Supplementary material

Modified ARCA analogs providing enhanced translational properties of capped mRNAs

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Chemical synthesis

All used reagents were purchased in the highest available purity from Sigma-Aldrich Chemical Co. and were used without any further treatment except for the benzyl azides, which were prepared by treating the corresponding benzyl bromides with NaN_3 in DMF. Triethylammonium bicarbonate (TEAB) buffer was prepared by bubbling CO_2 through an ice-cold aqueous solution of redistilled triethylamine. Intermediate nucleotides and final products were separated by ion-exchange chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) using a linear gradient of TEAB buffer (pH 7.6). Fractions containing products were combined, evaporated under reduced pressure with several additions of ethanol and isolated as triethylammonium salts (TEA salts). HPLC was performed on a Knauer instrument using Supelcosil LC-18-T RP column (4.6x250 mm, flow rate 1.0mL min^{-1}) with a linear gradient of methanol from 0 to 50% (v/v) in 0.05 M ammonium acetate (pH 5.9). UV detection was performed at 254 nm. Exact mass measurements were recorded on a Micromass QToF spectrometer using electrospray ionization. ^1H NMR spectra were recorded on a Varian INOVA 400 MHz spectrometer using tetramethylsilane (TMS) as the internal standard in $CDCl_3$ and sodium 3-trimethylsilyl-[2,2,3,3-D4]-propinonate (TSP) in D_2O. ^{31}P NMR spectra were recorded on a Varian INOVA 200 MHz.

2'-O-methylguanosine and 3'-O-methylguanosine were obtained as previously reported (Kuśmierek and Shugar 1978).

2',5'-di-O-acetyl-3'-O-methylguanosine was synthesized by adding acetic anhydride (0.37 mL, 4mmol) to cooled suspension of 3'-O-methylguanosine (260 mg, 0.9 mmol), triethylamine (1.1 mL, 8 mmol) and N,N-(dimethylamino)pyridine (12 mg, 0.1 mmol) in 7.4 mL of anhydrous acetonitrile. The mixture was stirred 3 hours at (-10˚C) and quenched with methanol (1.5 mL). The mixture was concentrated using rotary evaporator and to the residue diethyl ether was added. The resulting precipitate was filtered and dried overnight over P_2O_5 to yield 273 mg (0.7 mmol, 82%) of product.

3',5'-di-O-acetyl-2'-O-methylguanosine; was obtained in the same manner as 2',5'-di-O-acetyl-3'-O-methylguanosine.Obtained 268,6 mg (0.7 mmol, 87%)

2',5'-di-O-acetyl-3'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]guanosine; The mixture of 2',5'-di-O-acetyl-3'-O-methylguanosine (710 mg, 1.8 mmol), triphenylphosphine (730 mg, 2.7 mmol) and 2-(4-nitrophenyl)ethanol (450 mg, 2.7 mmol) in anhydrous toluene (12 mL) was stirred and diisopropylazodicarboxylate (0,427 ml, 2.16 mmol) was added dropwise. The reaction was carried out for 24 hours at RT and then the solvent was evaporated. The resulting oil was purified by column chromatography on silicagel with chloroform as eluent. Obtained 326 mg (0.6 mmol, yield 35%) of product as yellowish crystals.

3',5'-di-O-acetyl-2'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]guanosine; was obtained in the same manner as 2',5'-di-O-acetyl-3'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]guanosine to yield 240 mg (0.45 mmol, 19%).

N2-fluoro-2',5'-di-O-acetyl-3'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]inosine; 2',5'-di-O-acetyl-3'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]guanosine(326 mg, 0.6 mmol) was dissolved in anhydrous pyridine (2.3 mL, 28 mmol) in a polypropylene tube under nitrogen atmosphere. The reaction mixture was placed in a dry ice/acetonitile cooling bath (-35˚C) and 70% HF in pyridine (4.2 mL) was added dropwise over a period of 10 min. After 15 min, t-butyl nitrite (0.185 ml, 1.55 mmol) was added. The reaction mixture was stirred for 3 hours and then was quenched at 0˚C by slowly dropping the reaction mixture into an aqueous K_2CO_3 solution (9.8 g in 9 mL of water). The resulting solution was extracted three times with ethyl acetate, the

organic layers were collected, dried over anhydrous Na_2SO_4 and evaporated to dryness. Chromatographic isolation (silicagel, 60:1 CH_2Cl_2:MeOH) gave (250 mg, 0.45 mmol, 77%) of product as white crystals.

N2-fluoro-3',5'-di-O-acetyl-2'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]inosine was obtained in the same manner as N2-fluoro-2',5'-di-O-acetyl-3'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]inosine to yield 129 mg (0.2 mmol, 41%).

N2-benzyl-3'-methylguanosine (3) and N2-benzyl-2'-methylguanosine (5) N2-fluoro-2'(3'),5'-di-O-acetyl-3'(2')-O-methyl-O6-[2-(4-nitrophenyl)ethyl]inosine (215 mg, 0.4 mmol) was dissolved in 2.5 mL of anhydrous DMSO and benzylamine (0.215 mL, 2 mmol) was added. After 4 hours of stirring, the reaction was quenched with water and extracted three times with ethyl acetate. Organic layers were dried over anhydrous Na_2SO_4 and evaporated. Next, methylamine in ethanol (1 mL) was added and stirred at 50°C until the full deprotection of nucleoside was observed based on TLC (CH_2Cl_2/MeOH 25/1). The methylamine in ethanol was removed under high vacuum and the residual oil was treated with CH_2Cl_2 to cause precipitation of white crystals. The products were filtrated and washed several times with diethyl ether and dried over P_2O_5 to yield: **3** 96 mg (0.25 mmol, 62%) and **5** 25 mg (0.065 mmol, 27%).

