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Synthesis of N^2-modified 7-methylguanosine 5′-monophosphates as nematode translation inhibitors

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ABSTRACT

Preparative scale synthesis of 14 new N^2-modified mononucleotide 5′ mRNA cap analogues was achieved. The key step involved use of an S_NAr reaction with protected 2-fluoro inosine and various primary and secondary amines. The derivatives were tested in a parasitic nematode, *Ascaris suum*, cell-free system as translation inhibitors. The most effective compound with IC_{50} ~0.9 μM was a N^2-*p*-metoxybenzyl-7-methylguanosine-5′-monophosphate **35**.

1. Introduction

Methylation of the exocyclic amino group of guanosine is an important biological modification of RNAs. Modified nucleosides occur frequently in RNAs including N^2-methyl-(m^2G) and N^2,N^2-dimethylguanosine ($m_2^{2,2}G$) within ribosomal (rRNA)[1] and transfer (tRNA)[2] RNAs, respectively. N^2,N^2-dimethylG as a part of the RNA cap structure is also found at the 5′ end of small nuclear RNAs (U snRNA) and some messenger RNAs (mRNA). Eukaryotic mRNAs possess a 5′ terminal cap structure consisting of a 7-methylguanosine linked via a 5′,5′-triphosphate bridge to the first transcribed nucleotide (m^7GpppN, where N=G, A, C or U; MMG cap).[3] In nematodes, however, two different mRNAs co-exist in cells. In addition to mRNAs with the common MMG cap, about 70% of the mRNAs possess an atypical, hypermethylated cap. This cap (TMG-cap, $m_3^{2,2,7}$GpppG) has the N^7-methyl as well as N^2,N^2-dimethyl in its structure and is added along with a 22 nt spliced leader sequence during trans-splicing.[4]

The cap plays an essential role in several processes during gene expression[5] by interacting with proteins that specifically recognize its structure.[6] The best-characterized cap-binding protein is the translation initiation factor eIF4E. Recognition of the mRNA cap by eIF4E is the critical, rate limiting step for efficient translation initiation, and it is a major target for translational control.[7] It has been shown that eIF4E is a potent oncogene and its overexpression is associated with a variety of human cancers.[8] Consequently, cap analogs as specific inhibitors to counteract elevated eIF4E level in tumor cells have been explored.[9]

Many nematodes, including *Ascaris suum*, are parasitic, infect over 2 billion people, and remain a significant health problem. The role of trans-splicing as a mechanism of nematode gene expression and the effect of the TMG cap and a spliced leader addition on mRNA metabolism have been intensively investigated.[10] Ascaris cap-binding proteins must deal with two distinct populations of mRNA suggesting these proteins are unique and may be targets for the discovery of novel cap analogs that can specifically block parasite gene expression. We recently prepared and analyzed a guanosine derivative with a benzyl at N^2 position (bn^2m^7GMP, **32**) to examine the intermolecular interaction of MMG/TMG caps with Ascaris eIF4E-3.[11] The addition of an N^2-benzyl substituent on a monophosphate led to significant translation inhibition in an *Ascaris suum* embryo cell-free system. Therefore, we chose to extend these analyses to several other N^2 modified derivatives.

In the present study we designed and synthesized a series of N^2-substituted 7-methylguanosine 5′-monophosphates. This type of cap analog has not been widely studied due to difficulties with the development of a good method to efficiently introduce substituents at the N^2 position of guanosine. To achieve our goal we used a six step strategy starting from guanosine using an S_NAr reaction with fully protected 2-fluoro inosine and various primary and secondary amines. The synthesized mononucleotide TMG cap analogues were tested in a parasitic nematode, *Ascaris suum*, cell-free system as translation inhibitors.

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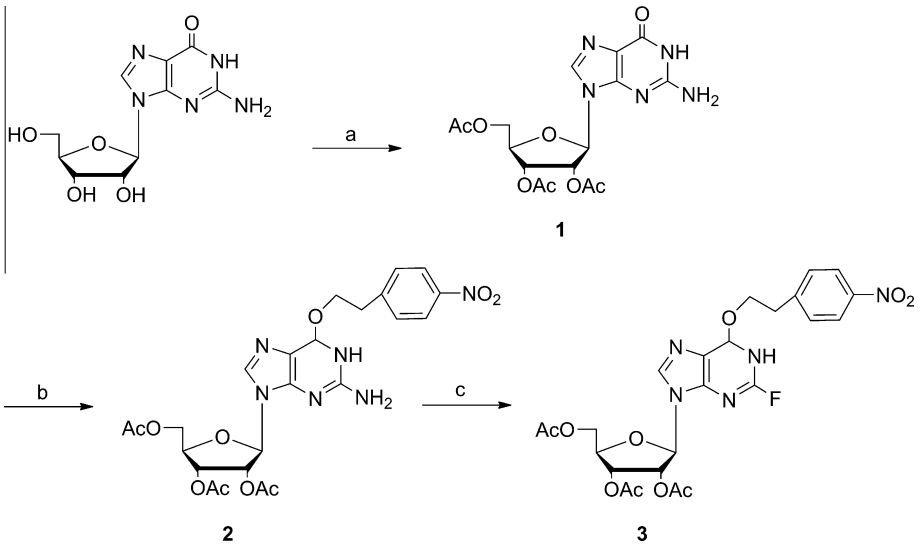
2. Results and discussion

2.1. Chemistry

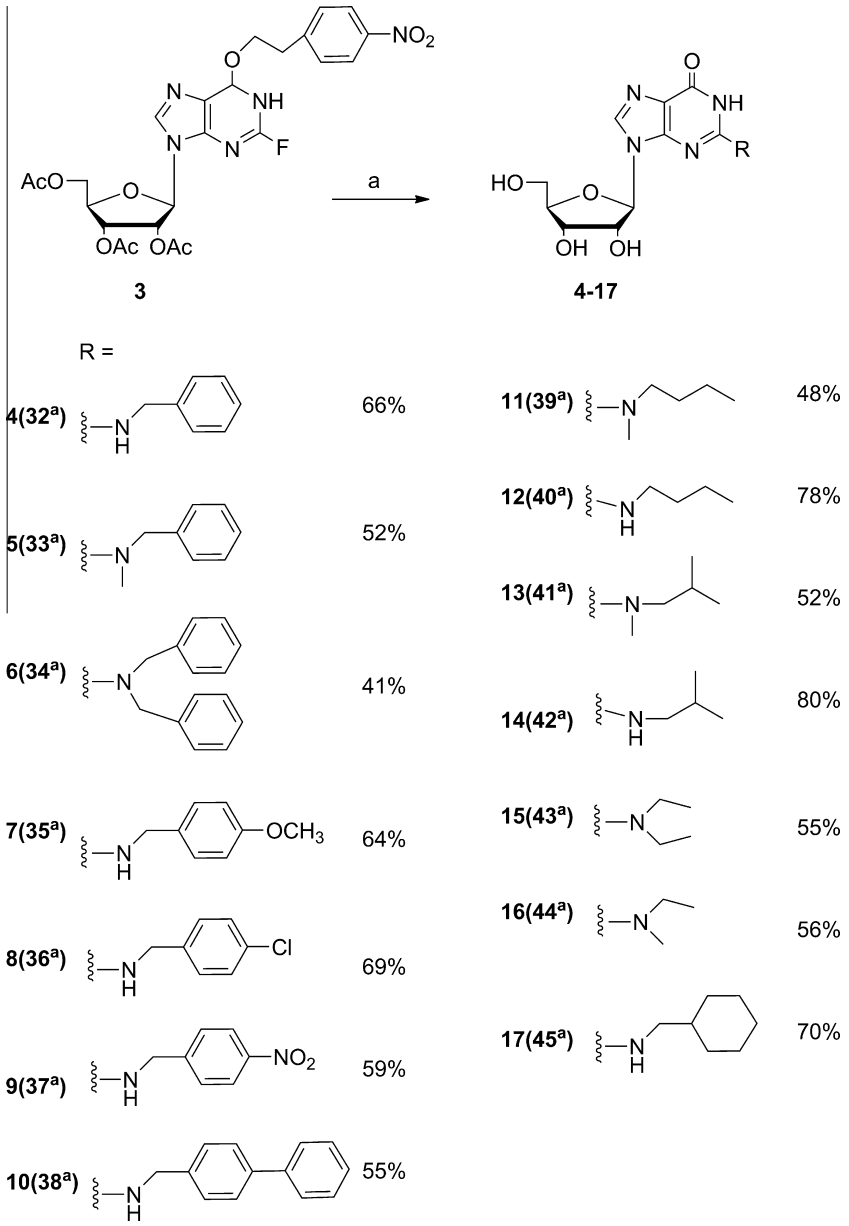
In order to synthesize N^2-substituted mononucleotide cap analogues, we adopted and modified a method for the preparation of the N^2,N^2-dimethylguanosine by Eritja and co-workers.[12] The synthesis was carried out in six steps using inexpensive, commercially available guanosine as the starting material (Scheme 1). The synthesis began with the triacetylation of guanosine using acetic anhydride (Ac_2O) in the presence of triethylamine (TEA) and *N*,*N*-(dimethylamino)pyridine (DMAP) using a procedure modified from Nair et al.[13] By carrying out the addition of the Ac_2O at 0 °C, *N*-acetylation was suppressed and 2′,3′,5′-tri-*O*-acetylguanosine (**1**) was obtained in a near quantitative yield (98%). In the next step, protection of the O^6 group of guanosine was carried out with a *p*-nitrophenylethyl (NPE) group as it is believed to be stable under mild acid and base hydrolyzes, and it can be easily cleaved by DBU or DBN in a β-elimination reaction. The reaction was carried out via the Mitsunobu reaction with *p*-nitrophenylethanol, diisopropyl azodicarboxylate (DIAD), and triphenylphosphine (TPP) in anhydrous toluene at room temperature.[14] A long standing problem with the Mitsunobu reaction is the purification of the desired product from the reaction mixture. We have tried to replace triphenylphosphine and diisopropyl azodicarboxylate (which are converted during the reaction into triphenylphosphine oxide and related dialkylhydrazinodicarboxylate, respectively) by other reagents to facilitate the isolation and purification processes.[15] Our observations showed that obtaining pure product was possible using standard reagents but the purification required a very long chromatography process. In order to shorten the isolation of the 2′,3′,5′-tri-*O*-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanosine (2), we partially purified the final product and then used **2** with some impurities (mainly triphenylphosphine oxide) in the next step. By-products remaining after the Mitsunobu reaction were easily removed during the purification of **3**. This faster procedure allowed us to obtain a pure fluorinated derivative with good yield (85%).

