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PAPER

Synthesis of a new class of ribose functionalized dinucleotide cap analogues for biophysical studies on interaction of cap-binding proteins with the 5′ end of mRNA†

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mRNAs of primitive eukaryotes such as *Caenorhabditis elegans* and *Ascaris summ* possess two different caps at their 5′ terminus. They have either a typical cap which consists of 7-methylguanosine linked *via* a 5′,5′-triphosphate bridge to the first transcribed nucleotide (MMG cap) or an atypical hypermethylated form with two additional methyl groups at the N2 position (TMG cap). Studies on interaction between the 5′ end of mRNA and proteins that specifically recognize its structure have been carried out for several years and they often require chemically modified cap analogues. Here, we present the synthesis of five novel dinucleotide MMG and TMG cap analogues designed for binding studies using biophysical methods such as electron spin resonance (ESR) and surface plasmon resonance (SPR). New analogues were prepared by derivatization of the 2′,3′-*cis* diol of the second nucleotide in the cap structure with levulinic acid, and coupling of the obtained acetal through its carboxylic group with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino TEMPO), ethylenediamine (EDA) or (+)-biotinyl-3,6,9-trioxaundecanediamine (amine-PEO_3-biotin).

Introduction

All cellular eukaryotic mRNAs have at their 5′ end a unique structure (Fig. 1) known as a "cap" which consists 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).[1] The cap plays a crucial role in several cellular processes. It promotes pre-mRNA splicing,[2] enables transport of RNA from the nucleus to the cytoplasm,[3,4] protects mRNA from degradation by 5′ exonucleases[5] and is required for efficient translation.[1,6] During the initiation step of translation, the cap is specifically recognized by eIF4E (eukaryotic initiation factor 4E), the smallest subunit of a larger complex, eIF4F, which also consists of a RNA helicase, eIF4A, and a scaffolding protein, eIF4G.[7] eIF4F complex assembly is rate limiting for translation initiation and is largely dependent on eIF4E availability. Numerous studies have shown that eIF4E is a potent oncogene.[8,9] Ever since the discovery that increased eIF4E expression is associated with a variety of human cancers, many research groups have been investigating the mechanism of translation initiation and the possibility of exploiting eIF4E as a target for anticancer therapy.[9]

Early studies on the 5′ end of mRNA and its interaction with eIF4E using chemically modified mono- and dinucleotide cap analogues[10–12] were focused on specific parts of the cap structure essential for its function. Multidimensional nuclear magnetic resonance[13] and X-ray crystallography,[14–16] in which m^7GDP, m^7GTP and m^7GpppG/A had been used as a model representing the functional 5′ terminus of mRNA, complemented with biophysical experiments,[16–18] showed that binding the MMG cap to mammalian eIF4E is facilitated by charge-related interactions inside the cap-binding center and protein conformational changes. The former are accomplished by: sandwich cation–π stacking of methylated guanine with protein aromatic side chains, hydrogen bonds between nitrogen atoms N1 and/or N2 of m^7G base and acidic residues, and salt bridges between the negative charged phosphate chain of the cap and basic residues of the eIF4E. Crystal structures of other proteins that specifically recognize the 5′ end of mRNA, *e.g.* nuclear Cap Binding Complex (CBC),[19,20] vaccinia viral cap-dependent 2′-*O*-methyltransferase VP39[21] and

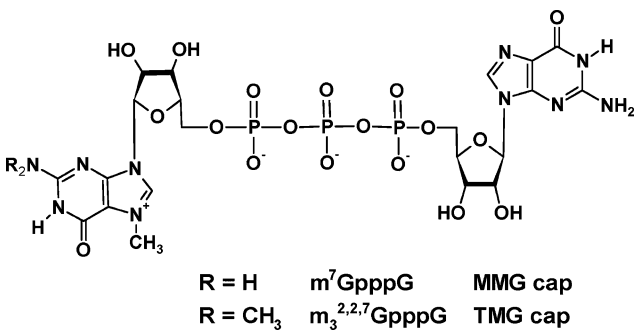

Fig. 1 mRNA cap structures.