N2-4-methoxybenzyl-3'-methylguanosine (4) N2-fluoro-3',5'-di-O-acetyl-2'-O-methyl-O6-[2-(4-nitrophenyl)ethyl]inosine(250 mg, 0.45 mmol) was dissolved in 2 mL of anhydrous DMSO and 4-methoxybenzylamine (0.300 mL, 2.25 mmol) was added. After 4 hours of stirring, the reaction was quenched with water and extracted three times with ethyl acetate. Organic layers were dried over anhydrous Na_2SO_4 and evaporated to dryness. Next, methylamine in ethanol (1 mL) was added and stirred at 50°C until the full deprotection of nucleoside was observed based on TLC (CH_2Cl_2/MeOH 25/1). The methylamine in ethanol was removed under high vacuum, and the residual oil was treated with CH_2Cl_2 to cause precipitation of white crystals. The product was filtrated and washed several times with diethyl ether and dried over P_2O_5 to yield 109 mg (0.3 mmol, 55%) of **4.**

Procedure for the synthesis of the N7 methylated N2 modified 5′-monophosphates

To the dried compounds **3-5** (60 mg, 0.15 mmol) phosphorus oxide trichloride ($POCl_3$) (1.7 mmol, 0.160mL) and trimethyl phosphate (15mmol, 1.8 mL) were added and cooled to 0°C.

The reaction mixtures were stirred for 3 h and then 1.0 M aqueous TEAB was added to maintain the pH as neutral. Products were isolated by ion-exchange chromatography on a DEAE-Sephadex A-25 column using a linear 0–1 M TEAB gradient to produce products as TEA salts.
3'-O-methyl-N^2-benzylguanosine-5'-monophosphate; 50 mg (0.1 mmol, 82%); ^{1}H NMR (400 MHz, D_2O) δ 8.01 (s, 1H, H-8'), 7.45 – 7.30 (m, 5H, Ph), 5,89 (d, 1H, H-1'), 4,77 (t, 1H, H-2'), 4.62 (d, 2H, CH_2Ph), 4,35-4,32 (m, 1H, H-3'), 4,06-3,94 (m, 3H, H-4', H-5', H-5''), 3.45 (s, 3H, 3'O-CH_3), ^{31}P NMR (283 MHz, D_2O) 0.60.
3'-O-methyl-N^2-(4-metoxybenzyl)guanosine-5'-monophosphate; 60 mg (0.12 mmol, 85%); ^{1}H NMR (400 MHz, D_2O) δ 8.39 (s, 1H, H-8'), 7.31 (d, 2H, Ph), 6.92 (d, 2H, Ph), 5,93 (d, 1H, H-1'), 4.74 (t, 1H, H-2'), 4.48 (d, 2H, CH_2Ph), 4.35 – 4,34 (m, 1H, H-3'), 4.15 – 4.10 (m, 1H, H-4'), 4.05 – 3,99 (m, 2H, H-5', H-5"), 3.76 (s, 3H, $PhOCH_3$), 3.41 (s, 3H, 3'O-CH_3), ^{31}P NMR (283 MHz, D_2O) 0,63.
2'-O-methyl-N2-benzylguanosine-5'-monophosphate; 16.4 mg (0.035 mmol, 55%);^{1}H NMR (400 MHz, D2O) δ 8.02 (s, 1H, H-8'), 7.43 – 7.27 (m, 5H, Ph), 5,95 (d, 1H, H-1'), 4.61 (d, 2H, CH2Ph), 4.44 (t, 1H, H-2'), 4.30 (t, 1H, H-3'), 4.24 – 4.20 (m, 1H, H-4'), 4.10 – 4.04 (m, 1H, H-5'), 4.02 – 3.96 (m, 1H, H-5''), 3.24 (s, 3H, 2'O-CH3), 31P NMR (283 MHz, D2O) 0.72.

Obtained compounds were further methylated at N7 position. To the suspensions of 1 mmol (50 mg) of mononucleotide derivatives in1.75 mL anhydrous DMSO, methyl iodide (0.300 mL, 5 mmol)was added and stirred at RT for 3 hours. Then reactions were quenched with water and extracted several times with diethyl ether. The aqueous layers were purified on DEAE Sephadex and eluted with linear 0 – 0.7 M gradient of TEAB. Fractions contained products were pooled and evaporated. Products were obtained as TEA salts:

