (s, 1H, H-8), 8.71 (s, 1H, H-2), 11.15 (br s, 1H,NH) ppm; MS-ESI *m*/*z* (%): 620.5 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{29}H_{47}O_6N_5PSi$: 620.30277, found: 620.30296.

9-[2-(Hydroxymethyl)-3-(diisopropoxyphosphorylmethoxy)propyl)]adenine (24). Compound **23** (1.14 g, 1.8 mmol) in dry THF (50 mL) was treated with TBAF (1M solution in THF, 4 mL) at room temperature overnight, evaporated, dissolved in EtOAc and washed with brine and dried over $MgSO_4$ to give a colorless oil: MS-ESI *m*/*z* (%): 506.16 (100) $[M+H]^+$. The crude product was dissolved in MeOH (15 mL) and treated with MeONa (1M solution in MeOH, 3 mL) at room temperature for 6 h. The reaction mixture was neutralized with AcOH, evaporated and purified by flash chromatography ($CHCl_3$/MeOH 0-5%). Compound **24** was obtained as a colorless oil (598 mg, 81%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.24 (d, *J*=6.1 Hz, 6H) and 1.22 (d, *J*=6.1 Hz, 6H, CH_3ipr.), 2.30 (m, 1H, H-2‘), 3.46 (m, 2H, H-3‘), 3.71 (m, 2H, PCH_2), 4.15 (m, 2H, H-1‘), 4.59 (m, 2H, CHipr.), 4.78 (t, *J*=5.0, 1H, OH), 7.22 (br s, 2H, NH_2), 8.08 (s, 1H, H-8), 8.13 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=24.17-24.31 (m, CH_3ipr.), 41.89 (C-2‘), 42.30 (C-1‘), 59.44 (C-4‘), 65.48 (d, *J*=164 Hz, PCH_2), 70.69 and 70.62 (2 x d, *J*=6.4 Hz, CHipr.), 71.46 (d, *J*=11.2 Hz, C-3‘), 119.10 (C-5), 141.81 (C-8), 150.14 (C-4), 152.80 (C-2), 156.39 (C-6) ppm; MS-ESI *m*/*z* (%): 402.2 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{16}H_{29}O_5N_5P$: 402.19008, found: 402.19016.

9-[2-(5-Chloroanthraniloyloxymethyl)-3-(diisopropoxyphosphorylmethoxy)propyl)]adenine (25). Prepared from derivative **24** (400 mg, 1 mmol) by GP1, colorless oil (410 mg, 74%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.21-1.24 (m, 12H, CH_3ipr.), 2.73 (m, 1H, H-2‘), 3.62 (d, *J*=5.3, 2H, H-3‘), 3.78 (d,

J=7.7 Hz, 2H, PCH_2), 4.18 (d, *J*=5.6 Hz, 2H, H-4‘), 4.28-4.30 (m, 2H, H-1‘), 4.57-4.62 (m, 2H, CHipr.), 6.77 (br s, 2H, NH_2), 6.79 (d, *J*=8.9 Hz, 1H, H-3‘‘), 7.19 (br s, 2H, NH_2), 7.27 (dd, *J*=2.6 Hz, *J*=8.9 Hz, 1H, H-4‘‘), 7.62 (d, *J*=2.6 Hz, 1H, H-6‘‘), 8.12 (s, 1H, H-8), 8.15 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=24.29 (m, CH_3ipr.), 39.09 (C-2‘), 42.32 (C-1‘), 63.07 (C-4‘), 65.52 (d, *J*=163.2 Hz, PCH_2), 70.64 (d, *J*=6.4 Hz, CHipr.), 70.97 (d, *J*=10.5 Hz, C-3‘), 109.74 (C-1‘‘), 118.22 (C-5‘‘), 118.87 (C-3‘‘), 119.13 (C-5), 129.89 (C-6‘‘), 134.41 (C-4‘‘), 141.57 (C-8), 150.21 (C-2‘‘), 150.68 (C-4), 152.91 (C-2), 156.44 (C-6), 166.62 (COO) ppm; MS-ESI *m*/*z* (%): 555.1 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{33}O_6N_6ClP$: 555.18822, found: 555.18838.