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the PB2 subunit of influenza virus polymerase,[22] show a lack of structural similarity but reveal the same strategy in binding to the cap structure. In some cases, interaction of 5′ capped mRNAs with cap-binding proteins may be affected by downstream base of the cap, *e.g.* the CBC binds dinucleotide cap analogue m^7GpppG with ~10-fold higher affinity than m^7GTP.[23]

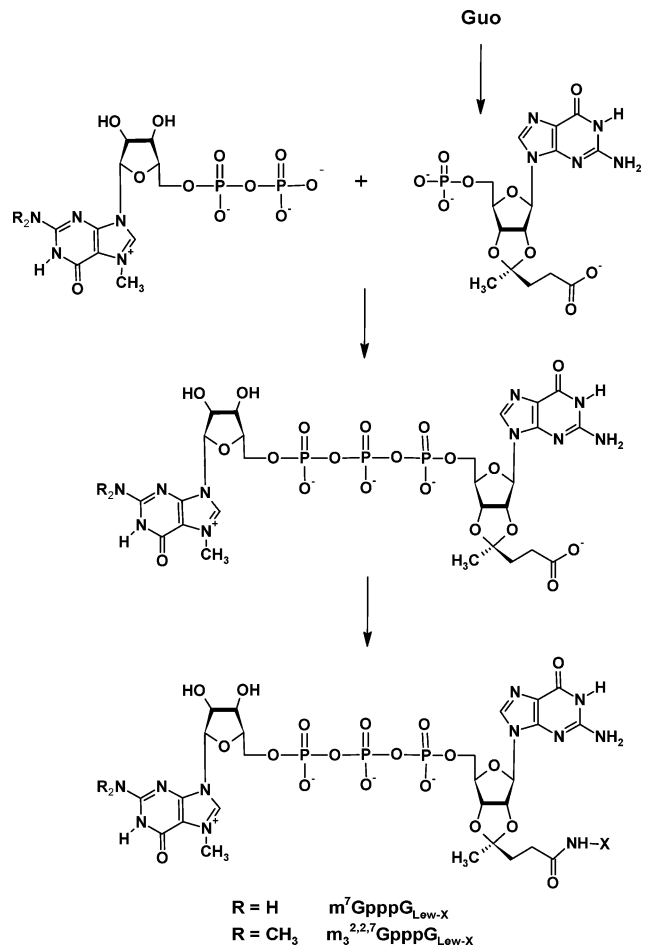
Some primitive eukaryotes such as *Caenorhabditis elegans* and *Ascaris suum*, possess at their 5′ terminus an atypical hypermethylated form of the cap (Fig. 1), with two additional methyl groups at the N2 position of 7-methylguanosine ($m_3^{2,2,7}$GpppN, TMG cap).[24,25] This atypical cap is added with a conserved splice leader sequence (SL) to pre-mRNA during *trans*-splicing to form the mature 5′ end of mRNA.[26,27] Consequently, two types of mRNA are present in the cell, *non-trans*-spliced with the MMG cap and *trans*-spliced with the 5′ spliced leader sequence capped with the TMG cap. In general, eIF4E proteins from higher eukaryotes are unable to effectively recognize and bind the TMG cap.[16] However, *C. elegans* and *A. suum* efficiently translate TMG capped mRNAs,[28,29] suggesting that eIF4E of these organisms may differ from that of other species. Identification and exploration of five eIF4E isoforms in *C. elegans*[30,31] provided evidence that the 4E isoforms vary in their ability to distinguish between MMG and TMG caps. Other parasitic nematodes, *Ascaris suum* and *Brugia malayi*, may contain fewer eIF4E isoforms (three have been found so far in their genome) but they still reveal adaptations that allow them to interact with the atypical TMG cap.[28] Although the problem of differential binding of 4E isoforms to MMG/TMG capped RNAs and their contribution to the translation of *trans*-spliced transcripts is intensively investigated,[28,29,32–34] the exact molecular mechanism describing the interaction of each isoform binding either cap remains unknown. Structural data provided by X-ray crystallography and NMR along with solution studies using various biophysical methods are necessary to elucidate 4E's dual specificity.