3'-O-methyl-N^2-benzyl-7-methylguanosine-5'-monophosphate (6): 19.3 mg(0.04 mmol, 47%); ^{1}H NMR (400 MHz, D_2O) δ 7.46 – 7.30 (m, 5H, Ph), 6,03 (d, 1H, H-1'), 4,75 – 4.72 (m, 1H, H-2'), 4.63 (d, 2H, CH_2Ph), 4,43 – 4,39 (m, 1H, H-3'), 4,24 – 4,22 (m, 1H, H-4'), 4,20 – 4,18 (m, 1H, H-5'), 4,07 (s, 3H, CH_3), 4,06 – 4,03 (m, 1H, H-5''), 3.41 (s, 3H, 3'O-CH_3), ^{31}P NMR (283 MHz, D_2O) 0,70;
3'-O-methyl-N^2-(4-metoxybenzyl)-7-methylguanosine-5'-monophosphate (7): 18.7 mg (0.03 mmol, 37%); ^{1}H NMR (400 MHz, D_2O) δ 9.01 (s, 1H, H-8'), 7.41 (d, 2H, Ph), 7,01 (d, 2H, Ph), 6,05 (d, 1H, H-1'), ca. 4.78 (signal for H-2' overlapped by water), 4.58 (d, 2H, CH_2Ph), 4.44 – 4,42 (m, 1H, H-3'), 4.27 – 4.26 (m, 1H, H-4'), 4,25 – 4,24 (m, 1H, H-5'), 4,09

(s, 3H, CH_3), 4.08 – 4,05 (m, 1H, H-5”), 3.82 (s, 3H, $PhOCH_3$), 3.43 (s, 3H, 3’O-CH_3), ^{31}P NMR (283 MHz, D_2O) 0,63;

2’-O-methyl-*N*^{2}-benzyl-7-methylguanosine-5’-monophosphate (8): 9.1 mg, (0.02 mmol, 54%); ^1H NMR (400 MHz, D_2O) δ 8.11 (s, 1H, H-8’), 7.46 – 7.33 (m, 5H, Ph), 5,99 (d, 1H, H-1’), 4.65 (d, 2H, CH_2Ph), 4.47 (t, 1H, H-2’), 4.34-4,33 (m, 1H, H-3’), 4.27 – 4.21 (m, 1H, H-4’), 4.10 – 4.07 (m, 1H, H-5’), 4,11 (s, 3H, CH_3), 4.03 – 3.99 (m, 1H, H-5’’), 3.24 (s, 3H, 2’O-CH_3), ^{31}P NMR (283 MHz, D_2O) 0,76;

Procedure for the preparation of dinucleotide cap analogs:

Guanosine 5’-diphosphate imidazolide (20 mg, 0.04 mmol) and $ZnCl_2$ (55 mg, 0.4 mmol) were stirred in anhydrous DMF (0.8 mL) with proper N2-modified ARCA nucleotides **6, 7** or **8** (18 mg, 0.04 mmol) at RT for 24 hours. The reactions were quenched with aqueous solution of EDTA (15 mg, 0.05 mmol in 1 mL) and purified by ion-chromatography on DEAE Sephadex with linear 0- 1.0 M gradient of TEAB. Fractions contained product were pooled and evaporated. Products were additionally purified by HPLC to give products as ammonium salts:

