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Clickable trimethylguanosine cap analogs modified within the triphosphate bridge: synthesis, conjugation to RNA and susceptibility to degradation

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The trimethylguanosine (m_3G) cap present at the 5′ end of small nuclear RNAs (snRNAs) has been proposed as an effective nuclear localization signal (NLS) for nucleus-targeting therapeutics such as antisense oligonucleotides. To provide novel tools for studies on m_3G-mediated transport and m_3G degradation, we synthesized a series of novel m_3G cap analogs that combine modifications potentially affecting its activity as NLS and stability *in vivo* with a modification enabling simple conjugation to biomolecules. The synthesized dinuclotide m_3G analogs carry a single phosphate-modification (phosphorothioate, methylenebisphosphonate or imidodiphosphate) at the selected position of the triphosphate bridge in order to increase their resistance to enzymatic cleavage and a (2-azidoethyl)-carbamoylmethyl group at the 2′-position of adenosine as a second nucleotide to enable conjugation to alkyne-containing biomolecules by copper catalyzed azide-alkyne cycloaddition (CuAAC). The susceptibility of m_3G cap analogs to non-specific and specific degradation was studied in fetal bovine serum and in an *in vitro* decapping assay with hNUDT16 enzyme, respectively. The susceptibility of m_3G cap analogs to hNUDT16 mediated decapping was also determined after their CuAAC-mediated conjugation to a model oligonucleotide bearing a 5′-alkyne group. Depending on the type and the position of introduced modifications, they modulate the susceptibility to specific and non-specific degradation of conjugated molecules to various extent, with O to NH substitution at the α/β position providing the greatest m_3G stability against hNUDT16.

Introduction

Hypermethylated 2,2,7-trimethylguanosine (m_3G) cap structures are present at the 5′ end of small nuclear RNAs that program mRNA splicing (U1, U2, U4 and U5 snRNAs) and some nucleolar RNAs (snoRNAs).[1, 2] For snRNAs the m_3G cap is formed post-transcriptionally by the cytoplasmic enzyme Tgs1, which catalyzes two successive methyl additions at the N^2 position of the m^7G cap in premature transcripts.[3, 4] Then, m_3G-capped snRNAs are recognized by snurportin, an adaptor protein that binds to importin β. This interaction mediates the import of matured snRNA back to the nucleus.[5-8] It has been shown that the presence of an m_3G cap is required for efficient nuclear import of snRNAs and for proper mRNA splicing.[8] Therefore, many m_3G cap analogs have been synthesized and applied in numerous biophysical and biological studies on the transport and function of small RNAs.[9-11] Importantly, they have been also proposed as a nuclear localization signal (NLS) for delivery of the splice-correcting antisense oligonucleotides (ONs)[12] designed to act inside the nucleus. As shown there, m_3G-capped oligonucleotides were efficiently imported into the nucleus, even as a part of large bioconjugates, and promoted splicing in model systems more efficiently than uncapped ones,[12] thus indicating their potential of use in experimental therapies for genetic disorders such as Duchenne Muscular Dystrophy (DMD)[13] or Spinal Muscular Atrophy (SMA).[14, 15] However, for future developments it would be beneficial to design m_3G cap analogs that not only interact with snurportin but can also be easily incorporated into RNA and are resistant to specific and non-specific enzymatic degradation. The unmodified m_3G cap structure is potentially susceptible to degradation by extra- and intracellular pyrophosphatases that cleave the triphosphate bridge between α/β or β/γ phosphates. Little is known about degradation pathways specific for m_3G-capped RNAs, but recent studies reveal involvement of NUDIX pyrophosphatases in the decapping process.[16] It has been shown that m_3G-capped RNAs are decapped by the NUDT16 enzyme *in vitro*. Recently, also Dcp2, known primarily as a major decapping enzyme targeting monomethylguanosine (m^7G) capped mRNAs, have been shown to hydrolyze m_3G-capped snRNAs as a part of a quality control mechanism.[16, 17] Both enzymes cleave the triphosphate bridge of the cap between α and β phosphates to release methylated GDP and 5′-phosphorylated RNA.[16, 17] Several modifications have been recently identified that protect m_3G and m^7G caps against degradation by hNUDT16 and Dcp2, respectively, as well as by other cap-specific and non-specific

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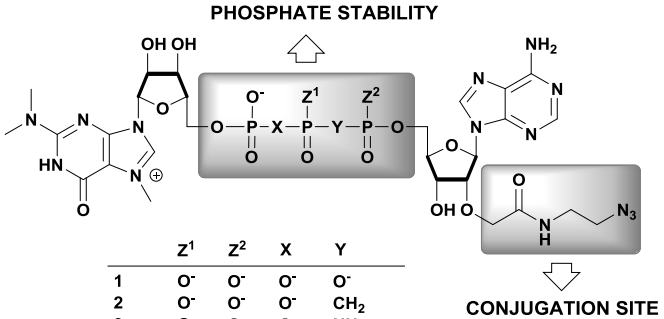
enzymes.[18-25] For example, introduction of β-phosphorothioate,[20] α,β-methylenebisphosphonate[26] or α,β-imidodiphosphate[22] groups into the triphosphate bridge of the m^{7}G cap significantly improve the cellular half-life of exogenously delivered capped-mRNAs.[19, 27, 28] In this work, we aimed at combining the phosphate modifications of m_3G cap with another beneficial feature, i.e. possibility of facile conjugation to oligonucleotides using click chemistry[29] as accomplished with unmodified caps.[30] Therefore, we synthesized a set of six novel phosphate-modified m_3G cap analogues (Fig. 1) that are equipped with 2-(azidoethyl)carbamoylmethyl handle at the 2'-position of the ribose moiety, which enables efficient conjugation of m_3G caps to alkyne modified RNAs by copper catalyzed azide-alkyne cycloaddition (CuAAC)[30] (Fig. 1).



	Z^1	Z^2	X	Y	
1	O^-	O^-	O^-	O^-	
2	O^-	O^-	O^-	CH_2	
3	O^-	O^-	O^-	NH	
4	O^-	O^-	CH_2	O^-	
5	O^-	O^-	NH	O^-	
6*a,b**	S^-	O^-	O^-	O^-	D1/D2
7*a,b**	O^-	S^-	O^-	O^-	D1/D2

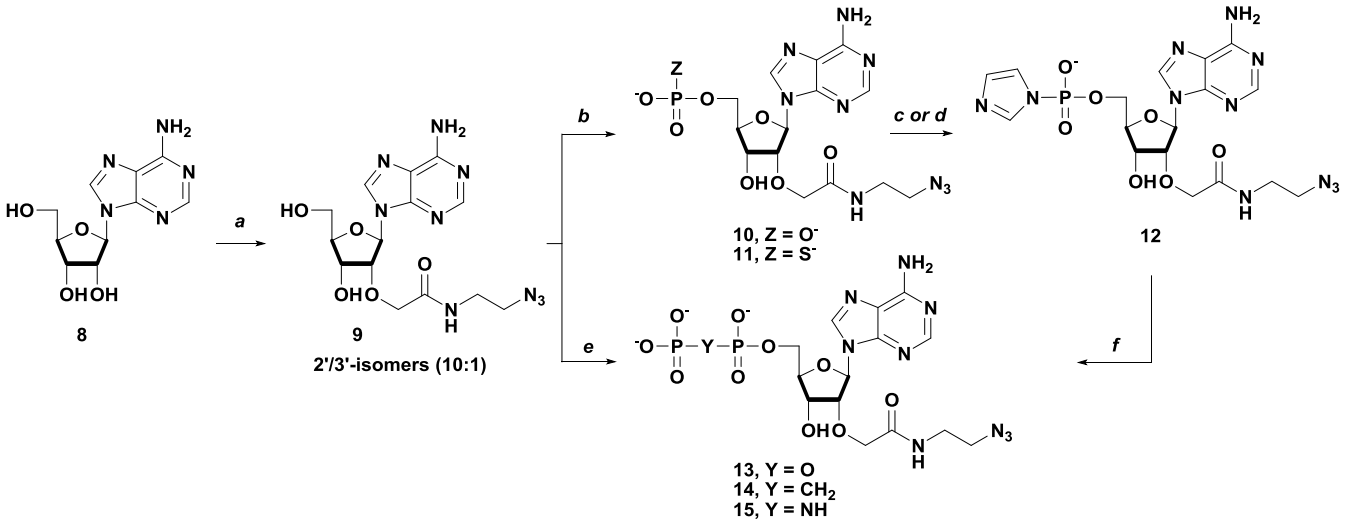
Fig. 1 Structures of m_3G-cap analogs synthesized in this study. * a and b refer to either of the two P-diastereomers of a given compound, D1 and D2. D1 denotes the isomer eluting faster from a reversed-phased HPLC column.

The analogs were modified at various positions of the 5',5'-triphosphate bridge by replacing either one of the bridging oxygens with CH_2 or NH groups, or one of the non-bridging oxygens with sulfur. All synthesized dinucleotide m_3G cap analogs were then used to study unspecific degradation in fetal bovine serum (FBS). To determine the influence of the modifications on susceptibility to decapping enzymes, the m_3G caps were then attached to model short RNA using "click chemistry" approach and studied for their susceptibility to decapping to recombinant hNUDT16 *in vitro*.