Bis(L-phenylalanine isopropyl ester) prodrug of ((3-(6-amino-9H-purin-9yl)-2-(5-chloroanthraniloyloxymethyl)propoxy)methyl)phosphonic acid (26). Prepared from **25** (110 mg, 0.2 mmol) by GP4 to give **26** (95 mg, 56%) as a white foam: ^{1}H NMR (500 MHz, [D_6]DMSO): δ=0.99-1.16 (m, 12H, CH_3ipr.), 2.60 (m, 1H, H-2‘), 2.75-2.94 (m, 4H, Ph-$\underline{CH_2}$), 3.07-3.40 (m, 4H, PCH_2, H-3‘), 3.88-4.05 (m, 2H, P-NH-$\underline{CH}$), 4.14-4.33 (m, 4H, H-1‘, H-4‘), 4.54-4.69 (m, 2H, P-NH), 4.74-4.86 (m, 2H, CHipr.), 6.78-6.81 (m, 3H, H-3‘‘, NH_2-Ph), 7.11-7.25 (m, 12H, 6-NH_2, Ph-H-2, Ph-H-3, Ph-H-4), 7.26-7.29 (m, 1H, H-4‘‘), 7.65 and 7.61 (d, *J*=2.6 Hz, *J*=2.7 Hz, 1H, H-6‘‘), 8.12-8.16 (m, 2H, H-2, H-8) ppm; ^{13}C NMR (125 MHz, [D_6]DMSO): δ=21.49-21.68 (m, CH_3ipr.), 39.00 (C-2‘), 39.9 ($\underline{CH_2}$-Ph), 41.79 and 41.64 (C-1‘), 53.96-54.40 (m, P-NH-$\underline{CH}$), 63.01 and 63.00 (C-4‘), 67.76 and 67.67 (d, *J*=135.5 Hz, *J*=135.6, PCH_2), 68.15, 68.13 and 68.00 (CHipr.), 70.29 and 70.09 (d, *J*=12.0 Hz, *J*=12.7 Hz, C-3‘), 109.51 and 109.48 (C-1‘‘), 117.98 and 117.97 (C-5‘‘), 118.66 and 118.64 (C-3‘‘), 118.84 and 118.81 (C-5), 126.64, 126.58 and 126.56 (C-4-Ph), 128.25, 128.23 and 128.21 (C-3-Ph), 129.61-129.68 (m, C-2-Ph, C-6‘‘), 134.18 (C-4‘‘), 137.34, 137.30 and 137.28 (C-1-Ph), 141.46 and 141.44 (C-8), 149.93 and 149.88 (C-2‘‘), 150.46 and 150.44 (C-4), 152.77 and 152.70 (C-2), 156.22 and 156.20 (C-6), 166.39 and 166.36 (1‘‘-COO), 172.38-172.58 (m, COOipr.) ppm; MS-ESI *m*/*z* (%): 849.5 (56) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{41}H_{51}O_8N_8ClP$: 849.32505, found: 849.32538.

((3-(6-Amino-9H-purin-9yl)-2-(5-chloroanthraniloyloxymethyl)propoxy)methyl)phosphonic acid diphosphate, sodium salt (27). Prepared from **25** (55 mg, 0.1 mmol) by GP5, white foam, yield 29 mg (41%): ^{1}H NMR (500 MHz, D_2O): δ=2.85 (m, 1H, H-2‘), 3.82-3.87 (m, 2H, H-3‘), 3.91-3.97 (m, 2H, PCH_2), 4.36 (dd, *J*=11.5, *J*=7.6, 1H, H-4’b), 4.45-4.51 (m, 3H, H-1‘, H-4‘a), 6.56 (d, *J*=2.6 Hz, H-6‘‘), 6.69 (d, *J*=8.9 Hz, 1H, H-3‘‘), 7.21 (dd, *J*=8.9 Hz, *J*=2.6 Hz, 1H, H-4‘‘), 7.93 (s, 1H, H-2), 8.24 (s, 1H, H-8) ppm; ^{13}C NMR (125 MHz, D_2O): δ=38.46 (C-2‘), 45.09 (C-1‘), 66.57 (C-4‘), 67.57 (d, *J*=163.5 Hz, PCH_2), 71.57 (d, *J*=11.8 Hz, C-3‘), 111.08 (C-1‘‘), 119.00 (C-5), 119.40 (C-3‘‘), 120.98 (C-5‘‘), 129.50 (C-6‘‘), 134.81 (C-4‘‘), 143.41 (C-8), 149.08 (C-2‘‘), 149.59 (C-4), 152.02 (C-2), 155.39 (C-6), 167.52 (COO) ppm; ^{31}P NMR (202 MHz, D_2O): δ=-22.24 (dd, *J*=20.0 and 26.3 Hz, P-β). -9.58 (d, *J*=20.0 Hz, P-α), 9.52 (d, *J*=26.3 Hz, P-γ) ppm; HRMS-ESI *m*/*z* $[M+H]^-$ calcd for $C_{17}H_{21}O_{12}N_6ClP_3$: 629.01243, found: 629.01283.