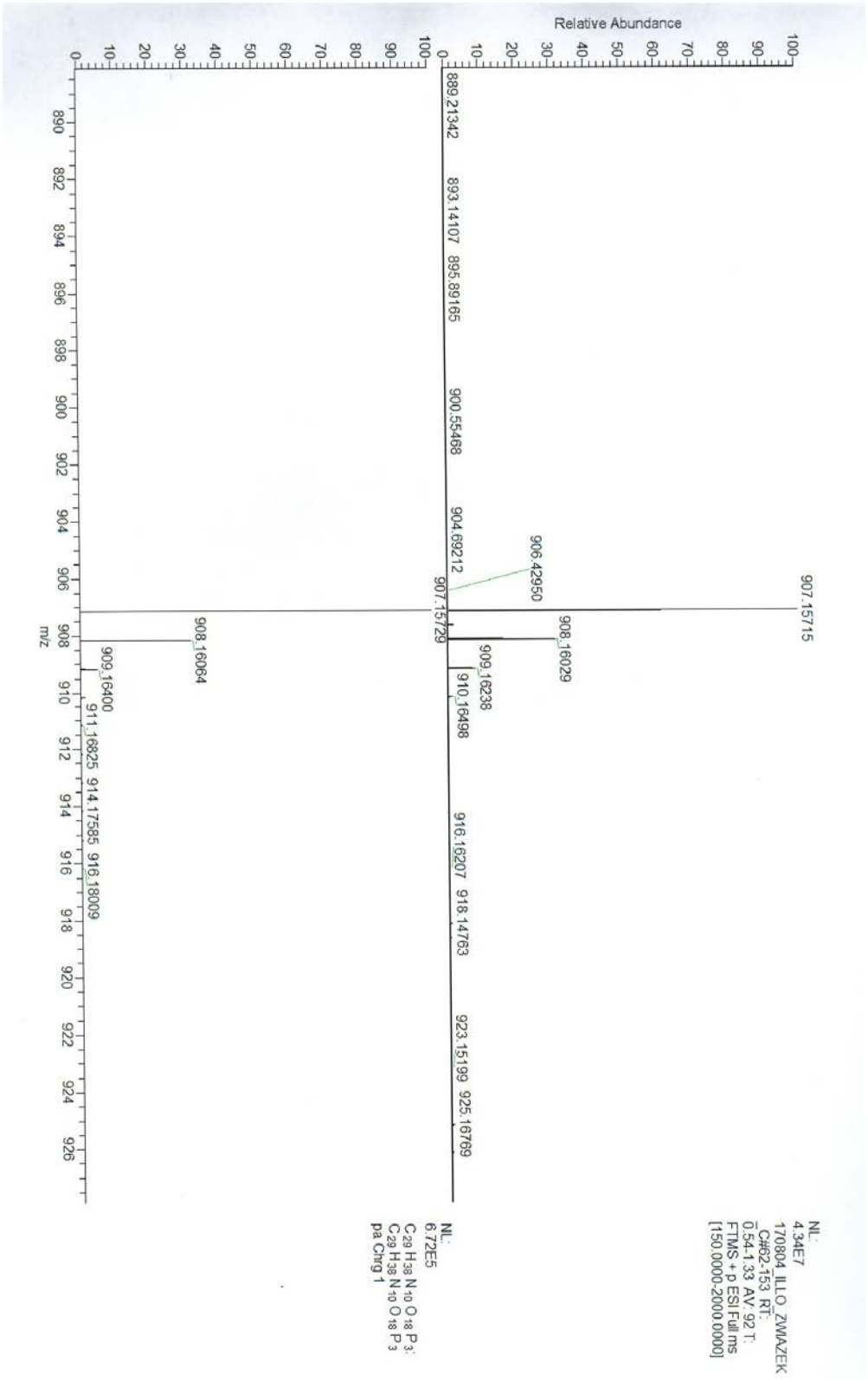
P^1-3’-O-methyl-N^2-benzyl-7-methylguanosine-5’-P^3-guanosine- 5’-triphosphate (9) 11,3 mg (0.012 mmol, 35%), R_T 21.5 min.; ^1H NMR (600 MHz, D_2O) δ 7.99 (s, 1H, H-8 (G)), 7.45–7.32 (m, 5H, Ph), 5.86 (s, 1H, H-1’(m^7G)), 5.78 (d, 1H, H-1’(G)), 4,70 – 4.65 (m, 1H, H-2’(G)), 4.64–4.60 (m, 2H, $PHCH_2$) , 4,59 – 4,52 (m, 3H, H-2’ (m^7G), H-3’(G), H-3’ (m^7G)), 4,47 – 4,36 (m, 4H, H-4’(G), H-4’ (m^7G), H-5’(G), H-5’ (m^7G)), 4,34 – 4,30 (m, 1H, H-5’’(G)), 4,06 (s, 3H, N7-CH_3), 4,01 – 3,98 (m, 1H, H-5” (m^7G)), 3,41 (s, 3H, 3’O-CH_3); ^{31}P NMR (283 MHz, D_2O) δ -14,38 (2P, $P_{\alpha,\gamma}$), -25,75 (1P, P_β).

P^1-3’-O-methyl-N^2-(4-metoxybenzyl)-7-methylguanosine-5’-P^3-guanosine-5’-triphosphate (10) 8 mg (0.09 mmol, 29%) R_T 23.0 min.; ^1H NMR (600 MHz, D_2O) δ 8.98 (s, 1H, H-8, (m^7G)) 8,00 (s, 1H, H-8 (G)), 7.38 (d, 2H, Ph), 6,98 (d, 2H, Ph) 5.90 (d, 1H, H-1’(m^7G)), 5.77 (d, 1H, H-1’(G)), 4,65 - 4,62, (m, 2H, $PhCH_2$), 4.61–4.57 (m, 1H, H-2’(G)) 4,49 – 4,41 (m, 4H, H-2’ (m^7G), H-3’(G), H-3’ (m^7G), H-4’(G)), 4.33–4.28 (m, 2H, H-4’ (m^7G), H-5’(G)), 4,25 – 4,18 (m, 2H, H-5’ (m^7G), H-5’’(G)), 4,06 (s, 3H, N7-CH_3), 4,04 – 4,02 (m. 1H, H-5” (m^7G)), 3,81 (s, 3H, $PhOCH_3$), 3,43 (s, 3H, 3’O-CH_3); ^{31}P NMR (283 MHz, D_2O) δ -14,38 (2P, $P_{\alpha,\gamma}$), -25,90 (1P, P_β).