Results and discussion

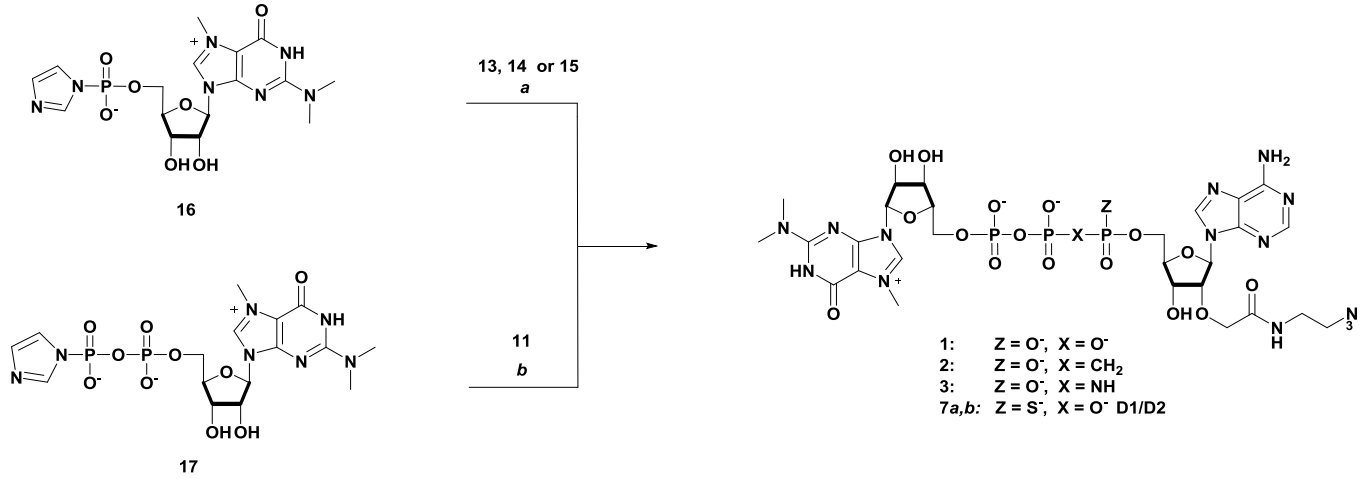
Synthesis of modified m_3G cap analogs 1-7

The syntheses of six phosphate-modified m_3G cap analogs functionalized with (2-azidoethyl)carbamoylmethyl group at the 2'-position of adenosine together with an unmodified parent compound described previously are shown in Schemes 1–3.[30, 31] The synthesis was accomplished by coupling two mononucleotide units taking advantage of P-imidazolide chemistry. The key starting materials in the synthesis of all m_3G cap analogs were an adenosine-derived building block bearing a "clickable" azido linker at 2'-position and a m_3G-derived building block, one of which had to be modified in the phosphate moiety and the other activated with imidazole.[30] 2'-*O*-(N-(2-Azidoethyl)carbamoyl)methyl adenosine **9** was synthesized by a minor modification of the previously described procedure[30] to avoid 5'-OH protection, since we observed that MMTr group removal in acidic conditions is accompanied with partial degradation of the amide bond yielding also 2'-O-(carboxymethyl)adenosine. Alkylation of unprotected adenosine (**8**) (Scheme 1) was performed in a one-pot two-step procedure involving NaH and allyl bromoacetate treatment followed by addition of 2-azidoethylamine in DMF to provide the desired 2'-alkylated product **9**[30] with a good regioselectivity (2′:3′ as 10:1) and in 80-90% yield. Next, modified Yoshikawa's phosphorylation procedure[32] was employed to convert **9** into its 5'-monophosphate (**10**)[30] or 5' monophosphorothioate (**11**) using $POCl_3$ or $PSCl_3$, to afford compounds **10** and **11** in, respectively, 85% and 65% isolated yields after ion-exchange chromatography (IEC).

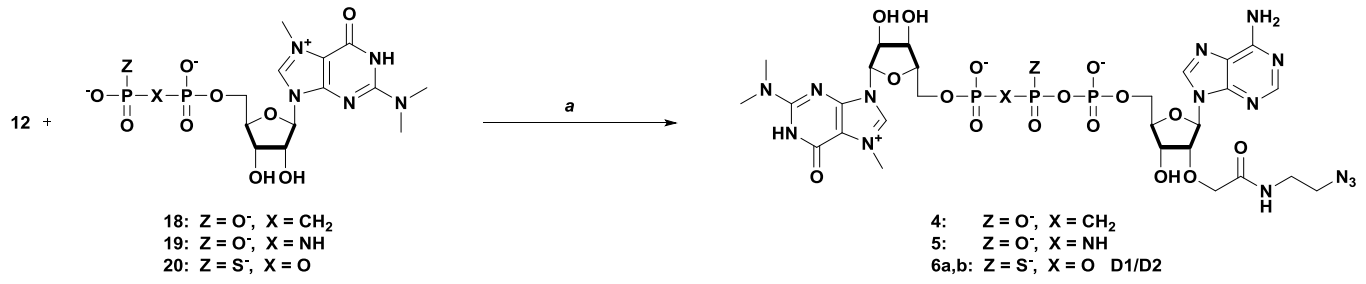


Scheme 1 Synthesis of 2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine (**9**) and its 5'-phosphate derivatives (**10**-**15**) a) i: NaH (4 equiv), dry DMF, 10 min, r.t; allyl bromoacetate (2 equiv), r.t, 1h; ii. 2-azidoethylamine (3 equiv), 24h, r.t.; b) i: $PXCl_3$ (X=O, S) (3 equiv), $PO(OMe)_3$, 3-4h, 0°C., ii: $NaHCO_{3aq}$ (9 equiv); c) i: Imidazole (10 equiv), 2,2'-dithiopyridine (3 equiv), TEA (3 equiv), PPh_3 (3 equiv); DMF, 24h, r.t.; ii: $NaClO_4$, acetone; d) *in situ*, CDI (5 equiv), DMF, MW (40°C, 5W, 20min), e) i: $Cl_2POCH_2POCl_2$ (3 equiv) for **14** or $Cl_3P{=}NP(O)Cl_2$, (3 equiv) $PO(OMe)_3$ for **15**, 0°C, 6-8h; ii: $NaHCO_{3aq.}$(15 equiv), e) PO_4^{3-}/TEA^+ (2 equiv) for **13**, $ZnCl_2$ (8 equiv), DMF, 6h, r.t.

Similarly, 2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-methylene-(bisphosphonate) (**14**) or imidodiphosphate (**15**) were obtained by reacting **9** with methylenebis(phosphonic chloride) or dichlorophosphorylphosphorimidoyltrichloride.[25, 33] Compounds **14** and **15** were obtained after IEC purification in 60% and 50% yields, respectively. The synthesis of the reactive nucleotide P-imidazolide **12** was achieved either by *in situ* activation with 1,1'-carbonyldiimidazole (CDI) accelerated by microwave irradiation in DMF[34, 35] or by the Mukaiyama-Hashimoto method employing imidazole, 2,2' dithiodipyridine, triethylamine and triphenylphosphine in DMF and subsequent precipitation by sodium perchlorate solution in acetone.[36] Noteworthy, we did not observe any azide reduction using PPh_3. The synthetic route to m_3G cap analogs unmodified **1**[30] or bearing a modification at the α/β position of triphosphate bridge **2-3**, and **7a**,**b** is depicted in Scheme 2. Compounds bearing the O to CH_2 or NH (**2** and **3**) substitutions at the α/β position of the 5',5'-triphosphate bridge (Scheme 2) were obtained by coupling N^2,N^2,N^7-trimethylguanosine 5'-monophosphate P-imidazolide (**16**)[19] with compounds **14** and **15**, respectively, in the presence of excess $ZnCl_2$ (Scheme 3). The role of $ZnCl_2$ is to improve the solubility of reactants, activate the imidazole as a leaving group, and act as a template coordinating both nucleotide subunits that form the pyrophosphate bond.[9] m_3G cap analogs bearing a CH_2 or NH substitution at the β/γ position of the 5',5'-triphosphate bridge **4**, **5** (Scheme 3) were obtained by coupling N^2,N^2,N^7-trimethylguanosine 5'-methylene(bisphosphonate) (**18**)[19] or P1-(N^2,N^2-dimethylguanosine-5'-yl)imidodiphosphate (**19**)[19] with **12** (obtained by CDI-mediated activation) under similar conditions. β-Thio-modified m_3G-cap analog **6** (**6a** – isomer D1 and **6b** – isomer D2) was obtained by coupling **12** (obtained by Mukaiyama-Hashimoto method) with trimethylguanosine 5'-*O*-(β-thiodiphosphate) (**20**)[19] leading to a mixture of two P-diastereoisomers of analog **6** (**6a** – isomer D1 and 6b – isomer **D2**)[19] The α-thio-modified m_3G-cap analogs **7a** and **b** (**7a** – isomer D1 and **7b** – isomer **D2**) were synthesized by coupling N^2,N^2,N^7- trimethylguanosine 5'-*O*-(diphosphate) P-imidazolide (**17**)[19] with **11**. All of the synthesized m_3G-caps **1-7** were isolated as triethylammonium salts using ion exchange chromatography with 40-60% yield. Further purification and, in the case of compounds **6** and **7**, separation of P-diastereoisomers was performed by semi-preparative RP-HPLC yielding m_3G caps **1-7** as ammonium salts. Final preparative yields ranged from 20 to 30%.



Scheme 2 Synthesis of dinucleotide m_3G cap analogs **1-3** and **7**; a) $ZnCl_2$, (10 equiv), DMF; b) $ZnCl_2$, (10 equiv) DMF.



Scheme 3 Synthesis of dinucleotide m_3G-cap analogs **4**-**6**; a) Imidazole (10 equiv), 2,2'-dithiopyridine (3 equiv), TEA (3 equiv), PPh_3 (3 equiv); DMF, 24h, r.t.; ii: $NaClO_4$, acetone or a.) CDI (5 equiv), DMF, MW (40°C, 5W, 20min), b.) H_2O (8 equiv) 5 min, ii) $ZnCl_2$ (10 equiv)

Stability of m_3G-cap analogs in Fetal Bovine Serum (FBS)

To determine the influence of various phosphate modifications on the general enzymatic susceptibility of the novel m_3G-cap analogs, we studied their degradation in fetal bovine serum (FBS). FBS contains various nucleases capable of hydrolyzing pyrophosphate and phosphodiester bonds and has been previously employed to study the general stability of modified nucleotides and oligonucleotides under physiological conditions.[37] Each m_3G-cap analog was incubated in 2.5% FBS at 37 ºC at a concentration of 10 µM. Samples taken at different time points were analyzed by RP-HPLC (Fig. 2).