9-[2-(5-Chloroanthraniloylaminomethyl)-3-(diisopropoxyphosphorylmethoxy)propyl)]adenine (28). The hydroxy derivative **24** (570 mg, 1.4 mmol) was converted to 9-[2-(azidomethyl)-3-(diisopropoxyphosphorylmethoxy)propyl)]adenine (280 mg, 46%) and further to 9-[2-(aminomethyl)-3-(diisopropoxyphosphorylmethoxy)propyl)]adenine (130 mg, 51%) by the same procedure as was described for compound **12**. 9-[2-(Azidomethyl)-3-(diisopropoxyphosphorylmethoxy)propyl)]adenine: ^{1}H NMR (400 MHz, $[D_6]$DMSO): δ=2.51 (m, 12H, CH_3ipr.), 3.43 (m, 2H, H-4‘), 3.51-3.46 (m, 2H, H-3‘), 3.74 (d, *J*=7.9 Hz, 2H, PCH_2), 4.18 (d, *J*=7.0, 2H, H-1‘), 4.56-4.65 (m, 2H, CHipr.), 7.23 (br s, 2H, NH_2), 8.11 (s, 1H, H-8), 8.14 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, $[D_6]$DMSO): δ=24.17-24.30 (m, CH_3ipr.), 39.24 (C-2‘), 39.98 (C-1‘), 50.39 (C-4‘), 65.50 (d, *J*=164 Hz, PCH_2), 70.66 and 70.65 (2 x d, *J*=6.4 Hz, CHipr.), 71.08 (d, *J*=11.0 Hz, C-3‘), 119.14 (C-5), 141.59 (C-8), 150.19 (C-4), 152.95 (C-2), 156.44 (C-6) ppm; MS-ESI *m*/*z* (%): 426.9 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{16}H_{28}O_4N_8P$: 427.19656, found:

427.19657. 9-[2-(Aminomethyl)-3-(diisopropoxyphosphorylmethoxy)propyl)]adenine: ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.24 (d, *J*=6.1 Hz, CH_3ipr.), 1.25 (d, *J*=6.2 Hz, 6H, CH_3ipr.), 2.14-2.20 (m, 1H, H-2‘), 2.47 (m, 2H, H-4‘), 3.44-3.51 (m, 2H, H-3‘), 3.72 (m, 2H, PCH_2), 4.16 (m, C-1‘), 4.56-6.65 (m, 2H, CHipr.), 7.20 (br s, 2H, NH_2), 8.09 (s, 1H, H-8), 8.13 (s, 1H, H-2) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=24.30-24.20 (m, CH_3ipr.), 42.19 (C-2‘), 42.59 (C-1‘), 65.44 (d, *J*=164, PCH_2), 70.62 and 70.64 (2 x d, *J*=6.4 Hz, CHipr.), 70.66 (d, *J*=1.8 Hz, CHipr.), 71.96 (d, *J*=11.1 Hz, C-3‘), 119.06 (C-5), 141.74 (C-8), 150.23 (C-4), 152.85 (C-2), 156.42 (C-6) ppm; MS-ESI *m*/*z* (%): 401.15 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{16}H_{30}O_4N_6P$: 401.20607, found: 401.20611. The amino derivative (120 mg, 0.3 mmol) was finally converted by GP3 to **28** (140 mg, 84%): ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.25 and 1.24 (2 x d, *J*=6.2 Hz, CH_3ipr.), 2.45 (m, 1H, H-2‘), 3.29 and 3.17 (m, 2 x 1H, H-4‘), 3.48 (d, *J*=5.4 Hz, 2H, H-3‘), 3.76 (d, *J*=7.6 Hz, 2H, PCH_2), 4.19 (d, *J*=6.7 Hz, 2H, H-1‘), 4.59-4.64 (m, 2H, CHipr.), 6.50 (br s, 2H, NH_2), 6.71 (d, *J*=8.8 Hz, 1H, H-3‘‘), 7.16 (dd, *J*=8.5 Hz, *J*=2.5 Hz, 1H, H-4‘‘), 7.23 (br s, 2H, NH_2), 7.60 (d, *J*=2.5 Hz, 1H, H-6‘‘), 8.14 (s, 1H, H-8), 8.14 (s, 1H, H-2), 8.47 (t, *J*=5.8 Hz, NH-CO) ppm; ^{13}C NMR (100 MHz, [D_6]DMSO): δ=23.73-23.85 (m, CH_3ipr.), 38.03 (C-4‘), 39.42 (C-2‘), 42.53 (C-1‘), 65.01 (d, *J*=163.4, PCH_2), 70.27 and 70.24 (2 x d, *J*=6.4 Hz, CHipr.), 71.33 (d, *J*=10.2 Hz, C-3‘), 115.45 (C-1‘‘), 117.73 (C-5‘‘), 117.97 (C-3‘‘), 118.65 (C-5), 127.40 (C-6‘‘), 131.42 (C-4‘‘), 141.31 (C-8), 148.49 (C-2‘‘), 148.71 (C-4), 152.36 (C-2), 155.99 (C-6), 167.88 (COO) ppm; MS-ESI *m*/*z* (%): 554.5 (100) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{34}O_5N_7ClP$: 554.20421, found: 554.20432.