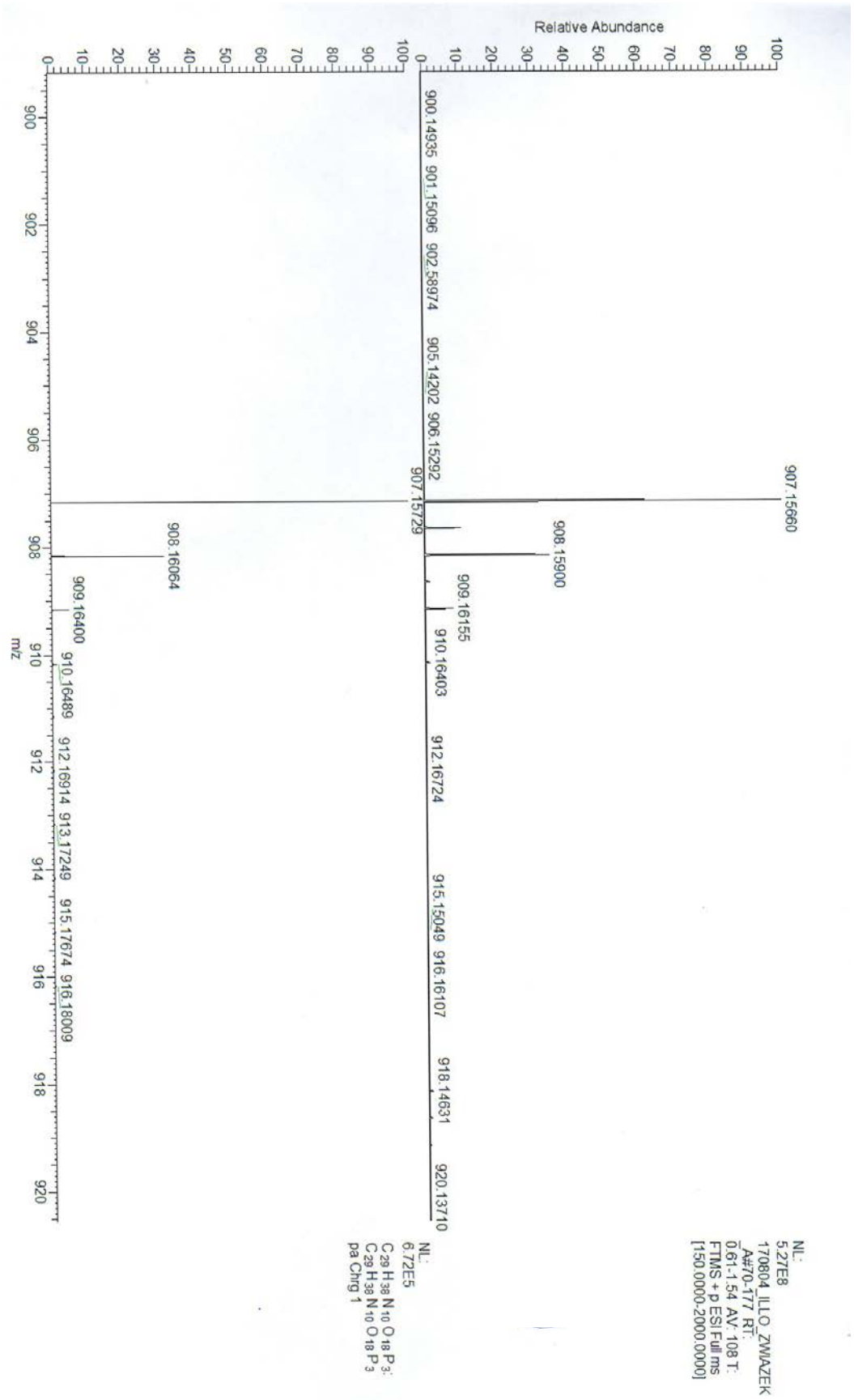
P^1-2'-O-methyl-N^2-benzyl-7-methylguanosine-5'-P^3-guanosine- 5'-triphosphate (11) 9 mg (0.01 mmol, 53%) R_T 21.3 min.; ^{1}H NMR (600 MHz, D_2O) δ 8.94 (s, 1H, H-8, (m^7G)) 7.99 (s, 1H, H-8 (G)), 7.40–7.30 (m, 5H, Ph), 5.86 (s, 1H, H-1'(m^7G)), 5.76 (d, 1H, H-1'(G)), 4.72–4.68 (m, 1H, H-2' (m^7G)), 4,62-4,54 (m, 3H, $PhCH_2$, H-2'(G)), 4.47–4.42 (m, 2H, H-3'(G), H-3' (m^7G)), 4,38 – 4,35 (m, 1H, H-4'(G)), 4,33 – 4,30 (m, 1H, H-4' (m^7G)), 4,28 – 4,20 (m. 3H, H-5'(G), H-5' (m^7G), H-5''(G)), 4,06 (s, 3H, N7-CH_3), 4,02-4,00 (m, 1H, H-5" (m^7G)), 3,29 (s, 3H, 2'O-CH_3); ^{31}P NMR (283 MHz, D_2O) δ -14,65 (2P, $P_{\alpha,\gamma}$), -26,13 (1P, P_β).

Mass spectra of the final compounds

P[1]-3'-O-methyl-N[2]-benzyl-7-methylguanosine-5'-P[3]-guanosine- 5'-triphosphate (9)



P[1]-2'-O-methyl-N[2]-benzyl-7-methylguanosine-5'-P[3]-guanosine- 5'-triphosphate (11)