The main route to N^2-substituted guanosine analogues is via nucleophilic displacement of a halogen at the 2 position of inosine. Since fluoro derivatives are more reactive toward nucleophilic displacement in a S_NAr reaction than other halogen derivatives,[16] the fully protected 2′,3′,5′-tri-*O*-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanosine (**2**) was transformed into N^2-fluoro-2′,3′,5′-*O*-triacetyl-O^6-[2-(4-nitrophenyl)ethyl]inosine (**3**). We tested three methods for diazotiation and fluorination of nucleosides. The first was to prepare the 2-fluoro derivative using aqueous diazotization of guanosine in the presence of fluoroboric acid.[17] These conditions, however, were too harsh and led to depurination. A second approach was the introduction of the fluorine atom under anhydrous conditions with *t*-butyl nitrite as the diazotizing agent and HF in pyridine as the fluoride source.[18] The reaction proceeded smoothly in very good yield (85%), and the main product remained fully protected. However, without careful monitoring, this reaction led to depurination or even failure to obtain product unless very high quality HF/pyridine reagent was used and a low temperature was maintained during the course of the reaction. Consequently, we also explored milder fluorination conditions using polyvinylpyridinium polyhydrogenfluoride (PVPHF) reagent developed by Olah and Li.[19] The advantages of this method were easy handling and an extremely convenient work-up, however, yields were limiting. Consequently, the most efficient way to prepare N^2-fluoro-2′,3′,5′-*O*-triacetyl-O^6-[2-(4-nitrophenyl)ethyl]inosine (**3**) is the procedure that involves using HF/pyridine for the substitution step.

The 2-fluoro intermediate was used directly in a S_NAr reaction by treating it with two-fold excess of primary or secondary, aliphatic or aromatic amines varying in size and steric branching in anhydrous DMSO (Scheme 2). Incorporated amines were chosen to produce several N^2 substituents (aliphatic, cyclic, or aromatic) in the cap to vary the size and steric branching of aliphatic N^2 substituents (compounds **11**–**17**) and to influence the electron density of the aromatic ring through the introduction of various substituents, such as metoxy (**7**), chloro (**8**), nitro (**9**), or phenyl (**10**), into the benzene ring. In addition to the mono substituted analogues, we also used some secondary amines and synthesized compounds bearing additional methyl (**5**, **11**, **13**, **16**), ethyl (**15**) or benzyl (**6**) groups at the N^2 position to check the effectiveness of these substituents on the cap as translation inhibitors. The nature of the nucleophile is the most critical parameter in the S_NAr reaction. Therefore, for the simple primary amines, the substitution reaction occurred rapidly and the fluorine derivative was used up in less than 30 min at temperature not higher than 50 °C. The reaction with sterically hindered and secondary amines such as dibenzylamine required longer time (in some cases even 2 days) and a



Scheme 1. Preparation of N^2-fluoro-2′,3′,5′-*O*-triacetyl-O^6-[2-(4-nitrophenyl)ethyl]inosine; (a) acetic anhydride, DMAP, Et_3N, AcCN, 4 °C to rt; (b) NPE, PPh_3, DIAD, toluene, rt; (c) HF/pyridine, *t*BuONO, pyridine, −40 °C.



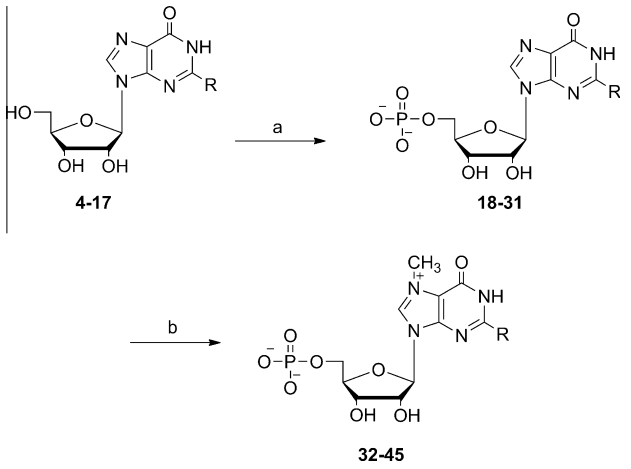
Scheme 2. Preparation of N^2-modified guanosine derivatives via a S_NAr reaction; (a) amine, DMSO, 50 °C to 65 °C. [a]Numbers in brackets correspond to compounds on Scheme 3.

higher temperature (65 °C). After completion of the substitution reaction 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was added to the reaction mixture to remove the NPE group followed by the addition of a mixture of THF/MeOH/$NaOH_{aq}$ in order to complete acetyl deprotection. Chromatographic purification on silica gel yielded pure nucleoside derivatives **4**–**17** in good yield. It is worth mentioning, that in course of the reaction progress, we observed that all protecting groups (acetyl and NPE) were slowly lost if the substitution reaction was carried out under prolonged heating in the presence of excess amine. This observation led us to a simpler purification procedure in which full deprotection took place without any DBU and final N^2-substituted guanosine derivatives were easily crystallized from the reaction mixture by the addition of CH_2Cl_2.

All the N^2-modified guanosine analogues were further 5′ phosphorylated using the Yoshikawa method[20] with phosphorus oxide trichloride in trimethyl phosphate at 4 °C and subsequently methylated at the N^7 position of the guanine ring with CH_3I in DMSO at RT[21] (Scheme 3). The reaction progress of the last step was monitored by HPLC to avoid over methylation. Purification of the analogues was done using ion-exchange chromatography on DEAE–Sephadex A-25 (HCO_3^- form) and the derivatives were subsequently converted into the sodium salt using Dowex 50WX8. The structure and homogeneity of final products were confirmed by HPLC, mass spectrometry, ^1H NMR and ^{31}P NMR.