A wide variety of physicochemical techniques have been applied to examine protein–ligand interactions. Some of these methods identify interaction of target molecules with specially prepared ligands bound covalently/non-covalently to the matrix which enable the analysis of biomolecular surface interactions using an appropriate physical method, for example surface plasmon resonance (SPR).[35,36] Others use neutral markers—for example in electron spin resonance (ESR), nonreactive radicals—attached to specific sites of the biological molecules thus providing information of the probes environment.[37,38] All such methods are suitable to gain a comprehensive insight into the dynamic nature of the 4E isoforms and explore their interaction with the RNA 5′ terminus.

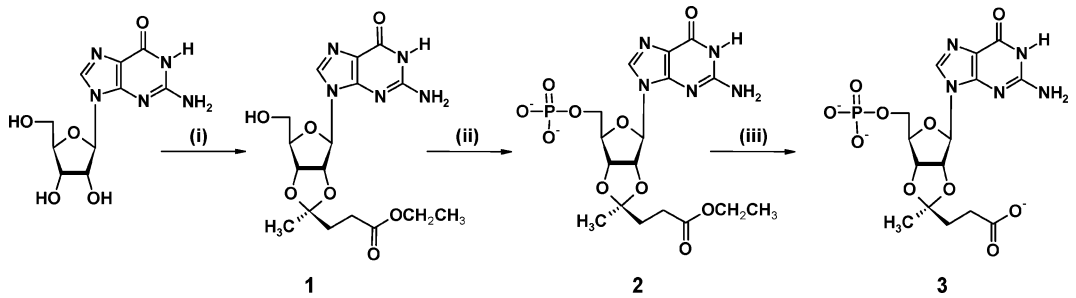
In order to examine the phenomenon of dual specificity of nematode cap-binding proteins for mono- and trimethylated 5′ terminal structures of mRNA, obtaining novel cap analogues, specially designed for different biophysical assays, has become crucial. Synthesis of 7-methylguanosine 5′-triphosphate (m^7GTP) with nitroxide radical TEMPO introduced into a mononucleotide through the γ-phosphate moiety, prepared for the needs of electron spin resonance spectroscopy, has been reported.[39] Here we describe the synthesis of the first dinucleotide cap analogues bearing various molecules connected within the cap structure which will be an excellent tool for cap–protein interaction studies. Our methodology is based on derivatization of the 2′,3′-*cis* diol of the second nucleotide in the cap structure with levulinic acid, and coupling of the obtained acetal through its carboxylic group with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino TEMPO), ethylenediamine (EDA) or (+)-biotinyl-3,6,9-trioxaundecanediamine (amine-PEO_3-biotin).

Results and discussion

Numerous biophysical studies on interaction between the 5′ end of mRNA and cap-binding proteins require specially designed dinucleotide cap analogues containing probes that are suitable for the given method. Reactive groups, linkers or markers can be incorporated into the cap structure through the heterocyclic base, phosphate, or sugar moiety. Taking into account that introduced modifications should not violate examined interactions and considering binding properties of the cap (see above),[14–20] we propose universal functionalization of the ribose moiety which makes a possible its further modification. Our methodology (Scheme 1) is based on derivatization of the 2′,3′-*cis* diol of one nucleoside, being exactly second in the cap structure, with ethyl levulinate, its 5′ phosphorylation, deprotection and coupling with an appropriate mono- or trimethylated diphosphate unit. Presence of the free carboxyl group in levulinic acid is further used for introduction of three different probes: 4-amino TEMPO (a paramagnetic nitroxide spin label for ESR studies), EDA and amine-PEO_3-biotin (for SPR immobilization).