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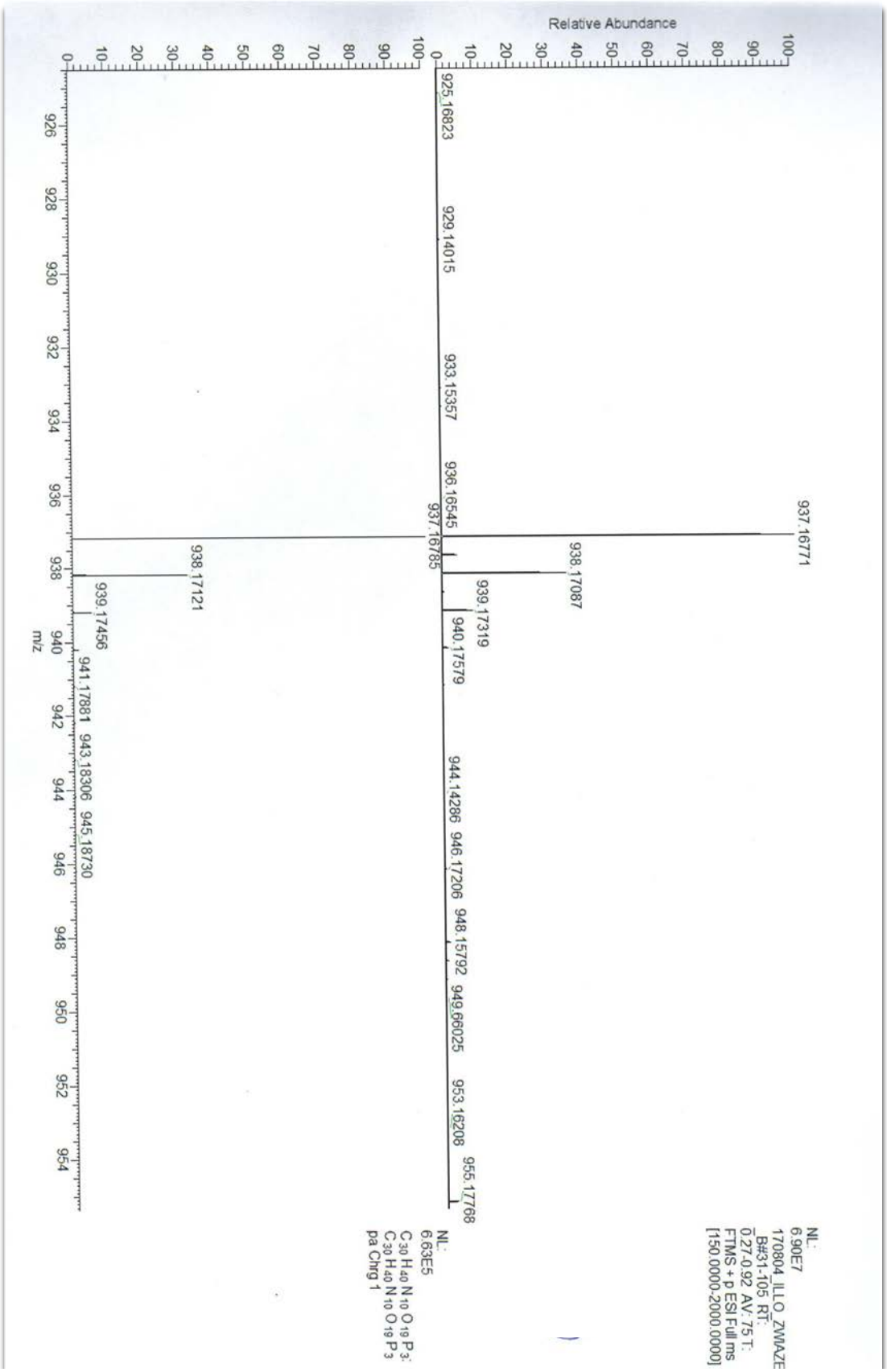
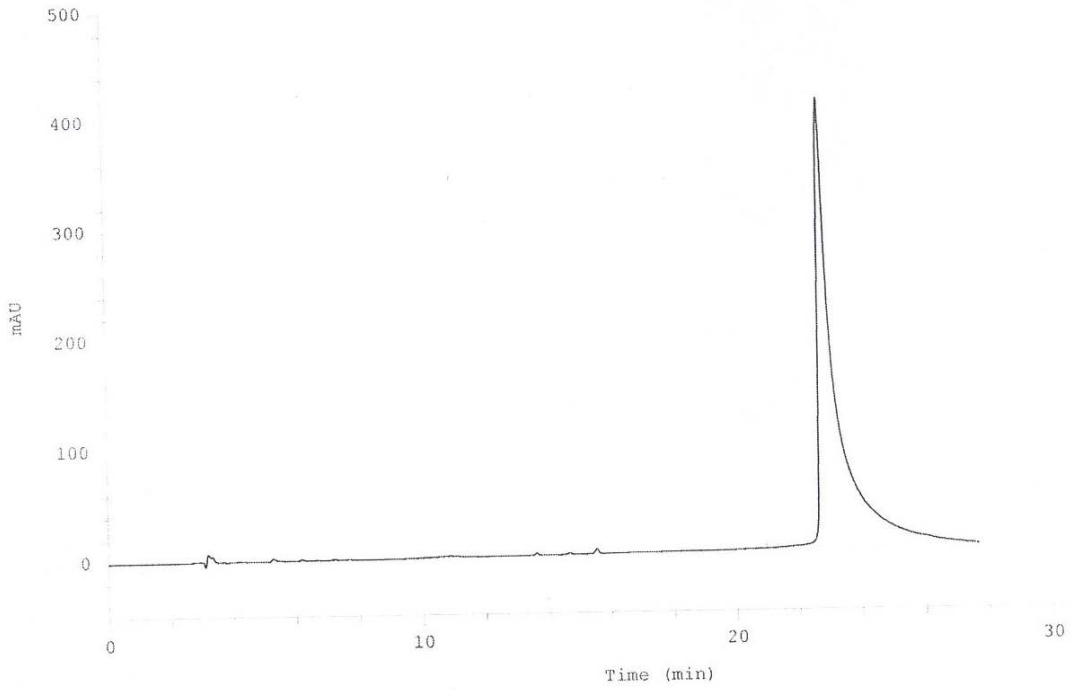
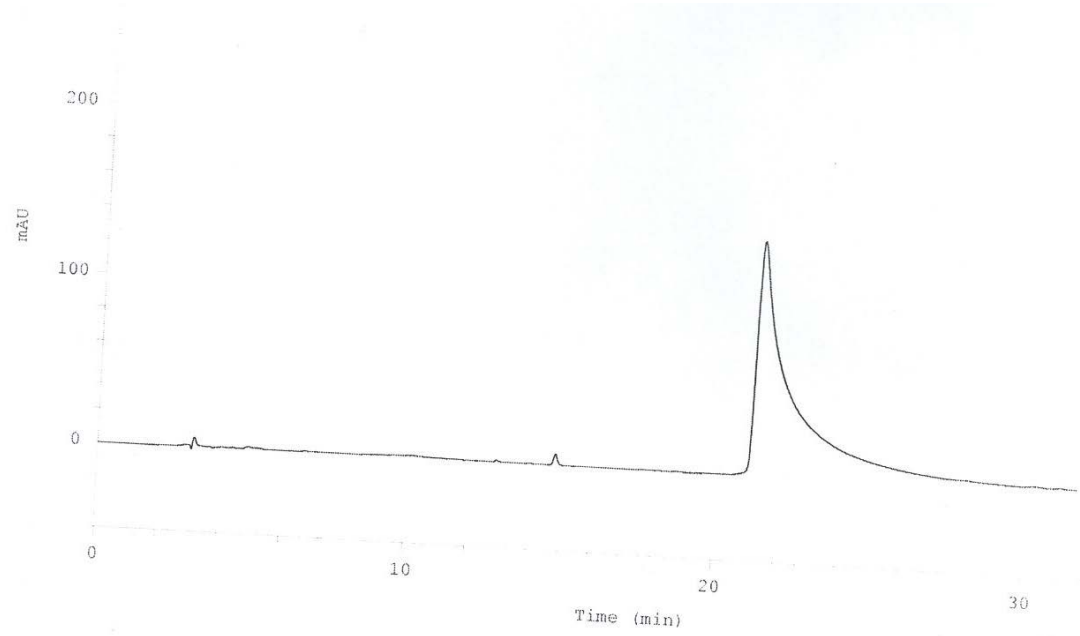