2.2. Biology

One of the synthesized compounds, bz^2m^7GMP (**32**), has been recently used in *Ascaris suum* eIF4E-3/m^7G- and $m_3^{2,2,7}G$-cap binding studies.[11] It was shown that bz^2m^7GMP binds to the protein with a 10-fold higher affinity compared with m^7GMP and is a strong inhibitor of either a m^7G- or $m_3^{2,2,7}G$-capped mRNA translation in a cell-free *Ascaris suum* translation system. To evaluate the ability of newly synthesized N^2-modified 7-methylguanosine 5′-monophosphates to inhibit cap-dependent translation in a cell-free



Scheme 3. Preparation of N^2-modified mononucleotide cap analogues (a) $POCl_3$, trimethyl phosphate, 4 °C; (b) methyl iodide, DMSO, rt. [a]R corresponds to various substituents at the N2 position of guanosine (see Scheme 2, numbers in brackets).

translation system where naturally occurring mono- and trimethylated mRNAs are translated, we tested 14 cap analogues (**32**–**45**) in the same manner as previously reported.[10c,11] Two standard cap analogues were used as internal controls in the experiments: m^7GTP as a positive control as it is an effective translation inhibitor in various cell-free systems (rabbit reticulocyte,[9a] wheat germ,[23] *Ascaris suum*[10c]) and ApppG as a negative control that have very little or no inhibitory efficacy due to lack of the positive charge on guanine that is generated by the N^7 methyl group. As previously shown, the inhibitory potency of cap analogues generally increases with the length of polyphosphate bridge.[9a] Therefore, in order to determine the strength of our new mononucleotide cap analogues we also compared them to m^7GMP which is known as a weak translation inhibitor. The results (Fig. 1, Table 1) indicated that any mono substitution at the N^2 position of guanine (alkyl–aliphatic, cyclic, or aryl) produced inhibitory compounds with IC_{50} similar to m^7GTP. All alkyl substituents led to similar levels of inhibition regardless of their size and steric branching. Aryl substituted compounds were more effective as translation inhibitors. The strongest inhibitor, about 7 times stronger than m^7GTP, was derivative **35** with an IC_{50} of 0.9 μM. The electron withdrawing properties of *para* substituents (NO_2 > Cl > Phe > OCH_3) were roughly correlated with the IC_{50} of the compounds (12.6 μM, 3.8 μM, 4.3 μM, 0.9 μM, respectively). The enhanced π electron density of the benzene ring increases the effectiveness of cap analogues as translational inhibitors, but other factors such as the size of substituent may also play an important role. Recent studies on the co-crystal structure of $m_3^{2,2,7}$GTP with Ascaris eIF4E-3[11] indicated that the two methyl groups at the N^2 position of the base are solvent exposed and do not interact directly with the protein. The N^2 substituents may form additional interactions with eIF4E influencing conformational changes known to form the binding pocket[24] or may change the thermodynamic aspects of the protein binding to the 5′ cap.

Our results also showed that the addition of a second substituent at the N^2 position of guanosine decreased the level of inhibition significantly (e.g. compounds **32** versus **33** or **39** versus **40**). These data are consistent with a comparison of the crystal structures of Ascaris eIF4E-3 with m^7GTP or $m_3^{2,2,7}$GTP.[11] These studies

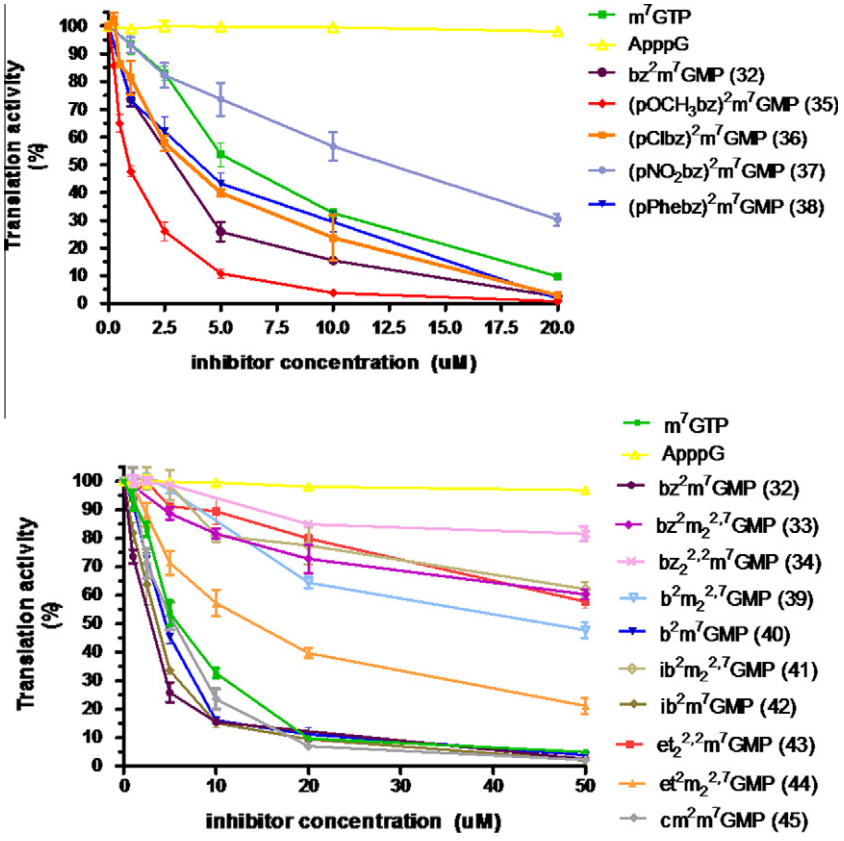
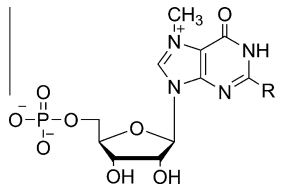
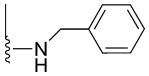
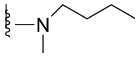
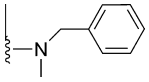
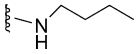
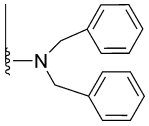
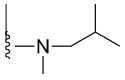
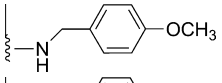
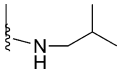
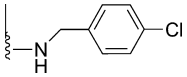
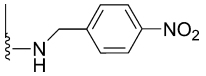
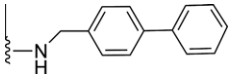
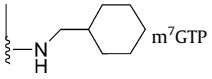


Figure 1. Translation inhibition assay in *A. suum* extract. The synthesized cap analogues were assayed for their ability to inhibit cap-dependent translation of an m^7GpppG-capped *Renilla* luciferase mRNA in an *Ascaris suum* embryo cell-free translation system. [22]The measurements were carried out as previously described and the% translation activity plotted against the inhibitor concentration.[10c] All measurements were made in triplicate in several preparations of extracts. Data presented are representative experiments.

Table 1
Inhibition of translation in *A. suum* extract by N^2-modified cap analogues



	R =	IC_{50} (μM)		R =	IC_{50} (μM)
32		2.9	**39**		>50
33		>50	**40**		3.9
34		>50	**41**		>50
35		0.9	**42**		4.5
36		3.8	**43**		>50
37		12.6	**44**		13.9
38		4.3	**45**		5.7
				m^7GTP	6.1
				m^7GMP	>50

IC_{50} values were extracted directly from the plotted of % translation activity values against compound concentration. IC_{50} values >50 μM—no measurable effect.

demonstrated that the addition of the second substituent at the N^2 position of guanosine leads to the loss of one hydrogen bond between the amine group of guanine and the carboxyl group of Glu116. This loss of a hydrogen bond likely influences the effectiveness of analogues as translation inhibitors.