Bis(L-phenylalanine isopropyl ester) prodrug of ((3-(6-amino-9H-purin-9yl)-2-(5-chloroanthraniloylaminomethyl)propoxy)methyl)phosphonic acid (29). Prepared from **28** (70 mg, 0.126 mmol) by GP4 to give **29** (70 mg, 65%) as a white foam: ^{1}H NMR (500 MHz, [D_6]DMSO): δ= 0.99-1.16 (m, 12H, CH_3ipr.), 2.33 (m, 1H, H-2‘), 2.76-2.92 (m, 4H, Ph-$\underline{CH_2}$), 3.11-3.31 (m, 6H, H-4‘, PCH_2, H-3‘), 3.91-4.05 (m, 2H, PNH-$\underline{CH}$), 4.15-4.18 (m, 2H, H-1‘), 4.32-4.37 (m, 1H, PNH), 4.66-4.73 (m, 1H, PNH), 4.74-4.87 (m, 2H, CHipr.), 6.54 (bs, 2H, 2‘‘-NH_2), 6.72 and 6.72 (d, *J*=8.8, 1H, H-3‘‘), 7.12-7.26 (m, 13H,

Ph-H-2, 3, 4, 6-NH_2, H-4''), 7.65 and 7.64 (d, *J*=2.5, 1H, H-6''), 8.15-8.16 (m, 2H, H-2, H-8), 8.56-8.60 (m, CO-NH) ppm; ^{13}C NMR (125 MHz, [D_6]DMSO): δ=21.47-21.67 (m, CH_3ipr.), 37.88 and 37.73 (C-4'), 39.7 (C-2'), 39.9 (Ph-$\underline{CH_2}$), 42.47 and 42.39 (C-1'), 54.00-54.50 (m, P-NH-$\underline{CH}$), 66.93-68.02 (m, PCH_2), 68.15 and 68.02 (CHipr.), 70.48-70.67 (m, C-3'), 115.64 and 115.53 (C-1''), 117.98 and 117.97 (C-5''), 118.20 and 118.18 (C-3''), 118.81 (C-5), 126.66 and 126.59 (Ph-C-4), 127.71 and 127.67 (C-6''), 128.28 and 128.25 (Ph-C-3), 129.68, 129.67 and 129.66 (Ph-C-2), 131.65 (C-4''), 137.37, 137.34, 137.29 and 137.27 (Ph-C-1), 141.63 and 141.57 (C-8), 148.79 and 148.76 (C-2''), 149.92 and 149.89 (C-4), 152.69 and 152.67 (C-2), 156.24 (C-6), 168.15 and 168.04 (CON), 172.40-172.58 (m, COO) ppm; MS-ESI *m*/*z* (%): 848.6 (42) $[M+H]^+$; HRMS-ESI *m*/*z* $[M+H]^+$ calcd for C41H52O7N9ClP: 848.34104, found: 848.34128.