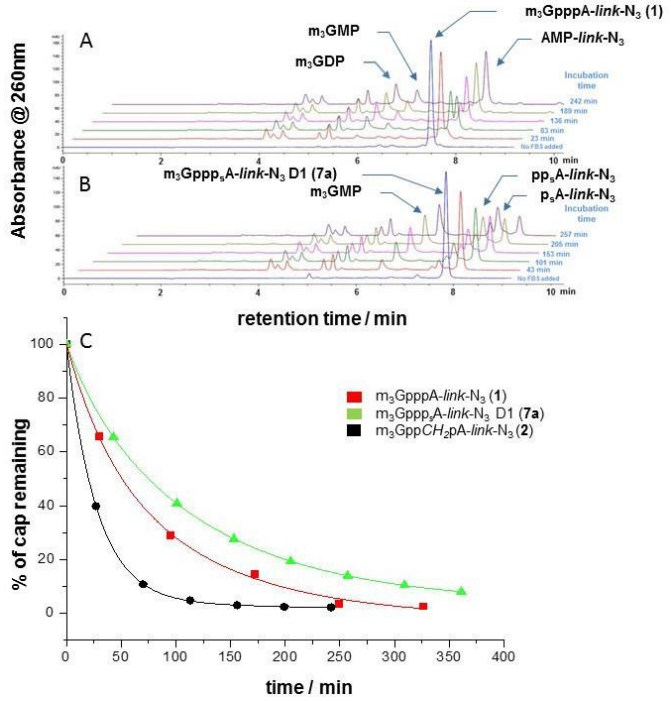


Fig. 2. Stability of m_3G-cap analogs **1** (A) and **7a** (B) in 2.5 % FBS monitored by RP-HPLC. (C) Comparison of the stability of cap analogs **1**, **2** and **7a** based on the HPLC assay.

Based on the chromatographic integration, a percentage of the remaining substrate was estimated and plotted against time. An exponential decay model was fitted to experimental data to determine half-life values. (Fig. 2) The phosphate unmodified analog **1** (m_3GpppAdo-link-N_3) and m_3GpppA without carbamoyl linker, had similar half-lives of 42.39±3.49 and 40.25±1.65 min, respectively, suggesting that carbamoyl substituent does not change the stability of cap analogs noticeably. Phosphate modifications influenced the stability of m_3G-caps to various extent. First, cap analogs **2** (m_3GppCH_2pAdo-link-N_3), **3** (m_3GppNHpAdo-link-N_3) and **4** ($m_3GpCH_2$ppAdo-link-N_3), had all shorter half-lives (21.63±1.31, 32.99±8.19, 31.49±2.62 min, respectively), indicating that bridging modifications at the α/β-phosphate decreased the stability in FBS. This is in contrast to previous finding on bridging methylene modifications in m_3G cap with 2'-OMe substituents where both modified caps where more stable than the unmodified cap in 10% FBS in cell media.[28] This suggests that the nature of the 2'-substituent does affect the relative cap stability. A stabilizing, effect was observed for β/γ imido modification **5** (m_3GpNHppAdo-link-N_3) with half-life of 58.81±2.11 min. m_3G caps bearing bridging modifications at β position **6a** ($m_3Gpp_spA$do-link-N_3, D1) and **6b** ($m_3Gpp_spA$do-link-N_3, D2) had half-life 52.03±2.90 and 40.47±1.10 min respectively showing a moderate effect of configuration on stability. Such effect is even more noticeable for compounds modified with phosphorothioate group at the α-position **7a** ($m_3Gppp_sA$do-link-N_3, D1) and **7b** ($m_3Gppp_sA$do-link-N_3, D2) with half-lives of 85.56±5.51 and 28.96±1.48 min respectively. This two-fold increased stability in FBS for the D1 isomer (**7b**) bearing sulfur modification at the α position indicate that the degradation of m_3GpppAdo-link-N_3 cap analogs in FBS occurs mainly through cleavage of the α,β-pyrophosphate bond which can also be concluded from our previous study where the α/β methylene modification showed greater stability in both FBS and cytosolic medium.[28] However, lower stability of compounds **2** and **3** suggests that other cleavage pathways are also possible.

Table 1 Stability of phosphate-modified m_3G-cap analogs in FBS. The stability was tested for 10 µM cap analogs in 2.5% FBS diluted with PBS (pH 7.2).

No.	Abbreviation	Half-life [min]
	m_3GpppA	40.25 ± 1.65
1	m_3GpppA-*link*-N_3	42.39 ± 3.49
2	m_3Gpp***CH₂***pA-*link*-N_3	21.63 ± 1.31
3	m_3Gpp***NH***pA-*link*-N_3	32.99 ± 8.19
4	m_3Gp***CH₂***ppA-*link*-N_3	31.49 ± 2.62
5	m_3Gp***NH***ppA-*link*-N_3	58.81 ± 2.11
6a	m_3Gpp_spA-*link*-N_3 D1	52.03 ± 2.90
6b	m_3Gpp_spA-*link*-N_3 D2	40.47 ± 1.10
7a	m_3Gppp_sA-*link*-N_3 D1	85.56 ± 5.51
7b	m_3Gppp_sA-*link*-N_3 D2	28.96 ± 1.48

Conjugation with RNA using CuAAC

Next, we tested whether the novel analogs can be attached to 5'-functionalized short RNAs using CuAAC. The model RNA functionalized with 5'-hexynylphosphate was synthesized using standard phosphoroamidite solid phase synthesis protocol and using commercially available 5'-hexynyl phoshoramidate as the 5'-phosphitylating reagent. The 5'-hexynylphosphate-RNA (6 nt, 5'-hex-GCUAAU, **21**) cleaved from the solid support, deprotected, and desalted by precipitation was pure enough to be used in "click" conjugation without additional purification. CuAAC reactions were carried out in H_2O/DMSO mixture at 37°C in the presence of catalytic amount of Cu-TBTA complex (0.05 equiv.) and excess sodium ascorbate at 37°C. The respective m_3G cap analog at 1 mM concentration was mixed with ~2-fold excess of 5'-hex-GCUAAU and the reaction progress was monitored by RP-HPLC. Six different clickable m_3G-cap analogs **1-5**, **7a**,**b** were successfully conjugated to model short RNA using this procedure. Due to relatively low reactant concentrations and a less reactive alkyne than in previous clicking of m_3G caps[30], the reactions proceeded slowly, reaching 50-90% conversions (based on the amount of m_3G cap-azide) within 3 to 5 days. The resulting m_3G-capped conjugates were purified using analytical RP-HPLC and subjected to biochemical studies. In the case of m_3G caps bearing a β-S modification **6a**,**b** low yields of product after purification resulted in that amounts of isolated conjugate was insufficient for biological assays. This resulted from very slow reaction progress and overlapping HPLC signals of starting material and conjugate product

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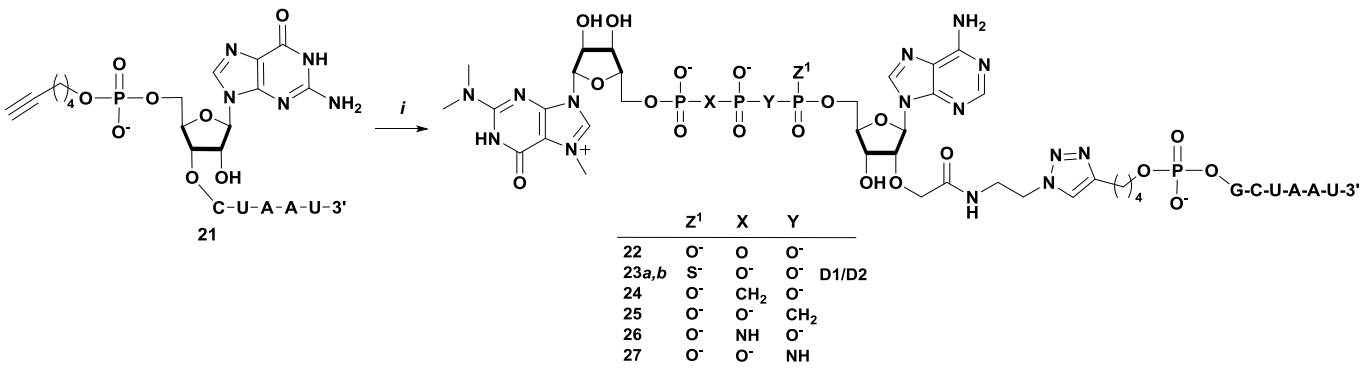

	Z^1	X	Y
22	O^-	O	O^-
23*a,b*	S^-	O^-	O^-
24	O^-	CH_2	O^-
25	O^-	O^-	CH_2
26	O^-	NH	O^-
27	O^-	O^-	NH

Scheme 4 Synthesis of m_3GpppA-*triazole*-GCUAAU conjugates (**22-27**), i.) **1-3**, **7a**,**b**, $CuSO_4$×TBTA (1:1 complex, 5 mol%), sodium ascorbate, DMSO:H_2O (1:1).

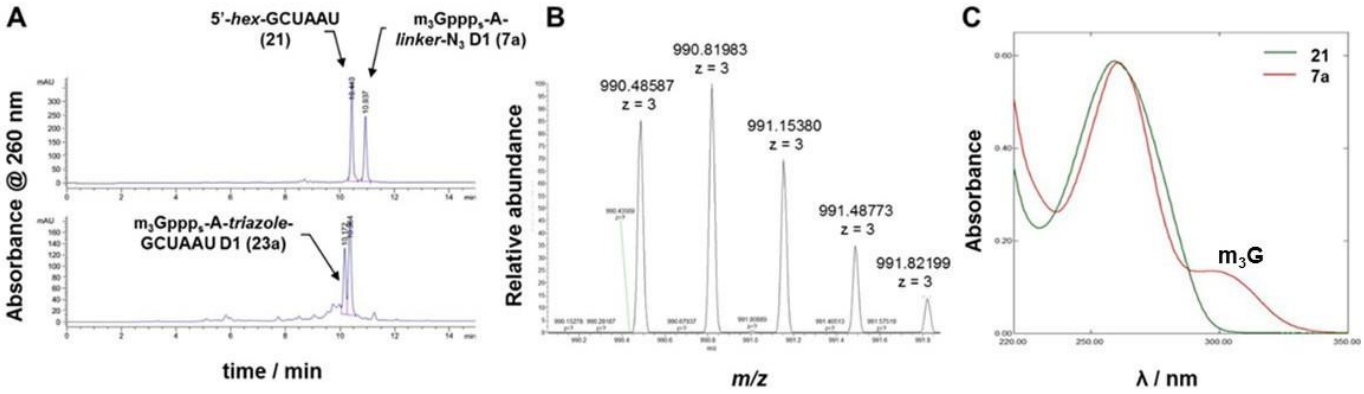

Fig. 3 Analysis of m_3G cap analogs and their conjugates. A) RP-HPLC profiles of the reaction mixture before (upper) and after 72h (lower) click conjugation of m_3G-cap **7a** with 5'-*hex*-GCUAAU **21**; B) HRMS spectrum of the product m_3Gppp_sA-*triazole*-GCUAAU D1 (**23a**); C) UV spectra of oligonucleotide **21** and dinucleotide **7a.**