3. Conclusions

We have prepared 14 novel N^2 modified trimethylguanosine cap analogues *via* a six-step synthesis from guanosine. This general strategy provides a new approach to scale-up the synthesis of a large number of modified cap analogues that should be useful in studying eIF4E function and cap-dependent translation. In addition the idea of exploring cap analogues that possess only one phosphate that typically have little inhibitory activity offers an opportunity to explore compounds that are not highly charged and can be good candidates for new drug development.

4. Materials and methods

All reagents were the highest available purity and purchased from Sigma–Aldrich Chemical Co. Triethylammonium bicarbonate (TEAB) buffer was prepared by bubbling CO_2 through an ice-cold aqueous solution of redistilled triethylamine. Intermediate nucleotides 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 and evaporated under reduced pressure with several additions of ethanol and isolated as triethylammonium salts (TEA salts) and subsequently converted into the sodium salt using Dowex 50WX8 (Na^+ form). Homogeneity of the final analogues was checked by reversed-phase analytical HPLC. HPLC was performed using a Supelcosil LC-18-T RP column (4.6 × 250 mm, flow rate 1.0 mL/min) with: Method A — a linear gradient of methanol from 0% to 50% (v/v) in 0.05 M ammonium acetate (pH 5.9) in 20 min, an isocratic elution of 50% methanol (v/v) in 0.05 M ammonium acetate (pH 5.9) till 30 min, Method B — a linear gradient of methanol from 0% to 50% (v/v) in 0.05 M ammonium acetate (pH 5.9) in 10 min and then an isocratic elution of 50% methanol (v/v) in 0.05 M ammonium acetate (pH 5.9) till 30 min, Method C — an isocratic elution of 50% methanol (v/v) in 0.05 M ammonium acetate (pH 5.9), on a Knauer instrument, with UV detection at 254 nm. MS spectra were acquired using Waters Micromass Q-TOF Premier spectrometer with positive electrospray ionization source. ^1H and ^{13}C NMR spectra of intermediate derivatives were obtained with a Varian UnityPlus 200 MHz spectrometer. ^1H and ^{31}P NMR spectra of the final compounds were recorded on a Varian INOVA 700 MHz spectrometer.

4.1. Experimental

4.1.1. 2′,3′,5′-Tri-*O*-acetylguanosine (1)

2.2 mL of acetic anhydride (22 mmol) was added dropwise to a suspension of guanosine (2 g, 7.0 mmol) (dried for 2 days over P_4O_{10} in high vacuum), triethylamine (7.7 mL, 55.2 mmol) and N,N-(dimethylamino)pyridine (92 mg, 0.75 mmol) in 27 mL of acetonitrile at 0 °C. The mixture was stirred until it became homogeneous and kept an additional 3 h at room temperature. The

reaction was quenched with methanol (2.3 mL). The volume was reduced to 1/3 using a rotary evaporator and diethyl ether was added dropwise to induce precipitation of a fine white powder. The product was collected by filtration, washed with diethyl ether, and then stirred for 2 hr with acetone (30 mL) at 50 °C. The filtrate produced 2.8 g (98%) of a fine white powder. ^{1}H NMR (200 MHz, DMSO) δ 10.54 (s, 1H, NH) 7.93 (s, 1H, H-8), 6.34 (br, 2H, NH_2), 5.98 (d, J = 6.1 Hz, 1H, H-1′), 5.79 (t, J = 5.8 Hz, 1H, H-2′), 5.51–5.48 (m, 1H, H-3′), 4.40–4.37 (m, 1H, H-4′), 4.33–4.31 (m, 1H, H-5′), 4.23–4.28 (m, 1H, H-5″), 2.11 (s, 3H, CH_3, acetyl), 2.05 (s, 3H, CH_3, acetyl), 2.04 (s, 3H, CH_3, acetyl); ^{13}C NMR (200 MHz, $CDCl_3$) δ 172.1 (C═O), 171.0 (C═O), 169.9 (C═O), 156.3 (C-6), 152.8 (C-2), 151.2 (C-4), 137.6 (C-8), 119.6 (C-5), 86.1 (C-1′), 82.3 (C-4′), 73.6 (C-2′), 72.3 (C-3′), 61.8 (C-5′), 21.1 (C, acetyl), 20.6 (C, acetyl), 20.2 (C, acetyl); m/z: calcd for $C_{18}H_{21}N_5O_5$ $(M+H)^+$: 410.1306, found: 410.1305.

4.1.2. 2′,3′,5′-Tri-*O*-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanosine (2)

A suspension of 2′,3′,5′-tri-*O*-acetylguanosine (2.37 g, 5.8 mmol), triphenylphosphine (2.28 g, 8.7 mmol) and 2-(4-nitrophenyl)ethanol (1.45 g, 8.7 mmol) in anhydrous toluene was stirred for 30 min and diisopropyl azodicarboxylate (1.4 mL) was added dropwise over a period of 45 min. The reaction mixture was kept for 12 h at rt. Then the solvent was evaporated and the residual oil was purified by column chromatography on silica gel with chloroform to produce a pure product of yellowish crystals, 2.26 g (70%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.17 (d, 2H, Ph), 7.72 (s, 1H, H-8), 7.49 (d, 2H, Ph), 6.05–5.91 (m, 2H, H-1′, H-2′), 5.87–5.75 (m, 1H, H-3′), 4.73 (t, J = 6.7 Hz, 2H, O–CH_2, NPE), 4.50–4.36 (m, 3H, H-4′, H-5′, H-5″), 3.28 (t, 6.7 2H, CH_2Ph, NPE), 2.14 (s, 3H, CH_3, acetyl), 2.09 (s, 3H, CH_3, acetyl), 2.08 (s, 3H, CH_3, acetyl); ^{13}C NMR (200 MHz, $CDCl_3$) δ 171.7 (C═O), 171.0 (C═O), 169.7 (C═O), 161.9, 155.5 (C-6), 154.5 (C-2), 148.8, 148.6, 138.9 (C-8), 129.3 (C, Ph), 124.8 (C, Ph), 113.6 (C-5), 86.8 (C-1′), 82.3 (C-4′), 73.4 (C-2′), 70.6 (C-3′), 67.1 (OCH_2, NPE), 62.5 (C-5′), 35.5 (CH_2Ph), 21.2 (C, acetyl), 20.6 (C, acetyl), 20.2 (C, acetyl); m/z: calcd for $C_{24}H_{26}N_6O_{10}$ $(M+H)^+$: 559.1783, found: 559.1784.

4.1.3. N^2-Fluoro-2′,3′,5′-*O*-triacetyl-O^6-[2-(4-nitrophenyl)ethyl] inosine (3)

Dry 2′,3′,5′-tri-*O*-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanosine (1 g, 1.79 mmol) in polypropylene tube under nitrogen was dissolved in anhydrous pyridine (6.75 mL, 0.082 mol). The tube was placed in a dry ice/acetonitrile cooling bath (−35 to −45 °C) and 70% HF/pyridine solution (12 mL, 0.42 mol) was added dropwise over a period of 5 min to 45% final HF. The reaction mixture was stirred for 15 min and *t*-butyl nitrite (0.54 mL, 4.5 mmol) was added. After 1hr, the reaction was quenched at 0 °C by slowly pouring the reaction mixture into an aqueous K_2CO_3 solution (28.5 g in 25 mL of water) and then extracted three times with ethyl acetate. The organic layers were collected, dried over anhydrous Na_2SO_4 and evaporated to dryness. Purification by column chromatography using as eluate 60:1 CH_2Cl_2: MeOH gave 0.85 g (85%) of product; TLC silica gel, CH_2Cl_2: MeOH, 60:1 R_F = 0.4; ^{1}H NMR (700 MHz, $CDCl_3$) δ 8.21–8.15 (m, 2H, Ph), 8.08 (s, 1H, H-8), 7.50 (d, 2H, Ph), 6.13 (d, J = 5.6 Hz, 1H, H-1′), 5.82 (t, J = 4.9 Hz, 1H, H-2′), 5.60–5.55 (m, 1H, H-3′), 4.84 (t, J = 6.7 Hz, 2H, O–CH_2, NPE), 4.46–4.42 (m, 2H, H-4′, H-5′), 4.38–4.36 (m, 1H, H-5″), 3.32 (t, J = 6.7 Hz, 2H, CH_2Ph, NPE), 2.15 (s, 6H, CH_3, acetyl), 2.08 (s, 3H, CH_3, acetyl). ^{13}C NMR (200 MHz, $CDCl_3$) δ 170.2 (C═O), 169.5 (C═O), 169.3 (C═O), 160.3, 159.3 (C-6), 154.2 (C-2), 147.1, 145.1, 140.8, 129.9 (Ph), 123.8 (Ph), 86.4 (C-1′), 80.5 (C-4′), 73.0 (C-2′), 72.4 (C-3′), 67.7 (OCH_2, NPE), 62.8 (C-5′), 34.9 (CH_2Ph), 20.7 (C, acetyl), 20.5 (C, acetyl), 20.3 (C, acetyl); m/z: calcd for $C_{24}H_{24}FN_5O_{10}$ $(M+H)^+$: 562.1579, found: 562.1581.