((3-(6-Amino-9H-purin-9yl)-2-(5-chloroanthraniloylaminomethyl)propoxy)methyl)phosphonic acid diphosphate, sodium salt (30). Prepared from **28** (55 mg, 0.1 mmol) by GP5, white foam, yield 12 mg (17%): ^{1}H NMR (500 MHz, D_2O): δ= 2.75 (m, 1H, H-2'), 3.34-3.43 (m, 2H, H-4'), 3.60-3.65 (m, 2H, H-3'), 3.77-3.81 (m, 2H, PCH_2), 4.29 (dd, *J*=14.7 Hz, *J*=9.1 Hz, 1H, H-1'b), 4.36 (dd, *J*=14.7, *J*=5.0 Hz, 1H, H-1'a), 6.66 (d, *J*=8.8 Hz, H-3''), 6.77 (d, *J*=2.4 Hz, 1H, H-6''), 7.13 (dd, *J*=8.8 Hz, *J*=2.4 Hz, 1H, H-4''), 8.04 (s, 1H, H-2), 8.14 (s, 1H, H-8) ppm; ^{13}C NMR (125 MHz, [D_6]DMSO): δ=38.57 (C-2'), 40.71 (C-4'), 45.37 (C-1'), 67.75 (d, *J*=162.9 Hz, PCH_2), 72.77 (d, *J*=11.4 Hz, C-3'), 118.10 (C-1''), 119.08 (C-5), 119.89 (C-3''), 122.21 (C-5''), 127.43 (C-6''), 132.79 (C-4''), 143.52 (C-8), 146.28 (C-2''), 149.66 (C-4), 152.77 (C-2), 155.89 (C-6), 169.89 (CON) ppm; ^{31}P NMR (202 MHz, D_2O): δ= -21.84 (dd, *J*=19.8 and 25.7 Hz, P-β), -7.60 (m, P-α), 9.40 (d, *J*=25.7, P-γ) ppm; HRMS-ESI *m*/*z* $[M+H]^-$ calcd for $C_{17}H_{21}O_{11}N_7ClNaP_3$: 650.01036; found: 650.00998.

Molecular Docking. Crystal structure of *Bordetella pertussis* adenylyl cyclase toxin with CAM and PMEApp (PDB code 1ZOT, resolution 2.2Å)[17] was prepared with MOE Ligx with default setup and structure was minimized to RMS gradient of 0.001. Structures of

all final compounds above were properly protonated and deprotonated and minimized to RMS gradient of 0.001. For docking studies rigid dock protocol was chosen with structure waters included and ligands rotate bonds was enabled. Default placement and refinement method was used with 50 retained structures after the first refinement and 20 retained structures after the second refinement. As positions of nucleobases in enzymes are typically highly conserved, pharmacophore restrains were applied for nucleobase (features volume in brackets): both aromatic rings (1.3), hydrogen donor to backbone oxygen of Thr300 (1.5), hydrogen acceptor from water1030 to nitrogen N1 of purine (1.3). For all calculations Amber12:EHT mixed forcefield was used with Generalized Born solvent model.

Biological assays

Effect on the viability of J774A.1 cells. J774A.1 cells were plated onto white 96-well assay plates at $5x10^4$ cells per well and allowed to attach overnight. Cells were then washed with HBSS and treated with 10 μM compounds for 5 h. Cell viability was then assessed with a Cell Titer-Glo Luminescent Cell Viability assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Measurement of luminescence signal was performed by use of a GENios microplate reader (Tecan Systems). Data were expressed as percent of control, represented by untreated cells.

Inhibition of ACT – cell-based assay. J774A.1 cells were seeded in a 96-well plate at $5x10^4$ cells per well and left to attach overnight. Prior to the experiment, cells were washed with HBSS (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM glucose, 10 mM HEPES [pH 7.4]) and pre-incubated with compounds at concentrations of 0.001–30 μM for 5 h. After that, cells were exposed to ACT (2 nM) from *B.pertussis* (Enzo Life Sciences, Palo Alto, CA; SA=115 μmol/min/mg) for 30 min. Finally, the cAMP content was determined by using the CatchPoint cAMP immunoassay kit (Molecular Devices, Wokingham, UK). After the addition of lysis buffer (50 μL per well) provided by

the manufacturer, the cellular content was extracted by shaking the plate at 250 rpm for 10 min. The plate was then centrifuged to remove cell debris, the supernatant was replaced to the assay plate, and immunoassays were carried out according to the manufacturer's instructions. Fluorescence signal was acquired using an Infinite M1000 plate reader (Tecan Systems Inc., San Jose, CA, USA).