HPLC traces

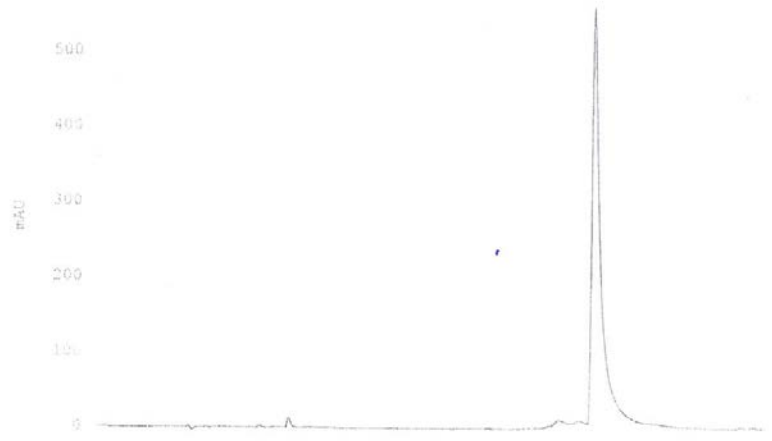
P^1-3'-O-methyl-N^2-benzyl-7-methylguanosine-5'-P^3-guanosine- 5'-triphosphate (9)



P^1-3'-O-methyl-N^2-(4-metoxybenzyl)-7-methylguanosine-5'-P^3-guanosine-5'-triphosphate (10)



P[1]-2'-O-methyl-N[2]-benzyl-7-methylguanosine-5'-P[3]-guanosine- 5'-triphosphate (11)