Table 2 HRMS data for m_3G-cap-RNA conjugates (**22-27**)

No.	Mol. Formula	*m*	*z*	Calc. m/z	Found m/z
21	$C_{63}H_{78}N_{22}O_{43}P_6$	2016.3029	2	1008.1514	1008.1533
22	$C_{90}H_{116}N_{36}O_{61}P_9$	2955.4720	3	985.1579	985.1605
23a	$C_{90}H_{116}N_{36}O_{60}P_9S$	2971.4491	3	990.4836	990.4859
23b	$C_{90}H_{116}N_{36}O_{60}P_9S$	2971.4491	3	990.4836	990.1499
24	$C_{91}H_{118}N_{36}O_{60}P_9$	2953.4927	3	984.4981	984.5003
25	$C_{91}H_{118}N_{36}O_{60}P_9$	2953.4927	3	984.4981	984.5000
26	$C_{90}H_{117}N_{37}O_{60}P_9$	2955.4958	3	985.1658	985.8324
27	$C_{90}H_{117}N_{37}O_{60}P_9$	2955.4958	3	985.1658	985.8324

Susceptibility of chemically capped short RNAs to degradation by hNUDT16

Next, we studied the susceptibility of m_3G cap-RNA conjugates obtained by CuAAC to degradation by recombinant human decapping enzyme, hNUDT16.[38] hNUDT16, belongs to the NUDIX family of phosphohydrolases[16], which contain metal binding acidic amino acids in the catalytic site and utilize substrates composed of a Nucleoside Diphosphate linked to a moiety X.[39] hNUDT16 cleaves both m^7G and m_3G capped RNAs between the α and β phosphates to produce m^7GDP or m_3GDP and 5'-phosphorylated RNA.[22] Here, we tested if the unmodified conjugate **22** bearing a triazole moiety (m_3GpppA-*triazole*-RNA) is recognized by hNUDT16 and whether phosphate modifications would modulate the susceptibility to enzymatic degradation. The 5'-capped short RNAs obtained by

click chemistry were incubated with hNUDT16 *in vitro* and the reaction products were analyzed on 20% denaturating polyacrylamide gel (Fig. 4). The susceptibility of the 5'-*hex*-RNA (**21**) to hNUDT16 was also tested. After resolving the gel was visualized by UV-shadowing at 254 nm (Fig. 4, left panel) to reveal both capped and decapped RNAs, and also using 302nm wavelength UV lamp (Fig. 4, right panel) that allows to detect (oligo)nucleotides containing an m_3G moiety (that have maximum absorption at 305 nm, as shown in Fig. 4, right). Since the capped products (5'-m_3Gppp-*triazole*-RNA) migrate slower than uncapped RNA (hex-GCUAAU) the decapping reactions were observed by visualization at 254 nm (Fig.4, left). Additionally, the m_3G mononucleotides produced as a result of decapping, can be observed as fast migrating bands upon visualization at 302 nm. As shown in Fig. 4, m_3GpppA-*triazole*-RNA (**22**) is cleaved by hNUDT16 releasing m_3GDP (Fig. 4 right), indicating that the cleavage specificity between α and β phosphates is retained in the modified "click-capped" RNA. This suggests that the linker moiety between the m_3G cap and the RNA does not disturb the recognition by hNUDT16. The O to NH substitution at the α/β produces RNA (m_3GppNHpA-*triazole*-RNA, **27** and m_3GppCH_2pA-*triazole*-RNA, **25**) that is resistant to decapping under conditions of our assay. In contrast, the m_3G capped RNAs modified at the β/γ position (m_3GpCH_2ppA-*triazole*-RNA, **24** and m_3GpNHppA-*triazole*-RNA **26**) were efficiently cleaved by hNUDT16 to release products migrating slower than m_3GDP and slightly faster than m_3GMP, which we assigned as m_3GpXp (where X is NH or CH_2). The findings for **25**, and **27** are in agreement with the regiospecificity of the enzyme observed for natural transcripts.[20, 21] It also fits well with the considerably higher stability of the α/β modification in cytosolic extract[28] However, surprisingly, transcripts modified at the α/β position with methylene group (m_3GppCH_2pA-*triazole*-RNA, **25**) were also decapped to some extent, apparently through β/γ-pyrophosphate bond cleavage and release of m_3GMP. This suggests that some modifications may direct the decapping to the β/γ-site, likely through a conformational change of the triphosphate bridge. That the decapping of conjugates **24** and **26** appears to be somewhat faster than for the unmodified **22** suggests also that the β/γ-modifications even activate decapping by hNUDT16. That observation was confirmed in an analogous assay carried out on unconjugated m_3G dinucleotide cap analogs and higher concentration of hNUDT16: the m_3GppNHpA-*link*-N_3 appeared to be fully resistant to hNUDT16 degradation, whereas the m_3GppCH_2pA-*link*-N_3 showed some susceptibility (**1-7**; Electronic Supporting Information, ESI Fig. S1-S3). The RNA capped with the m_3Gppp$_5$A D2 **23b** is resistant to hNUDT16 decapping activity under the reaction conditions, but for the other diastereoisomer **23a**, the degradation was observed. However, for its dinucleotide cap analogs **7a** and **7b** we observed higher stability of isomer D1. Most importantly, our results indicate that the O to NH substitution at the α, β-position is more efficient than the O to CH_2 substitution for stabilizing m_3G capped RNAs obtained by CuAAC, making this analog the best candidate for future studies on interaction with snurportin and intracellular transport.

Conclusions

In conclusion, we synthesized a set of m_3G cap dinucleotides bearing an azido functionalized linker at the second nucleoside and various modifications within the 5',5'-triphosphate bridge. The synthetic approach was based on phosphorimidazolide chemistry and enabled straightforward incorporation of either bridging (CH_2, NH) or non-bridging (S) substitutions at different positions of the 5',5'-triphosphate chain in combination with 2'-O-(N-(2-azidoethyl)carbamoyl)methyl group at the 2'-O-position of adenosine. The cap analogs were then successfully attached to short RNA using click chemistry approach. Despite the sterical hindrance introduced by the cap-RNA linker in m_3G-*triazole*-RNAs, the capped RNAs were correctly recognized by hNUDT16 enzyme, as indicated by α/β cleavage of phosphate-unmodifed m_3G cap in dacapping assay. Interestingly, modifications at both the α/β and β/γ positions modulated the susceptibility to degradation by decapping enzyme, however, with distinct effects. Importantly, among studied compounds, only the bridging α/β O to NH substitution made the RNA and dinucleotide cap analogs resistant to hNUDT16, designating the compounds as most promising candidates for an efficient NLS signal. The bridging α/β O to CH_2 substitution was also highly stabilizing but surprisingly, this analog was decapped slowly, presumably trough cleavage at the γ/β position. In contrast, the methylene or imido modifications at the β/γ position of triphosphate bridge appeared to increase the cleavage reaction rate. The influence of modification on non-specific degradation in fetal bovine serum was less pronounced. Nevertheless, the analogs containing the O to S substitution at the α-position showed increased stability. Therefore, the reported set of m_3G cap analogs represents a useful toolbox for future studies aimed at assessing the importance of both type of degradations (specific or unspecific) under *in vivo* conditions. Moreover, further studies to assess the full potential of novel m_3G cap analogs and their oligonucleotide conjugates, including affinity for snurportin and intracellular transport and stability are in progress.

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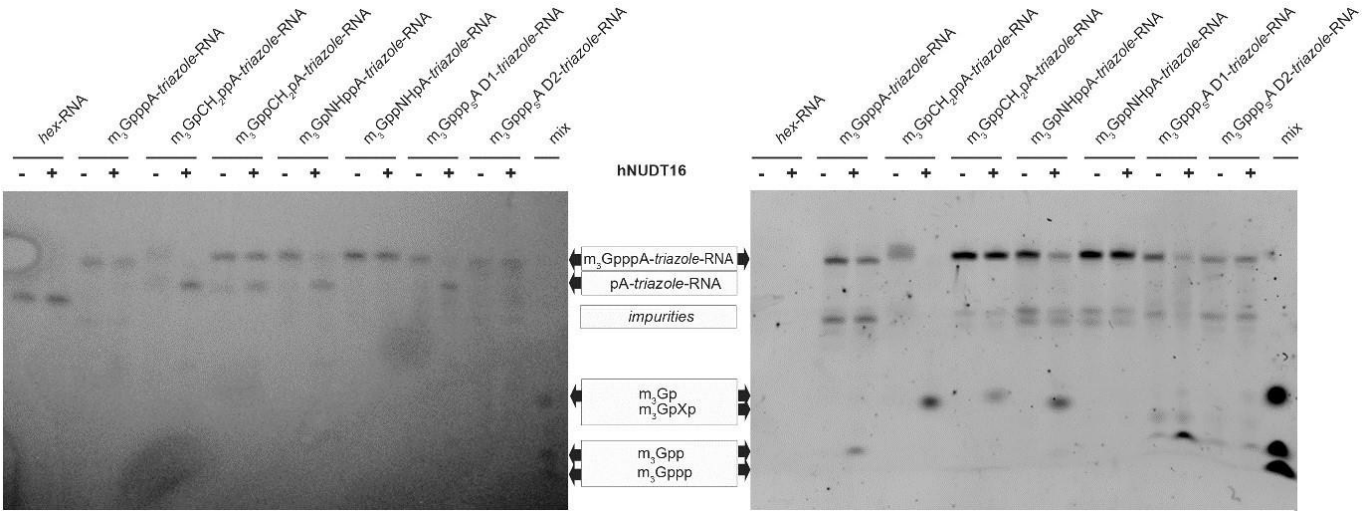

Fig. 4 Susceptibility of RNAs carrying variously modified m_3Gcap structures to degradation by hNUDT16. Short capped RNAs (m_3Gcap-*triazole*-GCUAAU) were synthesized by "click chemistry" as described in Experimental Section. Decapping reactions with hNUDT16 were incubated for 1 h at 37 °C in HCl-Tris pH 7.9. Thermally inactivated hNUDT16 was used in negative control reactions. Reaction products were separated on 20% denaturating polyacrylamide gel and visualized by UV 254 nm (left panel) and 302nm (right panel). Visualization at 302nm enables exclusive observation of m_3G-containing (oligo)nucleotides due to distinct absorption and fluorescence properties of m_3G (Fig. 4C). The capped RNAs that showed no difference for hNUDT16+ and hNUDT16– samples were assigned as resistant.