4.1.4. General procedure for the synthesis of N^2-substitutiuted derivatives 4-17

N^2-fluoro-2′,3′,5′-*O*-triacetyl-O^6-[2-(4-nitrophenyl)ethyl]inosine (250 mg, 0.45 mmol) was dissolved in 2 mL of anhydrous dimethylsulfoxide (DMSO) and then an amine (0.9 mmol) was added. The reaction mixture was stirred at 60 °C from 30 min to 2 days until the fluoronucleoside completely disappeared (based on TLC) followed by addition of 0.5 M NaOH in THF/MeOH/H_2O (5/4/2). The solvent was removed under high vacuum and the resulting oily residue was treated with CH_2Cl_2 to induce precipitation of a fine white powder. The product was collected by filtration and washed several times with methanol and dried over P_4O_{10} to yield:

4.1.4.1. N^2-Benzylguanosine (4). 134 mg, 66%; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.69 (s, 1H, H-8), 7.38–7.19 (m, 5H, Ph), 5.66 (d, J = 5.6 Hz, 1H, H-1′), 4.64–4.62 (m, 1H, H-2′), 4.55–4.47 (m, 2H, CH_2Ph), 4.12–4.10 (m, 1H, H-3′), 3.89–3.84 (m, 1H, H-4′), 3.69–3.57 (m, 2H, H-5′); ^{13}C NMR (50 MHz, $CDCl_3$) δ 157.4 (C-6), 153.8 (C-2), 150.6 (C-4), 140.4 (Ph) 134.6 (C-8), 128.0 (Ph), 127.2 (Ph), 126.2 (Ph), 117.1 (C-5), 87.5 (C-1′), 85.1 (C-4′), 73.0 (C-2′), 70.6 (C-3′), 61.7 (C-5′), 44.2 (N-CH_2Ph); m/z: calcd for $C_{17}H_{19}N_5O_5$ $(M+H)^+$: 374.1458, found: 374.1453.

4.1.4.2. N^2-Benzyl-N^2-methylguanosine (5). 109 mg, 52%; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.66 (s, 1H, H-8), 7.26–7.14 (m, 5H, Ph), 5.64 (d, J = 5.5 Hz, 1H, H-1′), 4.62–4.60 (m, 1H, H-2′), 4.50–4.42 (m, 2H, CH_2Ph), 4.13–4.11 (m, 1H, H-3′), 3.81–3.74 (m, 1H, H-4′), 3.62–3.51 (m, 2H, H-5′), 2.88 (s, 3H, N^7-CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 157.6 (C-6), 151.1 (C-2), 149.4 (C-4), 140.7, 137.2, 128.6 (Ph), 128.5 (Ph), 127.2 (Ph), 119.3 (C-5), 87.9 (C-1′), 84.6 (C-4′), 73.2 (C-2′), 71.9 (C-3′), 62.3 (C-5′), 44.8 (N-CH_2Ph), 37.0 (N-CH_3); m/z: calcd for $C_{18}H_{21}N_5O_5$ $(M+H)^+$: 388.1615, found: 388.1613.

4.1.4.3. N^2,N^2-Dibenzylguanosine (6). 103 mg, 41%,^{1}H NMR (200 MHz, $CDCl_3$) δ 7.59 (s, 1H, H-8), 7.36–7.29 (m, 10H, Ph), 5.67 (d, J = 5.6 Hz, 1H, H-1′), 4.68 (s, 4H, CH_2Ph), 4.61 (t, J = 5.0 Hz, 1H, H-2′), 4.16–4.13 (m, 1H, H-3′), 3.87–3.83 (m, 1H, H-4′), 3.60–3.43 (m, 2H, H-5′); ^{13}C NMR (50 MHz, $CDCl_3$) δ 155.8 (C-6), 152.2 (C-2), 150.3 (C-4), 139.8, 137.3, 128.5 (Ph), 128.2 (Ph), 127.1 (Ph), 119.6 (C-5), 88.9 (C-1′), 85.8 (C-4′), 74.4 (C-2′), 72.0 (C-3′), 62.3 (C-5′), 54.3 (N-CH_2Ph). m/z: calcd for $C_{24}H_{25}N_5O_5$ $(M+H)^+$: 464.1928, found: 464.1918.

4.1.4.4. N^2-*p*-Metoxybenzylguanosine (7). 140 mg, 64%,^{1}H NMR (200 MHz, $CDCl_3$) δ 8.02 (s, 1H, H-8), 7.27 (d, 2H, Ph), 6,85 (d, 2H, Ph), 5.92 (d, J = 5.4 Hz, 1H, H-1′), 4.73 (t, J = 4.9 Hz, 1H, H-2′), 4.48 (s, 2H, CH_2Ph) 4.21–4.18 (m, 1H, H-3′), 3.93–3.90 (m, 1H, H-4′), 3.72–3.68 (m, 2H, H-5′), 3,59 (s, 3H, O-CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 158.9 (C-6), 155.9, 154.1, 150.2, 137.8, 132.8, 127.9, 118.9, 113.9, 88.9 (C-1′), 86.1 (C-4′), 74.3 (C-2′), 72.0 (C-3′), 62.7 (C-5′), 56.0 (OCH_3), 44.6 (N-CH_2Ph). m/z: calcd for $C_{18}H_{21}N_5O_6$ $(M+H)^+$: 404.1564, found: 404.1560.

4.1.4.5. N^2-*p*-Chlorobenzylguanosine (8). 153 mg, 69%,^{1}H NMR (200 MHz, $CDCl_3$) δ 8.04 (s, 1H, H-8), 7.36–7.30 (m, 4H, Ph), 5.90 (d, J = 5.3 Hz, 1H, H-1′), 4.65 (t, J = 4.8 Hz, 1H, H-2′), 4.35 (s, 2H, CH_2Ph), 4.18–4.15 (m, 1H, H-3′), 3.91–3.88 (m, 1H, H-4′), 3.70–3.67 (m, 2H, H-5′); ^{13}C NMR (50 MHz, $CDCl_3$) δ 157.2 (C-6), 154.8 (C-2), 150.4 (C-4), 137.9, 136.7, 131.3, 129.2 (Ph), 128.3 (Ph), 119.8 (C-5), 88.6 (C-1′), 85.5 (C-4′), 74.3 (C-2′), 71.7 (C-3′), 62.4 (C-5′), 44.8 (N-CH_2Ph). m/z: calcd for $C_{17}H_{18}ClN_5O_5$ $(M+H)^+$: 408.1069, found: 408.1073.

4.1.4.6. N^2-*p*-Nitrobenzylguanosine (9). 134 mg, 59%,^{1}H NMR (200 MHz, $CDCl_3$) δ 8.04 (s, 1H, H-8), 8.22 (d, 2H, Ph), 7.63 (d, 2H,

Ph), 5.84 (d, J = 5.2 Hz, 1H, H-1′), 4.63 (s, 2H, CH_2Ph) 4.66–4.64 (m, 1H, H-2′), 4.18–4.15 (m, 1H, H-3′), 3.98–3.95 (m, 1H, H-4′), 3.75–3.71 (m, 2H, H-5′); ^{13}C NMR (50 MHz, $CDCl_3$) δ 156.6 (C-6), 153.7 (C-2), 150.9, 147.8, 146.4, 136.9 (C-8), 128.2 (Ph), 123.9 (Ph), 118.8 (C-5), 88.4 (C-1′), 86.8 (C-4′), 72.7 (C-2′), 71.2 (C-3′), 62.2 (C-5′), 43.8 (N-CH_2Ph). m/z: calcd for $C_{17}H_{18}N_6O_7$ $(M+H)^+$: 419. 1309, found: 419.1312.