NMR spectra of the final compounds

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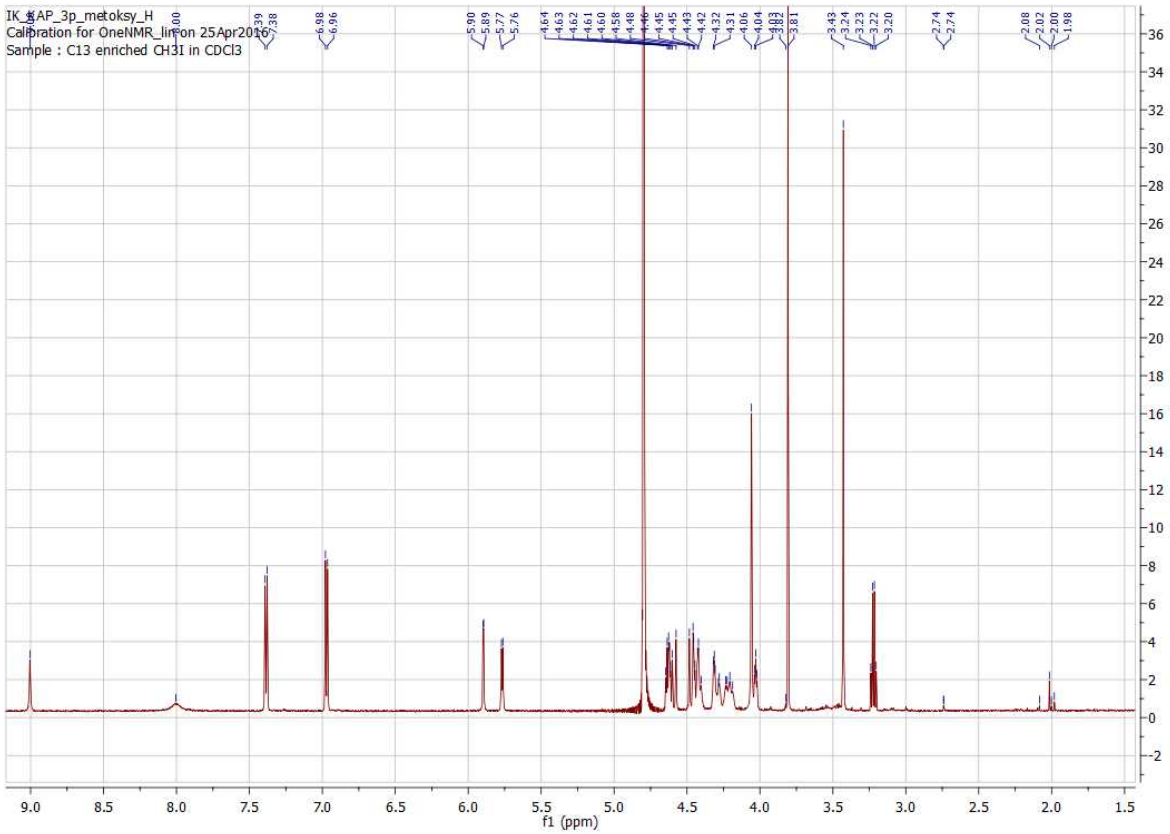
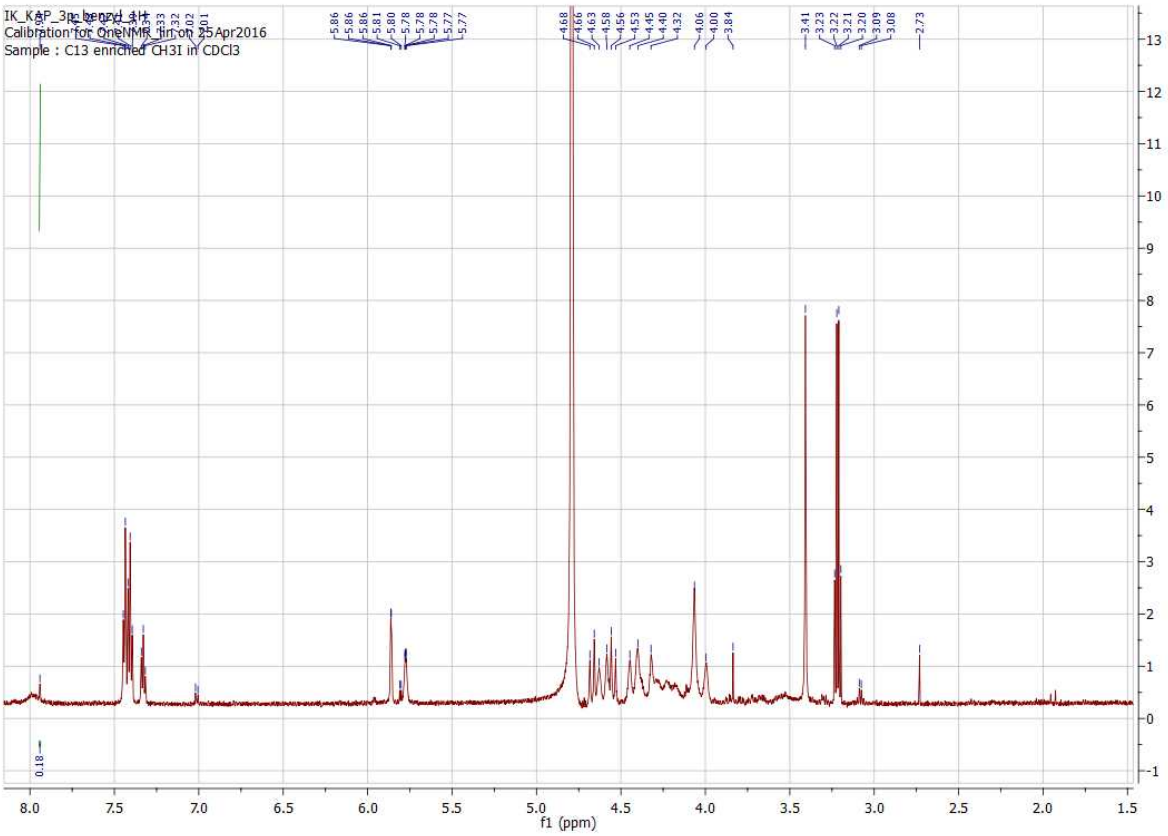


P1-3’-O-methyl-N2-benzyl-7-methylguanosine-5’-P3-guanosine- 5’-triphosphate (9)



P1-2’-O-methyl-N2-benzyl-7-methylguanosine-5’-P3-guanosine- 5’-triphosphate (11)