Inhibition of ACT – cell-free assay. In a cell-free assay, AC enzymatic activity was measured by conversion of [^{3}H] ATP to of [^{3}H]cAMP. The reaction was carried out at 30^{o}C for 30 min, with a final reaction volume of 50 μl. Each assay mixture contained 3 μM BSA, 20 mM HEPES (pH 7.4), 10 mM $MnCl_2$, 1 mM EDTA, 1 μM $CaCl_2$, 0.1 mM cold ATP, 20 μCi [2,8-^{3}H]ATP (ARC, St. Louis, MO, USA; specific activity 20 Ci/mmol), 1.2 μM calmodulin and tested compound at concentration of 0 – 100 μM. Inhibition of AC activity was determined in the presence of 3 different enzymes ACT (Sigma, specific activity 65 μmol/min/mg), ACT (Enzo, specific activity 115 μmol/min/mg) and EF (LBL, specific activity 830 μmol/min/mg) with the final enzyme concentration of 1.1 nM , 0.67 nM and 0.12 nM , respectively. The incubation was carried out for 30 min at 30^{o}C, in a final reaction volume of 50 μl. A 2 μL aliquot of the assay mixture was spotted on a polyethylenimine chromatographic sheet, and developed in 4M LiCl:1 M acetic acid (1:4). After developing, the spots containing ATP and cAMP were quantified using Radio-TLC scanner RITA (RAYTEST, Germany) with evaluation software GINA STAR TLC. Data were calculated from the percentage conversion of [^{3}H]ATP to [^{3}H]cAMP. Ki values were calculated using the Graphpad Prism 5 software (San Diego, CA, USA). All assays were performed in duplicate with three independent repetitions. In statistical analysis, Student's *t* test (two-sided) was used. Results are given as means ± SD.

Fluorescence spectroscopy. The measurements were carried out in black 96-well plates (Nunc) at 25°C using the Cytation 3 microplate reader (BioTek, VT, USA). The final assay volume was 75 μl. Reaction mixtures contained a buffer consisting of 100 μM $CaCl_2$, 100 mM KCl, 5 mM $MnCl_2$, and 75 mM HEPES, pH 7.4. Further, 2-(Cl-ANT)-

ANPpp compounds, ACT and CaM were added successively. In FRET experiments, 2-(CI-ANT)-ANPpps were used at final concentrations from 10 nM to 500 nM, and ACT and CaM were 300 nM each. Steady-state emission spectra were recorded at low speed with and λex= 280 nm (λem=320–500 nm) and λex= 295 nm (λem = 320–540 nm). In PMEApp displacement experiment, 100 nM 2-(CI-ANT)-ANPpps were displaced from ACT by PMEApp at concentrations of 5 nM to 1 μM. Direct fluorescence of MANTS was excited at 350 nm, and steady-state emission spectra were recorded from 380 to 550 nm. The final concentrations of ACT and CaM were 2.4 μM each. Saturation experiments were performed using MANTs at concentration range from 10 nM to 500 nM; ACT and CaM were 300 nM each. Saturation curves were obtained by subtracting the fluorescence intensity at 430 nm after the addition of ACT from the maximal fluorescence (FRET) after the addition of ACT/CaM. Half-saturation concentration EC_{50} was calculated using the Graphpad Prism 5 software (San Diego, CA, USA). Basal fluorescence in the presence of buffer alone was subtracted.

Assays with mACs. HEK cells stably expressing AC1, AC2, or AC5 were cultured and frozen as previously described.[40,41] Cells were thawed and plated in white bottom 384-well plates (PerkinElmer, Shelton, CT). Inhibitor compounds (at concentration of 30 μM) were added to cells and incubated at room temperature for 30 min. Then, the specific mAC stimulator (3 μM A23187 for AC1, 100 nM PMA for AC2, and 300 nM forskolin for AC5) in 500 μM 3-isobutyl-1-methyxanthine was added to the cells. Cells were incubated at room temperature for 1 h and cAMP accumulation was measured using Cisbio's dynamic 2 kit (Cisbio Bioassays, Bedford, MA) according to the manufacturer's instructions.