4.1.4.7. N^2-*p*-Phenylbenzylguanosine (10). 134 mg, 55%,^{1}H NMR (200 MHz, $CDCl_3$) δ 7.98 (s, 1H, H-8), 7.75–7.71 (m, 9H, Ph), 5.89 (d, J = 5.3 Hz, 1H, H-1′), 4.69–4.65 (m, 2H, CH_2Ph), 4.61 (t, J = 4.9 Hz, 1H, H-2′), 4.16–4.14 (m, 1H, H-3′), 3.89–3.81 (m, 1H, H-4′), 3.64–3.41 (m, 2H, H-5′); ^{13}C NMR (50 MHz, $CDCl_3$), δ 158.7 (C-6), 153.2 (C-2), 151.8 (C-4), 140.9, 140.8, 138.1, 137.5, 129.1 (Ph), 128.5 (Ph), 127.7 (Ph), 127.6 (Ph), 127.3 (Ph), 117.4 (C-5), 88.3 (C-1′), 87.5 (C-4′), 75.6 (C-2′), 72.8 (C-3′), 63.7 (C-5′), 43.7 (N-CH_2Ph); m/z: calcd for $C_{23}H_{23}N_5O_5$ $(M+H)^+$: 450.1771, found: 450.1773.

4.1.4.8. N^2-Butyl-N^2-methylguanosine (11). 92 mg, 48%,^{1}H NMR (200 MHz, $CDCl_3$) δ 8.11 (s, 1H, H-8), 5.72 (d, J = 4.9 Hz, 1H, H-1′), 4.69 (t, J = 5.3 Hz, 1H, H-2′), 4.13 (t, J = 3.9 Hz, 1H, H-3′), 3.76–3.73 (m, 1H, H-4′), 3.63–3.61 (m, 2H, N-CH_2), 3.58–3.46 (m, 2H, H-5′), 3.09 (s, 3H, N-CH_3), 1.60–1.52 (m, 2H, CH_2 butyl), 1.38–1.31 (m, 2H, CH_2CH_3 butyl), 0.90 (t, J = 7.3 Hz, 3H, CH_3 butyl); ^{13}C NMR (50 MHz, $CDCl_3$), δ 158.2 (C-6), 152.3 (C-2), 140.3 (C-4), 136.9 (C-8), 118.5 (C-5), 87.9 (C-1′), 85.6 (C-4′), 74.3 (C-2′), 71.4 (C-3′), 62.4 (C-5′), 51.2 (N-CH_2 butyl), 38.5 (N-CH_3), 27.8 (CH_2 butyl), 20.3 (CH_2 butyl), 13.7 (CH_3 butyl). m/z: calcd for $C_{15}H_{23}N_5O_5$ $(M+H)^+$: 354. 1771, found: 354.1768.

4.1.4.9. N^2-Butylguanosine (12). 144 mg, 78%, ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.09 (s, 1H, H-8), 5.68 (d, J = 4.8 Hz, 1H, H-1′), 4.64–4.59 (m, 1H, H-2′), 4.11–4.08 (m, 1H, H-3′), 3.76–3.73 (m, 1H, H-4′), 3.63 (t, J = 6.2, 2H, N-CH_2), 3.58–3.46 (m, 2H, H-5′), 1.63–1.54 (m, 2H, CH_2 butyl), 1.28–1.25 (m, 2H, CH_2CH_3 butyl), 0.93 (t, J = 7.3 Hz, 3H, CH_3 butyl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 157.6 (C-6), 154.4 (C-2), 150.3 (C-4), 137.8 (C-8), 117.8 (C-5), 86.8 (C-1′), 84.9 (C-4′), 74.0 (C-2′), 72.3 (C-3′), 62.1 (C-5′), 42.3 (N-CH_2 butyl), 30.7 (CH_2 butyl), 20.4 (CH_2 butyl), 14.3 (CH_3 butyl). m/z: calcd for $C_{14}H_{21}N_5O_5$ $(M+H)^+$: 340. 1615, found: 340.1619.

4.1.4.10. N^2-*iso*Butyl-N^2-methylguanosine (13). 100 mg, 52%, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.55 (s, 1H, H-8), 5.64 (d, J = 5.2 Hz, 1H, H-1′), 4.64 (t, J = 5.0 Hz, 1H, H-2′), 4.16 (t, J = 3.8 Hz, 1H, H-3′), 3.86–3.81 (m, 1H, H-4′), 3.74–3.71 (m, 2H, N-CH_2), 3.60–3.45 (m, 2H, H-5′), 2.97 (s, 3H, N-CH_3), 1.89–1.85 (m, 1H, CH isobutyl), 0.94 (d, J = 6.9, 6H, CH_3 isobutyl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 156.8 (C-6), 152.4 (C-2), 141.9 (C-4), 136.7 (C-8), 119.8 (C-5), 89.3 (C-1′), 85.1 (C-4′), 74.0 (C-2′), 72.3 (C-3′), 62.8 (C-5′), 52.5 (N-CH_2 isobutyl), 38.6 (N-CH_3), 25.2 (CH isobutyl), 20.4 (CH_3 isobutyl). m/z: calcd for $C_{15}H_{23}N_5O_5$ $(M+H)^+$: 354. 1771, found: 354.1777.

4.1.4.11. N^2-*iso*Butylguanosine (14). 147 mg, 80%,^{1}H NMR (200 MHz, $CDCl_3$) δ 7.64 (s, 1H, h-8), 5.61 (d, J = 5.5 Hz, 1H, H-1′), 4.64–4.61 (m, 1H, H-2′), 4.15 (t, J = 3.9 Hz, 1H, H-3′), 3.87–3.83 (m, 1H, H-4′), 3.61–3.58 (m, 2H, N-CH_2), 3.58–3.47 (m, 2H, H-5′), 1.90–1.86 (m, 1H, CH isobutyl), 0.92 (d, J = 6.5, 6H, CH_3 isobutyl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 155.9 (C-6), 151.9 (C-2), 149.2 (C-4), 137.5 (C-8), 119.8 (C-5), 87.7 (C-1′), 85.6 (C-4′), 74.6 (C-2′), 72.8 (C-3′), 62.5 (C-5′), 50.3 (N-CH_2 isobutyl), 28.7 (CH isobutyl), 19.2 (CH_3 isobutyl). m/z: calcd for $C_{14}H_{21}N_5O_5$ $(M+H)^+$: 340.1615, found: 340.1615.

4.1.4.12. N^2,N^2-Diethylguanosine (15). 101 mg, 55%,^{1}H NMR (200 MHz, $CDCl_3$) δ 7.58 (s, 1H, H-8), 5.65 (d, J = 5.0 Hz, 1H, H-1′), 4.63 (br, 1H, H-2′), 4.15 (br, 1H, H-3′), 3.83–3.78 (m,,3H, H-4′, H-5′), 3.69–3.57 (m, 4H, CH_2 ethyl), 1.06 (t, 6H, CH_3 ethyl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 156.8 (C-6), 151.8 (C-2), 141.6 (C-4), 136.9 (C-8), 117.3 (C-5), 87.5 (C-1′), 86.2 (C-4′), 74.2 (C-2′), 71.9 (C-3′), 61.8 (C-5′), 42.6 (N-CH_2 ethyl), 13.1 (CH_3 ethyl). m/z: calcd for $C_{14}H_{21}N_5O_5$ $(M+H)^+$: 340.1615, found: 340.1610.