Table 3 Susceptibility to hNUDT16

No.	Compound at the 5' end of RNA	Susceptibility to hNUDT16
22	m_3GpppA-*triazole*	hydrolyzed
23a	m_3Gpp***p$_s$***A-*triazole D1*	hydrolyzed
23b	m_3Gpp***p$_s$***A-*triazole D2*	resistant
24	m_3Gp***CH$_2$***ppA-*triazole*	slowly hydrolyzed
25	m_3Gpp***CH$_2$***pA-*triazole*	hydrolyzed
26	m_3Gp***NH***ppA-*triazole*	resistant
27	m_3Gpp***NHp***A-*triazole*	hydrolyzed

Experimental

All chemicals and solvents were purchased from Sigma-Aldrich and used as received. For syntheses under anhydrous conditions solvents were additionally dried over 4Å molecular sieves. $POCl_3$ and $PSCl_3$ were distilled prior to use. Silica gel column chromatography was performed on Kieselgel 60Å (230-400 mesh, 40-63µm) Thin Layer Chromatography (TLC) analysis was carried out on pre-coated Silica Gel 60Å on aluminum foil with fluorescence indicator 254 nm (Sigma-Aldrich). N^2,N^2,N^7-Trimethylguanosine 5'-monophosphate P-imidazolide (m_3GMP-Im) (**16**) and N^2,N^2,N^7-trimethylguanosine 5'-diphosphate P-imidazolide (m_3GDP-Im, **17**) were prepared as described by Honcharenko *et al.*[28] N^2,N^2,N^7-Trimethylguanosine 5'-methylene(bisphosphonate) (m_3GpCH$_2$p, **18**), P1-(N^2,N^2-dimethylguanosine-5'-yl)imido-diphosphate (m^3GpNHp, **19**) and N^2,N^2,N^7-trimethylguanosine 5'-(2-thiodiphosphate), (m_3GDPβS, **20**) were prepared as described recently[19] Dichlorophosphorylphosphorimidoyl trichloride (PCl_3NPOCl_2) was prepared as described previously by Tomasz *et al.*[33] with the exception that it was used in a liquid form for further reactions as problems with its crystallization occurred.[33] 2 Azidoethylamine[31] and allyl bromoacetate[31] were synthesized as previously described. Synthesized nucleotides were purified by ion-exchange chromatography on DEAE-Sephadex A-25 (HCO_3^-form) column. A column was loaded with reaction mixture and washed through with excess of water to remove metal (II) salt/EDTA complex. Then, the nucleotides were eluted using a linear gradient of triethylammonium bicarbonate (TEAB) in deionized water. After evaporation under reduced pressure with repeated additions of ethanol to decompose TEAB, compounds were isolated as triethylammonium (TEA) salts. Yields were calculated on the basis of either sample weight or (preferably) optical milliunits (opt.mu) of the product. Optical unit measurements were performed in 0.1 M phosphate buffer (pH 7 or pH 6 for m_3G nucleotides) at 260 nm. Analytical HPLC was performed on Agilent Tech. Series 1200 using Supelcosil LC-18-T HPLC column (4.6 x 250 mm, flow rate 1.3 mL/min) with a linear gradient 0–100% of methanol in 0.05 M ammonium acetate buffer (pH 5.9) in 15 min, UV-detection at 260 nm and

fluorescence detection (excitation at 260 nm and detection at 370 nm). Semi preparative HPLC was performed on the same apparatus equipped with Discovery RP Amide C-16 HPLC column (25cm x 21.2 mm, 5µm, flow rate 5.0 mL/min) with linear gradients of acetonitrile in 0.05 M ammonium acetate buffer (pH 5.9) and UV-detection at 260 nm. The structure and homogenity of each final product was confirmed by RP HPLC, high resolution mass spectrometry HRMS (ESI-), $^{1}H/^{31}P$ NMR and FTIR spectroscopy. Intermediate products were characterized by low resolution MS (ESI-) or NMR. FTIR spectra were recorded on IRPrestige-21 spectrophotometer (Shimadzu Scientific Instruments) using ATR mode (4 cm^{-1} spectral resolution). Mass spectra were recorded on Thermo Scientific LTQ Orbitrap Velos (high resolution) and AB Sciex API 3200 (low resolution) spectrometers. ^{1}H NMR and ^{31}P spectra were recorded at 25 °C on a Varian UNITY-plus spectrometer at 399.94 MHz and 161.90 MHz, respectively. ^{1}H NMR chemical shifts were reported to sodium 3-trimethylsilyl-[2,2,3,3-D4]-propionate (TSP) in D_2O as an internal standard. ^{31}P NMR chemical shifts were reported to 20% phosphorus acid in D_2O as an external standard. Solvents and chemical reagents were purchased from Sigma-Aldrich and used without any pre-treatment unless otherwise stated. The raw NMR files were processed using ACD/NMR processor Academic Edition, version 12.01, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2014.

Chemical syntheses

2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine (9).[30] Adenosine (**8**) (500 mg, 1.87 mmol) was dried with vacuum pumping prior to use. The nucleoside was dissolved in dry DMF (50 ml) at r.t. Sodium hydroxide (NaH, 4 eq. 179.6 mg, 7.48 mmol) was added and after 10 min also allyl bromoacetate (2 eq, 669.5 mg, 3.74 mmol) Stirring was continued for 1h and then 2-azidoethylamine (3 eq, 258.2 mg, 5.61 mmol) was added and the reaction mixture was stirred for 24h after which reaction mixture was concentrated on rotary evaporator. Crude product 9 was purified by column chromatography using a gradient of methanol in dichloromethane (from 0-10 %). Fractions containing product were collected and concentrated yielding compound **9** as a yellow solid. Yield **9** (70-80 %). R_t = 12.09 min; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.35 (1H, s), 8.25 (1H, s), 6.22 (d, 1H, J = 6.5 Hz), 4.68 (dd, 1H, J = 6.2, 5.2 Hz), 4.59 (dd, 1H, J = 5.1, 3.1 Hz), 4.34 (q, 1H, J = 3.1 Hz), 4.24 and 4.11 (ABq, 2H, J = 15.22 Hz), 3.88 (ddd, 2H, J = 2.74, 3.49, 12.95 Hz), 3.36-3.24 (m, 4H); FTIR-ATR, ν [cm^{-1}]: 2104.34 (N_3), HRMS (ESI^-) m/z 392,14309, calculated for $C_{14}H_{18}N_9O_5$; found 392.04209, $[M-H]^-$

2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-monophosphate (10).[30] 2'-O-(N-(2-azidoethyl)carbamoyl)-methyladenosine (**9**) (200 mg, 0.51 mmol) dried overnight in a vaccum dessicator over P_4O_{10} was suspended in trimethyl phosphate (10 ml) and place on ice-bath. After cooling to 0 °C freshly distilled $POCl_3$ (3 eq, 1.53 mmol, 234.85 mg) was added into the mixture and reaction was stirred at 0 °C until the disappearance of the starting material as determined by RP-HPLC (usually 3-4 h). Then, reaction was stopped by addition of 0.7 M TEAB (pH 7) or 1 M $NaHCO_{3aq}$ until neutral pH was reached. The crude product was purified by DEAE-Sephadex and isolated as TEA salts. Yield **10**: (4691 opt.mu, 255 mg, 75%) R_t = 9.34 min;^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.57 (s, 1H), 8.38 (s, 1H), 6.34 (d, 1H J = 4.5 Hz), 4.69-4.65 (m, 2H), 4.48-4.44 (m, 1H), 4.30 and 4.21 (ABq, 2H, J = 15.44 Hz), 4.23-4.11 (m, 2H), 3.43-3.33 (m, 4H); ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm] 0.59; (s, 1P); FTIR-ATR, ν [cm^{-1}]: 2104.34 (N_3); HRMS (ESI-) m/z 472.10942, calculated for $C_{14}H_{19}N_9O_8P$; found 472.10938 $[M-H]^-$.

2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-monothiophosphate (11). 2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine (**9**) (200 mg, 0.51 mmol) dried overnight in vaccum dessicator over P_4O_{10} was suspended in trimethyl phosphate (10 ml) and place on ice-bath. After cooling to 0 °C freshly distilled $PSCl_3$ (3 eq, 1.53 mmol, 259.33mg) was added into the mixture and reaction was stirred at 0 °C until the disappearance of the starting material as determined by RP-HPLC (usually 2-3 h). Then, reaction was stopped by addition of 0.7 M TEAB (pH 7) or 1 M $NaHCO_{3aq}$ until neutral pH was reached. The crude product was purified by DEAE-Sephadex and isolated as TEA salts. Yield **11**: (4043 opt.mu, 225 mg, 65%); R_t = 8.83 min; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.66 (1H, s), 8.36 (s, 1H), 6.32 (d, 1H, J = 5.23 Hz), 4.7 (dd, 1H, J = 5.15, 3.85 Hz), 4.65 (dd, 1H, J = 5.5, 4.8 Hz), 4.50-4.46 (m, 1H), 4.21 and 4.30 (ABq, 2H, J = 15.44 Hz), 4.26-4.17 (m, 2H), 3.44-3.33 (m, 4H); ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm] 51.34; (s, 1P); FTIR-ATR, ν [cm-1] 2106.27 (N_3); HRMS (ESI-) m/z 488.08658, calculated for $C_{14}H_{19}N_9O_7PS$; found 488.08654 $[M-H]^-$.