4.1.4.13. N^2-Ethyl-N^2-methylguanosine (16). 100 mg, 56%,^{1}H NMR (200 MHz, $CDCl_3$) 7.79 (s, 1H, H-8), 5.67 (d, J = 5.1 Hz, 1H, H-1′), 4.72–4.69 (t, J = 5.2, 1H, H-2′), 4.16 (br, 1H, H-3′), 3.83–3.78 (m, 1H, H-4′), 3.69–3.57 (m, 2H, H-5′), 3.62 (q, J = 6.8 Hz, 2H, CH_2 ethyl), 3.12 (s, 3H, N-CH_3), 1.22 (t, J = 7.1 Hz, 3H, CH_3 ethyl); δ ^{13}C NMR (50 MHz, $CDCl_3$) δ 157.7 (C-6), 152.4 (C-2), 140.2 (C-4), 137.5 (C-8), 119.2 (C-5), 88.8 (C-1′), 85.9 (C-4′), 75.0 (C-2′), 71.3 (C-3′), 63.4 (C-5′), 44.4 (N-CH_2 ethyl), 37.5 (N-CH_3), 11.9 (CH3 ethyl). m/z: calcd for $C_{13}H_{19}N_5O_5$ $(M+H)^+$: 326.1458, found: 326.1463.

4.1.4.14. N^2-Cyclohexylmethylguanosine (17). 144 mg, 70%,^{1}H NMR (200 MHz, $CDCl_3$) δ 7.82 (s, 1H, H-8),5.68 (d, J = 3.8 Hz, 1H, H-1′), 4.72 (t, J = 4.9 Hz, 1H, H-2′), 4.15 (t, J = 5.7 Hz, 1H, H-3′), 3.85–3.80 (m, 1H, H-4′), 3.61–3.52 (m, 2H, H-5′), 3.32–3.28 (m, 2H, N-CH_2), 1.82–0.98 (m, 11H, cyclohexyl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 156.1 (C-6), 153.2 (C-2), 150.4 (C-4), 137.9 (C-8), 120.1 (C-5), 87.9 (C-1′), 86.6 (C-4′), 73.9 (C-2′), 71.9 (C-3′), 62.3 (C-5′), 44.9 (N-CH_2), 35.7 (cyclohexyl), 29.9 (cyclohexyl), 25.8 (cyclohexyl), 25.1 (cyclohexyl). m/z: calcd for $C_{17}H_{25}N_5O_5$ $(M+H)^+$: 380.1928, found: 380.1925;

4.1.5. General procedure for the synthesis of N^2-substitutiuted 5′-monophosphates 32–45

Phosphorus oxide trichloride ($POCl_3$) (3.5 equiv) in trimethyl phosphate (0.05 equiv) was cooled to 0 °C and added to the dried over P_4O_{10} compound **4**–**17** (1 equiv). The reaction mixture was stirred at 0 °C. After 2 h, 1 M aqueous TEAB was added to neutralize the pH of the reaction mixture. The product was purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column using a linear 0–1 M TEAB gradient to produce products **18**-**31** as TEA salts. Methyl iodide (7 equiv) was added to a suspension of mononucleotide derivative (**18**–**31**) in 1.5 mL anhydrous dimethylsulfoxide (DMSO) and stirred at room temperature for 2 h. The mixture was poured into water and extracted three times with diethyl ether. The aqueous phase was purified on DEAE–Sephadex using a linear 0–0.8 M gradient of TEAB. Final products were converted into sodium salts using DOWEX 50WX8.

4.1.5.1. N^2-Benzyl-7-methylguanosine-5′-monophosphate (32). 129 mg, 65%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 7.46–7.32 (m, 5H, Ph), 6.07 (d, J = 3.4 Hz, 1H, H-1′), 4.61 (s, 2H, CH_2Ph), 4.58 (t, J = 4.7 Hz, 1H, H-2′), 4.43 (t, J = 5.7 Hz, 1H, H-3′), 4.35–4.33 (m, 1H, H-4′), 4.15–4.11 (m, 1H, H-5′), 4.09 (s, 3H, N^7-CH_3), 4.00–3.97 (m, 1H, H-5″); ^{31}P NMR (283 MHz, D_2O) 3.234; m/z: calcd for $C_{18}H_{23}N_5O_8P$: 468.1127, found: 468.1131; HPLC (Method B) t_R 9 min.

4.1.5.2. N^2-Benzyl-N^2,7-dimethylguanosine-5′-monophosphate (33). 110 mg, 68%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 7.45–7.31 (m, 5H, Ph), 6.11 (d, J = 3.5 Hz, 1H, H-1′), 4.68 (s, 2H,CH_2Ph), 4.62 (t, J = 4.7 Hz, 1H, H-2′), 4.44 (t, J = 5.4 Hz, 1H, H-3′), 4.35–4.32 (m, 1H, H-4′), 4.15–4.11 (m, 1H, H-5′), 4.10 (s, 3H, N^7-CH_3), 4.01–3.97 (m, 1H, H-5″), 3.17 (s, 3H, N^2-CH_3); ^{31}P NMR (283 MHz, D_2O) 3.139; m/z: calcd for $C_{19}H_{25}N_5O_8P$: 482.1284, found: 482.1287; HPLC (Method B) t_R 13 min.

4.1.5.3. N^2,N^2-Dibenzyl-7-dimethylguanosine-5′-monophosphate (34). 84 mg, 58%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 7.42–7.28 (m, 10H, 2 × Ph), 6.07 (d, J = 3.6 Hz, 1H, H-1′), 4.82 (s,

4H, CH_2Ph), 4.56 (t, J = 4.9 Hz, 1H, H-2′), 4.42 (t, J = 5.6 Hz, 1H, H-3′), 4.31–4.28 (m, 1H, H-4′), 4.09 (s, 3H, N^7-CH_3), 4.08–4.06 (m, 1H, H-5′), 3.97–3.93 (m, 1H, H-5″); ^{31}P NMR (283 MHz, D_2O) 3.085; *m/z*: calcd for $C_{25}H_{29}N_5O_8P$: 558.1597, found: 558.1592; HPLC (Method B) t_R 20.5 min.

4.1.5.4. N^2-*p*-Metoxybenzyl-7-methylguanosine-5′-monophosphate (35). 134 mg, 66%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 7.39 (d, 2H, Ph), 6.95 (d, 2H, Ph), 6.08 (d, J = 3.4 Hz, 1H, H-1′), 4.60 (t, J = 4.7 Hz, 1H, H-2′), 4.51 (s, 2H, CH_2Ph), 4.44 (t, J = 5.7 Hz, 1H, H-3′), 4.36–4.34 (m, 1H, H-4′), 4.17–4.13 (m, 1H, H-5′), 4.07 (s, 3H, N^7-CH_3), 4.02–3.98 (m, 1H, H-5″), 3.79 (s, 3H, OCH_3); ^{31}P NMR (283 MHz, D_2O) 3.011; *m/z*: calcd for $C_{19}H_{25}N_5O_9P$: 498.1233, found: 498.1237; HPLC (Method B) t_R 12.25 min.

4.1.5.5. N^2-*p*-Chlorobenzyl-7-methylguanosine-5′-monophosphate (36). 119 mg, 53%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 7.40 (s, 4H, Ph), 5.90 (d, J = 3.6, 1H, H-1′), 4.66–4.62 (m, 1H, H-2′), 4.50 (s, 2H, CH_2Ph), 4.35–4.32 (m, 1H, H-3′), 4.29–4.26 (m, 1H, H-4′), 4.09 (s, 3H, N^7-CH_3), 4.07–3.97 (m, 2H, H-5′, H-5″); ^{31}P NMR (283 MHz, D_2O) 2.590; *m/z*: calcd for $C_{18}H_{22}N_5O_8PCl$: 502.0894, found: 502.0897; HPLC (Method B) t_R 15 min.

4.1.5.6. N^2-*p*-Nitrobenzyl-7-methylguanosine-5′-monophosphate (37). 96 mg, 49%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 8.25 (d, 2H, Ph), 7.62 (d, 2H, Ph), 6.01 (d, J = 3.5 Hz, 1H, H-1′), 4.68 (s, 2H, CH_2Ph), 4.52–4.50 (m, 1H, H-2′), 4.41–4.39 (m, 1H, H-3′), 4.32–4.29 (m, 1H, H-4′), 4.10–4.09 (m, 1H, H-5′), 4.07 (s, 3H, N^7-CH_3), 3.98–3.94 (m, 1H, H-5‴); ^{31}P NMR (283 MHz, D_2O) 3.339; *m/z*: calcd for $C_{18}H_{22}N_6O_{10}P$: 513.0978, found: 513.0976; HPLC (Method C) t_R 4.5 min.

4.1.5.7. N^2-*p*-Phenylbenzyl-7-methylguanosine-5′-monophosphate (38). 114 mg, 60%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 7.84–7.41 (m, 9H, Ph–Ph), 6.09 (d, J = 3.5 Hz, 1H, H-1′), 4.69 (s, 2H, CH_2Ph), 4.64 (t, J = 4.7 Hz, 1H, H-2′), 4.46–4.44 (m, 1H, H-3′), 4.39–4.32 (m, 1H, H-4′), 4.19–4.16 (m, 1H, H-5′), 4.08 (s, 3H, N^7-CH_3), 4.05–4.01 (m, 1H, H-5″); ^{31}P NMR (283 MHz, D_2O) 3.121; *m/z*: calcd for $C_{24}H_{25}N_5O_8P$: 542.1440, found: 542.1443; HPLC (Method C) t_R 15.4 min.

4.1.5.8. N^2-Butyl-N^2,7-dimethylguanosine-5′-monophosphate (39). 78 mg, 55%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 6.11 (d, J = 3.2 Hz, 1H, H-1′), 4.69 (t, J = 4.7 Hz, 1H, H-2′), 4.46 (t, J = 5.9 Hz, 1H, H-3′), 4.38–4.35 (m, 1H, H-4′), 4.22–4.18 (m, 1H, H-5′), 4.09 (s, 3H, N^7-CH_3), 4.06–4.03 (m, 1H, H-5″), 3.68–3.60 (m, 2H, CH_2 butyl), 3.15 (s, 3H, N^2-CH_3), 1.66–1.60 (m, 2H, CH_2 butyl), 1.37–1.28 (m, 2H, CH_2CH_3 butyl), 0.92 (t, J = 7.4 Hz, 3H, CH_3 butyl). ^{31}P NMR (283 MHz, D_2O)1.548; *m/z*: calcd for $C_{16}H_{27}N_5O_8P$: 448.1440, found: 448.1445; HPLC (Method A) t_R 18.7 min.

4.1.5.9. N^2-Butyl-7-methylguanosine-5′-monophosphate (40). 134 mg, 60%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 6.11 (d, J = 3.0 Hz, 1H, 1H, H-1′), 4.69 (t, J = 4.8 Hz, 1H, H-2′), 4.46 (t, J = 5.8 Hz, 1H, 1H, H-3′), 4.40–4.36 (m, 1H, H-4′), 4.24–4.20 (m, 1H, H-5′), 4.08 (s, 3H, N^7-CH_3), 4.07–4.05 (m, 1H, H-5″), 3.45 – 3.40 (m, 2H, CH_2 butyl), 1.64–1.55 (m, 2H, CH_2 butyl), 1.42–1.32 (m, 2H, CH_2CH_3 butyl), 0.91 (t, J = 7.4 Hz, 3H, CH_3 butyl); ^{31}P NMR (283 MHz, D_2O) 0.770; *m/z*: calcd for $C_{15}H_{25}N_5O_8P$: 434.1284, found: 434.1285; HPLC (Method A) t_R 13.2 min.

4.1.5.10. N^2-*iso*Butyl-N^2,7-dimethylguanosine-5′-monophosphate (41). 78 mg, 51%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 6.11 (d, J = 3.2 Hz, 1H, H-1′), 4.67 (t, J = 4.8 Hz, 1H, H-2′), 4.46 (t, J = 5.9 Hz, 1H, H-3′), 4.37–4.35 (m, 1H, H-4′), 4.22–4.18 (m, 1H, H-5′), 4.09 (s, 3H, N^7-CH_3), 4.07–4.03 (m, 1H, H-5″), 3.45–3.40 (m, 2H, N-CH_2), 3.17 (s, 3H, N^2-CH_3), 2.15–2.05 (m, 1H, CH isobutyl), 0.92 (d, J = 6.5 Hz, 6H, CH_3 isobutyl); ^{31}P NMR (283 MHz, D_2O) 1.646; *m/z*: calcd for $C_{16}H_{27}N_5O_8P$: 448.1440 found: 448.1442; HPLC (Method A) t_R 21.5 min.

4.1.5.11. N^2-*iso*Butyl-7-methylguanosine-5′-monophosphate (42). 130 mg, 57%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 6.12 (d, J = 3.3 Hz, 1H, H-1′), 4.67 (t, J = 4.9 Hz, 1H, H-2′), 4.47 (t, J = 5.6 Hz, 1H, H-3′), 4.38–4.36 (m, 1H, H-4′), 4.20–4.16 (m, 1H, H-5′), 4.09 (s, 3H, N^7-CH_3), 4.06–4.02 (m, 1H, H-5″), 3.32–3.20 (m, 2H, N-CH_2), 1.98–1.89 (m, 1H, CH isobutyl), 0.93 (d, J = 6.7 Hz, 6H, CH_3 isobutyl); ^{31}P NMR (283 MHz, D_2O) 2.061; *m/z*: calcd for $C_{15}H_{25}N_5O_8P$: 434.1284, found: 434.1287; HPLC (Method A) t_R 16 min.

4.1.5.12. N^2,N^2-Diethyl-7-methylguanosine-5′-monophosphate (43). 83 mg, 52%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 6.12 (d, J = 3.1 Hz, 1H, H-1′), 4.70 (t, J = 4.9 Hz, 1H, H-2′), 4.46 (t, J = 5.5 Hz, 1H, H-3′), 4.37–4.35 (m, 1H, H-4′), 4.22–4.18 (m, 1H, H-5′), 4.08 (s, 3H, N^7-CH_3), 4.07–4.04 (m, 1H, H-5″), 3.60 (q, J = 6.1 Hz, 4H, CH_2 ethyl), 1.22 (t, J = 7.1 Hz, 6H, CH_3 ethyl); ^{31}P NMR (283 MHz, D_2O) 1.299; *m/z*: calcd for $C_{15}H_{25}N_5O_8P$: 434.1284, found: 434.1291; HPLC (Method A) t_R 14.2 min.

4.1.5.13. N^2-Ethyl-N^2,7-dimethylguanosine-5′-monophosphate (44). 109 mg, 70%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 6.12 (d, J = 3.4 Hz, 1H, H-1′), 4.70 (t, J = 4.8 Hz, 1H, H-2′), 4.46 (t, J = 5.8 Hz, 1H, H-3′), 4.38–4.35 (m, 1H, H-4′), 4.23–4.19 (m, 1H, H-5′), 4.08 (s, 3H, N^7-CH_3), 4.07–4.05 (m, 1H, H-5″), 3.64 (dd, J = 14.3, 7.1 Hz, 2H), 3.15 (s, 3H), 1.20 (t, J = 7.1 Hz, 3H); ^{31}P NMR (283 MHz, D_2O) 0.866; *m/z*: calcd for $C_{14}H_{23}N_5O_8P$: 420.1127, found: 420.1128; HPLC (Method A) t_R 13.9 min.

4.1.5.14. N^2-Cyclohexylmethyl-7-methylguanosine-5′-monophosphate (45). 122 mg, 56%, sodium salt; ^{1}H NMR (700 MHz, D_2O) δ 6.12 (d, J = 3.6 Hz, 1H, H-1′), 4.66 (t, J = 4.8 Hz, 1H, H-2′), 4.47 (t, J = 5.7 Hz, 1H, H-3′), 4.38–4.35 (m, 1H, H-4′), 4.16–4.12 (m, 1H, H-5′), 4.10 (s, 3H, N^7-CH_3), 4.02–3.98 (m, 1H, H-5″), 3.30–3.27 (m, 2H, N-CH_2), 1.79–0.95 (m, 11H, cyclohexyl); ^{31}P NMR (283 MHz, D_2O) 3.218; *m/z*: calcd for $C_{18}H_{29}N_5O_8P$: 474.1597, found: 474.1592; HPLC (Method A) t_R 15.6 min.

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Supplementary data

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