2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-phosphorimidazolide (12). 2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-monophosphate (10) (TEA salt, 100 mg, 0.15 mmol), imidazole (102.12 mg, 1.5 mmol, 10 eq.), and 2,2'-dithiodipyridine (99.14 mg, 0.45 mmol, 3 eq.) were mixed in 1.5 mL anhydrous DMF. Triethylamine (33.06 µL, 45.53 mg, 0.45 mmol, 3 eq.) and triphenylphosphine (118.03 mg, 0.45 mmol, 3 eq.) were added, and the mixture was stirred for 3 h. Reaction progress was monitored by ESI-MS. The product was precipitated from reaction mixture with anhydrous $NaClO_4$ (183.66 mg 1.5 mmol, 10 eq) solution in dry acetone (15 mL). After cooling at 4°C, the white precipitate was filtered, washed repeatedly with cold, dry acetone, and dried in vacuum dessicator over P_4O_{10}. Yield: 72 mg (93%): R_t = 13.48 min; FTIR-ATR, ν [cm^{-1}]: 2108.20 (N_3); HRMS (ESI-) m/z 522.13630, calculated for $C_{17}H_{21}N_{11}O_7P$; found 522.13762 $[M-H]^-$.

2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-diphosphate (13). 2'-O-(N-(2-azidoethyl)carbamoyl)-methyladenosine 5'-phosphorimidazolide (**12**) (100 mg, 0.18 mmol) was dissolved in anhydrous DMF (2 mL), and tris(triethylammonium)-phosphate (100 mg, 0.26 mmol) was added, followed by addition of $ZnCl_2$ (278.72 mg, 2.08 mmol), and the mixture was stirred at room temperature until the disappearance of the starting material as determined by RP-HPLC. Then, the reaction was stopped by addition of a solution of EDTA (607.36 mg, 2.08 mmol) in water (50 mL) and neutralized with 1M $NaHCO_3$. The crude product was purified by DEAE-Sephadex and isolated as TEA salts. Yield **13**: (1406

opt.mu, 97 mg, 61 %), R_t = 8.38 min; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.54 (s, 1H), 8.30 (s, 1H), 6.30 (d, 1H, J = 5.5 Hz), 4.74-4.70 (m, 1H), 4.66-4.62 (m, 1H), 4.47-4.43 (m, 1H), 4.29 and 4.17 (ABq, 2H, J = 15.73 Hz), 4.26-4.23 (m, 2H), 3.40-3.30 (m, 4H); ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm] -10.19 (s, 1P α); -10.98 (s, 1P β) FTIR-ATR, ν [cm-^{1}] 2106.27 (N_3); HRMS (ESI-) m/z 552.07575, calculated for $C_{14}H_{20}N_9O_{11}P_2$; found 552.07470 $[M-H]^-$.

2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-methylene(bisphosphonate) (14). 2'-O-(N-(2-azidoethyl)-carbamoyl)methyladenosine (**9**) (200 mg, 0.51 mmol) dried overnight in vaccum dessicator over P_4O_{10} was suspended in trimethyl phosphate (10 ml) and place on ice-bath. After cooling to 0 °C methylene bis(phosphonic dichloride) $(CH_2(POCl_2)_2$, 3 eq, 1.53 mmol, 382.16 mg) was added into the mixture and reaction was stirred at 0°C until the disappearance of the starting material as determined by RP-HPLC (usually 2-3 h). Then, reaction was stopped by addition of 0.7 M TEAB (pH 7) or 1 M $NaHCO_{3aq}$ until neutral pH was reached. The crude product was purified by DEAE-Sephadex and isolated as TEA salts. Yield: (3727 opt.mu, 257 mg, 59 %), R_t = 7.77 min; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.64 (s, 1H), 8.42 (s, 1H), 6.31 (d, 1H, J = 4.5 Hz), 4.71 (dd, 1H, J = 5.23, 4.98 Hz), 4.63 (dd, 1H, J = 4.90, 4.73 Hz), 4.46-4.42 (m, 1H), 4.31 and 4.26 (ABq, 2H, J = 15.44 Hz), 4.32-4.18 (m, 2H), 3.47-3.37 (m, 4H), 2.24 (t, 2H, J = 19.67 Hz); ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm] 18.64,(bs, P-α) 15.59; (m, P-β); FTIR-ATR, ν [cm^{-1}] 2106.27 (N_3); HRMS (ESI-) m/z, 550.09649 calculated for $C_{15}H_{22}N_9O_{10}P_2$; found 550.09657 $[M-H]^-$.

2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine 5'-imidodiphosphate (15). 2'-O-(N-(2-azidoethyl)carbamoyl)-methyladenosine (**9**) (200 mg, 0.51 mmol) dried overnight in vaccum dessicator over P_4O_{10} was suspended in trimethyl phosphate (10 ml) and place on ice-bath. After cooling to 0 °C dichlorophosphoryl-phosphorimidoyl trichloride ($Cl_3PNP(O)Cl_2$, 3 eq, 1.53 mmol, 871.33 mg) was added into the mixture and reaction was stirred at 0 °C until the disappearance of the starting material as determined by RP-HPLC (usually 4-5 h). Then, reaction was stopped by addition of 0.7 M TEAB (pH 7) or 1 M $NaHCO_{3aq}$ until neutral pH was reached. The crude product was purified by DEAE-Sephadex and isolated as TEA salts. Yield: (3089 opt.mu, 213 mg, 49 %), Rt = 8.26 min; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.61 (s, 1H), 8.35 (s, 1H), 6.31 (d, 1H, J = 4.98 Hz), 4.73 (dd, 1H, J = 4.73, 4.48 Hz), 4.64 (t, 1H, J = 4.98 Hz), 4.48-4.44 (m, 1H), 4.32 and 4.23 (ABq, 2H, J = 15.44 Hz), 4.24-4.18 (m, 2H), 3.44-3.33 (m, 4H), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm] 0.45,(s, P α), -1.07; (s, P β); FTIR-ATR, ν [cm^{-1}] 2104.34 (N_3); HRMS (ESI-) m/z, 551.09173 calculated for $C_{14}H_{21}N_{10}O_{10}P_2$; found 551.09150 $[M-H]^-$.

1. General procedure using m_3G-imidazolides (Scheme 2)

m_3GMP-Im, (**16**) or m_3GDP-Im, (**17**) (1.5 eq) and appropriate phosphates **13**-**15** or **11** were suspended in anhydrous DMF (1.0 mL) followed by addition of anhydrous $ZnCl_2$ (38.88 mg, 10 eq, 0.29 mmol). The mixture was vigorously shaken until the reagents dissolved. The reaction progress was monitored by RP-HPLC. After completion, (24 h) appropriate amount of EDTA solution (Na_2EDTA, 0.25 M, 1.16 mL, 0.29 mmol) was added to disassociate the nucleotide-metal complex, adjusted to pH 6 with solid $NaHCO_3$, followed by purification by DEAE-Sephadex and isolated as TEA salts. Triethylammonium salts were then repurified by semipreparative RP-HPLC and after repeated freeze-drying, were isolated as ammonium salts.

2. General procedure using imidazolide 12 or *in situ* activation with CDI (Scheme 3)

a) *in situ* activation using CDI

AMP-link-N_3 (**10**), (460 opt.mu, TEA salt, 25 mg, 0.037 mmol) was placed in a 10 mL microwave tube and suspended in anhydrous DMF (1.0 ml) followed by addition of carbodiimidazole (CDI, 5eq, 30.11 mg, 0.185 mmol). The tube was heated for 20 min in the microwave oven using dynamic power mode (parameters: P_{max} = 5 W and T_{max} 40 ± 1°C). After the reaction completion an excess of CDI was decomposed by addition of water (5eq, 3,35 µl, 0.185 mmol).

Then, to compound **12** or *in situ* activated compound **10** appropriate nucleotide **18, 19**, or **20** followed by anhydrous $ZnCl_2$ (75.65 mg, 10 eq, 0.55 mmol) were added. The reaction progress was monitored by RP-HPLC. After completion, (24h) appropriate amount of EDTA solution (Na_2EDTA, 0.25 M, 2.2 mL, 0.55 mmol) was added to disassociate the nucleotide-metal complex, adjusted to pH 6 with solid $NaHCO_3$, followed by purification by DEAE-Sephadex and isolated as TEA salts. Triethylammonium salts were then repurified by semipreparative RP-HPLC and after repeated freeze-drying, were isolated as ammonium salts.

P1-(2,2,7-trimethylguanosin-5'-yl) P3-[2'-O-(N-(2-azidoethyl)-carbamoyl)methyl-adenosine-5'-yl] -triphosphate; m_3GpppAdo-link-N_3 (1).[30] (469 opt.mu, 24 mg, 65 %, TEA salt), (237 opt.mu, 9.7 mg, 51 %, NH_4^+ salt), was obtained starting from (ADP-link-N_3, TEA salt, 362 opt.mu, 25 mg, 0.029 mmol) and m_3GMP-Im, (Na salt, 21.5 mg, 0.044 mmol, 1.5 eq) following the general procedure 1. R_t = 7.54 min; ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 8.90 (s, 1H), 8.24 (s, 1H), 8.15 (s, 1H), 6.01 (d, 1H, J= 5.2 Hz), 5.82 (d, 1H, J = 3.2 Hz), 4.69-4.63 (m, 2H), 4.53-4.48 (m, 2H), 4.44 (bs, 1H), 4.39–4.19 (m, 7H), 4.09 (s, 3H); 3.31-3.21, (m, 4H), 3.06 (s, 6H), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: -11.43 (m, 2P, P-α, P-γ), -23.05 (t, 1P, P-β, J= 17.2 Hz); FTIR-ATR, ν [cm^{-1}] 2106.27 (N_3); HRMS (ESI-) m/z, 939.17014 calculated for $C_{27}H_{38}N_{14}O_{18}P_3$; found 939.16821 $[M-2H]^-$.

P1-(2,2,7-trimethylguanosin-5'-yl) P3-[2'-O-(N-(2-azidoethyl)-carbamoyl)methyladenosine-5'-yl]-1,2-methylene-triphosphate; m_3GppCH_2pAdo-link-N_3 (2). (450 opt.mu, 23 mg, 63 %, TEA salt); (149 opt.mu, 6.1 mg, 26 %, NH_4^+ salt), was obtained starting from (pCH_2p-Ado-linker-N_3, TEA salt, 359 opt.mu, 25 mg, 0.029 mmol), and m_3GMP-Im, (Na salt, 21.5 mg, 0.044 mmol, 1.5 eq) following the general procedure 1. R_t = 9.38 min; ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 9.11 (s, 1H), 8.53

(s, 1H), 8.33 (s, 1H), 6.19 (d, 1H, J= 4.0 Hz), 5.98 (d, 1H, J = 3.5 Hz), 4.65-4.59 (m, 2H), 4.47-4.41 (m, 3H), 4.40–4.19 (m, 7H), 4.10 (s, 3H); 3.41-3.37, (m, 4H), 3.16 (s, 6H), 2.43 (td, 2H, J = 20.42, 3.49 Hz), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: 17.08 (m, 1P, P-β), 8.74 (d, 1P, J = 16.11 Hz, P-γ), -10.79 (m, 1P, J = 23.19 Hz, P-α); FTIR-ATR, ν [cm^{-1}] 2110.12 (N_3); HRMS (ESI-) m/z, 937.19870 calculated for $C_{28}H_{40}N_{14}O_{17}P_3$; found 937.18983 [M-2H]$^-$.

P1-(2,2,7-trimethylguanosin-5'-yl) P3-[2'-O-(N-(2-azidoethyl)-carbamoyl)methyladenosine-5'-yl]-1,2-imidotriphosphate; m_3GppNHpAdo-link-N_3 (3). (293 opt.mu, 15 mg, 40 %, TEA salt) (81 opt.mu, 3.3 mg, 27 %, NH_4^+ salt), was obtained starting from (pNHp-AMP-link-N_3, TEA salt, 395 opt.mu, 25 mg, 0.029 mmol) and m_3GMP-Im, (Na salt, 21.5 mg, 0.044 mmol, 1.5 eq) following the general procedure 1. R_t = 9.33 min; ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 9.02 (s, 1H), 8.43 (s, 1H), 8.25 (s, 1H), 6.11 (d, 1H, J= 4.73 Hz), 5.92 (m, 1H), 4.61-4.55 (m, 2H), 4.45-4.33 (m, 4H), 4.28–4.08 (m, 6H), 4.05 (s, 3H); 3.34-3.29, (m, 4H), 3.12 (s, 6H), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: -0.8 (m, 1P, P-α), -10.79 (m, 2P, P-β, P-γ); FTIR-ATR, ν [cm^{-1}] 2108.20 (N_3); HRMS (ESI-) m/z, 938.18612 calculated for $C_{27}H_{39}N_{15}O_{17}P_3$; found 938.18545 [M-2H]$^-$.

P1-(2,2,7-trimethylguanosin-5'-yl) P3-[2'-O-(*N*-(2-azido-ethyl)carbamoyl)methyladenosine-5'-yl]-2,3-methylene-triphosphate; m_3GpCH_2ppAdo-link-N_3 (4) (547 opt.mu, 28 mg, 60 % TEA salt); (152 opt.mu, 6.2 mg, 27.7 %, NH_4^+ salt) was obtained starting from m_3GpCH_2p, (**18**) (707 opt.mu, TEA salt, 43.56 mg, 0.056 mmol, 1.5 eq) and AMP-link-N_3, (**10**) (460 opt.mu, TEA salt, 25 mg, 0.037 mmol) following the general procedure 2. R_t = 9.39 min;^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 9.29 (s, 1H), 8.55 (s, 1H), 8.37 (s, 1H), 6.23 (d, 1H, J= 4.5 Hz), 6.03 (d, 1H, J = 3.2 Hz), 4.69-4.63 (m, 2H), 4.53-4.48 (m, 2H), 4.44 (bs, 1H), 4.39–4.19 (m, 7H), 4.09 (s, 3H); 3.43-3.36, (4H, m), 3.18 (s, 6H), 2.43 (t, 2H, J = 20.17 Hz), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: 17.29 (m, 1P, P-β), 7.76 (m, 1P, P-α), -10.93 (m, 1P, P-γ); FTIR-ATR, ν [cm^{-1}] 2101.34 (N_3); HRMS (ESI-) m/z, 937.19087 calculated for $C_{28}H_{40}N_{14}O_{17}P_3$; found 937.19045 [M-2H]$^-$.

P1-(2,2,7-trimethylguanosin-5'-yl) P3-[2'-O-(N-(2-azidoethyl)-carbamoyl)methyladeno-sine-5'-yl]-2,3-imidotriphosphate; m_3GpNHppAdo-link-N_3 (5). (371 opt.mu, 19 mg, 40 %, TEA salt); (132 opt.mu, 5.4 mg, 35 %, NH_4^+ salt), was obtained starting from m_3GpNHp, (**19**) (707 opt.mu, TEA salt, 43.62 mg, 0.056 mmol, 1.5 eq) and AMP-link-N_3, (**10**) (460 opt.mu, TEA salt, 25 mg, 0.037 mmol) following the general procedure 2. R_t = 9.73 min; ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 9.13 (s, 1H), 8.36 (s, 1H), 8.24 (s, 1H), 6.11 (d, 1H, J = 4.73 Hz), 5.91 (m, 1H), 4.62-4.55 (bs, 2H), 4.48-4.10 (m, 10H), 4.08 (s, 3H); 3.39-3.31, (m, 4H), 3.13 (s, 6H), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: -0.87 (m, 1P, P-α), -11.00 (m, 2P, P-β, P-γ); FTIR-ATR, ν [cm^{-1}] 2108.20 (N_3); HRMS (ESI-) m/z, 938.18612 calculated for $C_{27}H_{39}N_{15}O_{17}P_3$; found 938.18215 [M-2H]$^-$.

P1-(2,2,7-trimethylguanosin-5'-yl) P3-[2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine-5'-yl]-2-thio-triphosphate; m_3Gpp_spAdo-link-N_3 (6a,b) (480 opt.mu, 24.9 mg, yield 53 %, mixture of diastereoisomers as a triethylammonium salt); D1 (212 opt.mu, 8.8 mg, 44 %, NH4+ salt) and D2 (159 opt.mu, 6.6 mg, 33 %, NH_4^+ salt) were obtained starting from (Im AMP-link-N_3, Na salt, 30.5 mg, 0.056 mmol, 1.5 eq) and m_3GDP-βS, (TEA salt, 30.0 mg, 0.037 mmol) following the general procedure 2. **D1**: ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 8.33 (s, 1H), 8.18 (s, 1H), 6.07 (d, 1H, J= 5.48 Hz), 5.89 (d, J= 3.52 Hz), 4.59 (dd, 1H, J=4.70, 4.30 Hz), 4.52 (dd, 1H, J=4.30, 3.91 Hz), 4.45-4.39 (m, 4H), 4.37–4.22 (m, 4H), 4.17 and 4.05 (ABq, 2H, J= 15.26 Hz), 4.02 (s, 3H), 3.31-3.32, (m, 4H), 3.09 (s, 6H), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: 29.89 (m, 1P, P-β), -12.46 (t, 2P, J = 27.88, P-α, P-γ); FTIR-ATR, ν [cm^{-1}] 2104.34 (N_3); HRMS (ESI-) m/z, 955.14729 calculated for $C_{27}H_{38}N_{14}O_{17}P_3S$; found 955.14670 [M-2H]$^-$. **D2**: ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 8.30 (s, 1H), 8.16 (s, 1H), 6.04 (d, 1H, J= 5.48 Hz), 5.84 (d, J= 3.91 Hz), 4.57 (dd, 1H, J=4.30, 4.70 Hz), 4.52 (t, 1H, J=4.30), 4.43-4.33 (m, 4H), 4.29–4.21 (m, 4H), 4.17 and 4.05 (ABq, 2H, J= 15.26 Hz), 4.03 (s, 3H), 3.32-3.23, (m, 4H), 3.08 (s, 6H), ^{31}P NMR (162 MHz, D_2O, 25 °C): δ [ppm]: 29.50 (t, 1P, J= 24.94 Hz, P-β), -12.71 (dd, 2P, J = 24.94 Hz, P-α, P-γ); FTIR-ATR, ν [cm^{-1}] 2106.27 (N_3); HRMS (ESI-) m/z, 955.14729 calculated for $C_{27}H_{38}N_{14}O_{17}P_3S$; found 955.14711 [M-2H]$^-$.

P1-(2,2,7-trimethylguanosin-5'-yl) P3-[2'-O-(N-(2-azidoethyl)carbamoyl)methyladenosine-5'-yl]-3-thiotriphosphate; m_3Gppp_sAdo-link-N_3. (7a,b) (542 opt.mu, 28.1 mg, yield 60%, mixture of diastereoisomers as a triethylammonium salt); D1 (152 opt.mu, 6.3 mg, 28.0 %, NH_4^+ salt) and D2 (111 opt.mu, 4.6 mg, 20.5 %, NH_4^+ salt) were obtained starting from (AMPS-link-N_3, TEA salt, 446 opt.mu, 25.0 mg, 0.036 mmol) and m_3GDP-Im, (Na salt, 31.0 mg, 0.054 mmol, 1.5 eq) following the general procedure 1. **D1**: ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 9.01 (s, 1H), 8.43 (s, 1H), 8.24 (s, 1H), 6.12 (d, 1H, J= 5.45 Hz), 5.95 (d, J= 3.44 Hz), 4.64, (t, 1H, J=4.48), 4.58 (t, 1H, J=4.23 Hz), 4.47-4.27 (m, 8H), 4.23 and 4.11 (ABq, 2H, J= 15.44 Hz), 4.08 (s, 3H); 3.40-3.28, (m, 4H), 3.14 (s, 6H), ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: 43.67 (d, 1P, J = 21.63 Hz, P-α), -11.45 (d, 1P, J = 16.60 Hz, P-γ), -23.87, -24.01 (2d, 1P, J = 20.75, 21.97 Hz, P-β); FTIR-ATR, ν [cm^{-1}] 2106.27 (N_3); HRMS (ESI-) m/z, 955.14729 calculated for $C_{27}H_{38}N_{14}O_{17}P_3S$; found 955.14762 [M-2H]$^-$. **D2**: ^{1}H NMR (400 MHz, D_2O, 25°C): δ [ppm] 9.00 (s, 1H), 8.46 (s, 1H), 8.24 (s, 1H), 6.12 (d, 1H, J= 5.23 Hz), 5.92 (d, J= 3.49 Hz), 4.60, (dd, 1H, J=4.48, 4.73 Hz), 4.55 (dd, 1H, J=4.23, 3.98 Hz), 4.53-4.35 (m, 6H), 4.34-4.23 (m, 2H), 4.22 and 4.12 (ABq 2H, J= 15.69 Hz), 4.07 (s, 3H); 3.39-3.28, (m, 4H), 3.13 (s, 6H); ^{31}P NMR (162 MHz, D_2O, 25°C): δ [ppm]: 43.95 (d, 1P, J = 23.68 Hz, P-α), -11.47 (d, 1P, J = 16.36 Hz, P-β), -23.76, -23.89 (2d, 1P, J = 22.95, 20.02 Hz, P-γ) FTIR-ATR, ν [cm^{-1}] 2108.20 (N_3); HRMS (ESI-) m/z, 955.14729 calculated for $C_{27}H_{38}N_{14}O_{17}P_3S$; found 955.14770 [M-2H]$^-$.

Synthesis of 5'-*hexynyl*-GCUAAU (21)

RNA synthesis was performed on AKTA Oligopilot plus 10 synthesizer (GE Healthcare) on a 5 µmol scale using a 1.2 mL column filled with commercially available solid support – Custom Primer Support™ Ribo U 40 from GE Healthcare (Primer support 200 at 39 µmol/g). The detritilation reagent was 3% (v/v) dichloroacetic acid in toluene (Novabiochem® Deblocking Reagent from Merck). In the coupling step, the 20 equivalents

of appropriate acetyl base protected 2'-O-TBDMS phosphoramidate (rA^{Ac}, rC^{Ac}, rG^{Ac}, rU from ChemGenes) and 0.30 M 5-(benzylthio)-1H-tetrazole in acetonitrile (Novabiochem® Activator Reagent from Merck) were recirculated through the column for 15 minutes. The last coupling was performed in the same conditions using hexynyl phosphoramidate (ChemGenes), yet the detritilation step was omitted due to lack of DMT-tag in this phosphoramidate. As an oxidizing reagent, 0.05M solution of iodine in pyridine (Novabiochem® Oxidizing Reagent from Merck) was employed. Finally, RNA still on the solid support was treated with 20% (v/v) diethylamine in acetonitrile (Novabiochem® Merck) to remove 2-cyanoethyl protecting groups. Solid support was washed with acetonitrile, dried with air and transferred to a 50 mL plastic tube. Functionalized oligonucleotide was released from solid support and base protecting groups were removed using 5 mL of 1:1 mixture of 25% aqueous ammonia and 40% aqueous methylamine (AMA). After 60 minutes of incubation at 60°C the solution was filtered off and the support was washed with 50% ethanol, twice. Combined filtrates were evaporated, lyophilized from water and re-dissolved in 500 µL of DMSO. 625 µL of triethylammonium trihydrofluoride was added and the sample was shaken and incubated at 65 °C for 2.5 hours. Then, 125 µL of 3M sodium acetate was added followed by 5 mL of 1-butanol and the mixture was placed at -80°C for 30 minutes. Precipitate of oligonucleotide sodium salt was centrifuged, lyophilized from water and used for click reaction without additional purification.

Conjugation of the ''clickable'' m_3G-cap constructs (1-7) to the 5'-*hexynyl*-GCUAAU (21)

5'-*hexynyl*-GCUAAU (1.0 OD, 28 µg, 14 nmol, 3 eq), dissolved in (in 15.5 µL of H_2O:DMSO (1:1) was mixed with m_3G-cap **1-7** (0.1 OD, 4.5 µg, 4.6 nmol, 1.5 ml, 3 mM). Then Cu-TBTA complex (1.0 ml, 10 mM in 55 vol % DMSO to final concentration of 0.5 mM) and sodium ascorbate (2.0 ml, 5 mM in H_2O to final concentration of 0.5 mM). The solution was gently agitated on a vortex, centrifuged and termostated at 37 °C for few days (3-5). Purification was done by analytical RP-HPLC using a linear gradient of buffer B in A from 0 to 100% B in 15 min, detection at 260 nm.

FBS stability assay

For the serum stability assay, 100 µM stock solutions of compounds (18–27) in MilliQ water were prepared. Then, 100 µL of respective stock solution, 890 µL of PBS buffer (pH 7.2, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and 10 µL of FBS were mixed in 1.5 mL HPLC vials, so each solution contained 2.5 % of FBS and the final compound concentration was 10 µM. Immediately after FBS addition, the reaction mixtures were set up in a thermostated compartment maintained at 37 °C, and 100 µL injections were made with an automated sample injector at various time points. Usually, a single sample set covered unmodified cap analog **1** and a pair of α/β and β/γ isomers, or D1 and D2 isomers bearing the same modification, so that each compound could be injected at 8 different time points at 60 min intervals. Afterwards, each assay column was washed with methanol for at least 1h before the next analysis. Based on the integration of the remaining cap analog and resultant products, substrate conversion for each compound was estimated using the equation: $\Sigma(AUCp/\varepsilon)/\Sigma[(AUCp/\varepsilon)+AUCs/\varepsilon]\varepsilon$ where AUC(p,s) – area under curve of products (p) and starting material (s), ε-molar extinction coefficient. Extinction coefficient values used for calculations were as follows: for trimethylated products ε = 12640 $M^{-1}cm^{-1}$, for adenosine analogs ε = 12400 $M^{-1}cm^{-1}$ and for the m_3G-cap analogs ε = 24250 $M^{-1}cm^{-1}$. The obtained data were analyzed using OriginPro software v. 9.1

Protein expression and purification

Human Nudt16 protein (hNudt16, 1-199 aa, MW 22 kDa) was expressed in E. coli Rosetta2 (DE3) as a C-terminally His-tagged protein using a pET16b_Nudt16 vector, where the coding sequence of Nudt16 was cloned at the NcoI-BamHI restriction sites (verified further by sequencing). A sequence encoding four additional histidines was introduced just after the two terminal histidines of Nudt16 what resulted in a 6xHis C-terminal His-tag. Expression was induced at OD600 ~ 0.6 with 0.2 mM IPTG and after 4 hours incubation at 37°C bacterial cells were pelleted and washed in PBS buffer. Subsequently, the pelleted cells were resuspended in a lysis buffer (20 mM HEPES-KOH pH 8.0, 300 mM NaCl, 300 mM Urea, 10 mM imidazole, 10% glycerol, 1% Triton 100) supplemented with lysozyme, incubated on ice for 30 minutes and then disrupted by sonication. After centrifugation (17 500G for 30 min), the supernatant was added to NTA-agarose equilibrated with 20 mM HEPES buffer (20 mM HEPES-KOH pH 8.0, 300 mM NaCl, 10% glycerol and 5 mM imidazole) and incubated for 1 hour in a cold room with gentle stirring. Unbound proteins were removed by washing with 20 mM Tris HCl pH 8.0, 300 mM NaCl (10 x 2mL). The His-tagged hNudt16 protein was eluted with the increasing concentrations of imidazole (20 mM to 300 mM) in wash buffer (20 mM Tris HCl pH 8.0, 300 mM NaCl). The collected fractions of pure hNudt16 protein were dialyzed against 50 mM Tris-HCl pH 8.0, 150 mM KCl and 20% glycerol. Aliquots of purified hNudt16 were supplemented with 1 mM DTT, frozen in liquid nitrogen and stored at -80ºC.

Analysis of susceptibility of modified m_3G dinucleotides and capped ribooligonucleotides towards hNUDT16 decapping

Short m_3G-capped RNAs (m_3G-cap-GCUAAU) containing various cap structures (24-29) were chemically synthesized by using click chemistry approach. Decapping reactions with hNUDT16 of clicked ribooligonucleotides and cap dinucleotides were performed in a final volume of 10 µL in 40 mM Tris-HCl buffer (pH 7.9, 6 mM $MgCl_2$, 10 mM NaCl, 10mM DTT and 2 mM spermidine) for 1 hour at 30°C. Final concentration of hNudt16 in the reaction was 0.35 µM. To negative control reactions the same concentration of thermally inactivated hNUDT16 enzyme (for 8 minutes at at 100°C) was added. In the case of capped hex-oligo-RNA, around 500 pmoles of each substrate was present in the reaction (concentration of capped hex-oligo-RNA was estimated using PicoDrop spectrophotometer set up to oligo mode). In the case of dinucleotide analogs each substrate

was added to ~0.3mM concentration. Reactions were stopped by addition of equal volume of formazol and incubation for 5 min at 55°C. Reaction products were separated on 20 % denaturating polyacrylamide gel with 7M urea, and visualized by two methods: first by UV-shadowing using 254nm UV hand lamp and digital camera, and second - using ChemiDoc (Biorad) gel visualization system (with 302 nm UV lamp) set up to ethidium bromide mode.

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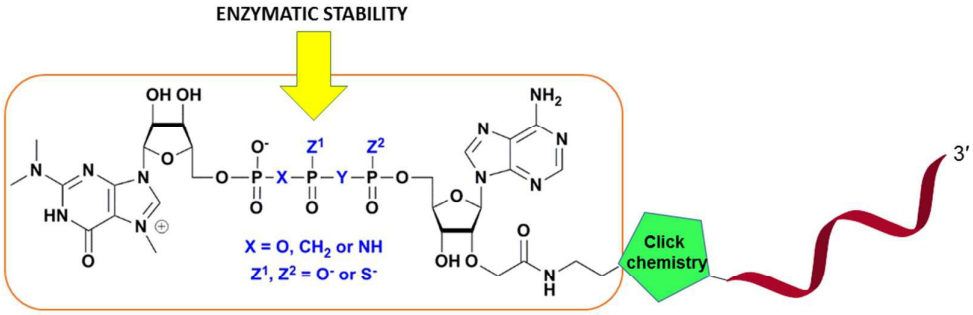

Phosphate-modified m3G cap analogs were synthesized, conjugated to RNA using “click chemistry”, and studied for susceptibility to hNUDT16 enzyme.