Scheme 1 Synthetic strategy for the preparation of levulinate derivatives of dinucleotide cap analogues modified with different probes (X).



Scheme 2 Synthesis of the 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate (LewGMP); (i) ethyl levulinate, *p*-toluenenesulfonic acid, triethyl orthoformate, (ii) $POCl_3$, trimethyl phosphate, (iii) 1 M NaOH.

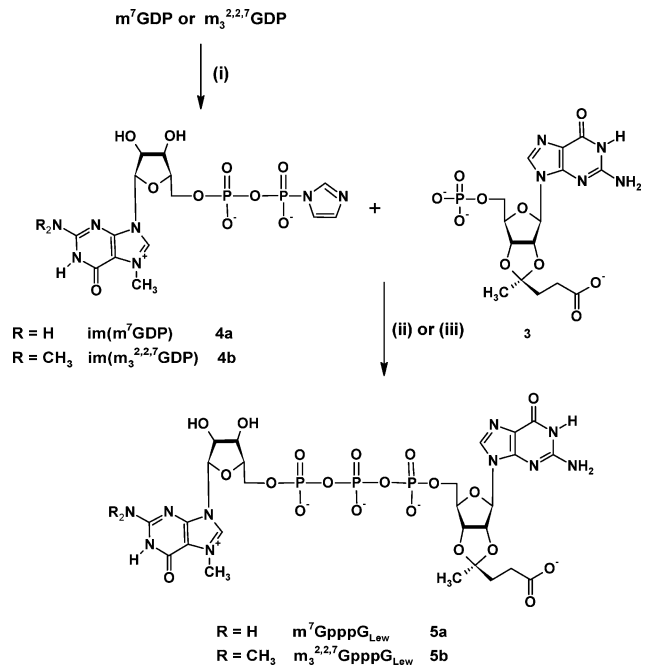
Synthesis of the 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate (LewGMP)

Our preliminary attempts to synthesize 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate were based on the procedure previously published by Seela and Waldek.[40] In this method, guanosine is converted to 2′,3′-*O*-[1-[2-(ethoxycarbonyl)ethyl]ethylidene]guanosine (Lew(OEt)Guo) by its condensation with ethyl levulinate in DMF using hydrochloric acid and triethyl orthoformate as catalyst. Unfortunately, despite several attempts, we were not able to obtain the desired product in a satisfactory yield (maximum 30% *vs*. lit. 63%). Here we describe a modified procedure (Scheme 2), in which guanosine is treated with ethyl levulinate in the presence of *p*-toluenesulfonic acid and triethyl orthoformate without any additional solvents at room temperature. Those modifications allowed us to obtain Lew(OEt)Guo (**1**) with higher yield (88%), shorter time (6 h), using an easier purification procedure in which crystallization is avoided.

Condensation of guanosine with ethyl levulinate leads to formation of diastereoisomers differing in the configuration at the acetal carbon. The ^{1}H NMR spectra of the obtained product **1** showed that both diastereoisomers were formed with large excess of one isomer (10 : 1), similar to its previous report.[40] Based on NMR[41] and X-ray crystallographic[42] studies the *endo* conformation had been assigned to the methyl group and the *exo* conformation to the side chain of the major product of guanosine derivative. Due to the large excess of the desired diastereoisomer, the mixture was not separated and was used directly in the next step. Phosphorylation of Lew(OEt)Guo was accomplished by Yoshikawa's method[43] using phosphorus trichloride oxide in trimethyl phosphate at 4 °C. The product Lew(OEt)GMP (**2**) was not isolated from the reaction mixture but directly converted into carboxylic acid derivative LewGMP (**3**) by alkaline hydrolysis. It is worth mentioning that the deprotection step leading to the final product **3** cannot be conducted at any other stage of the m^7GpppG_{Lew} (**5a**) or $m_3^{2,2,7}GpppG_{Lew}$ (**5b**) synthesis due to instability of the 7-methylated purine ring under alkaline conditions.[44]

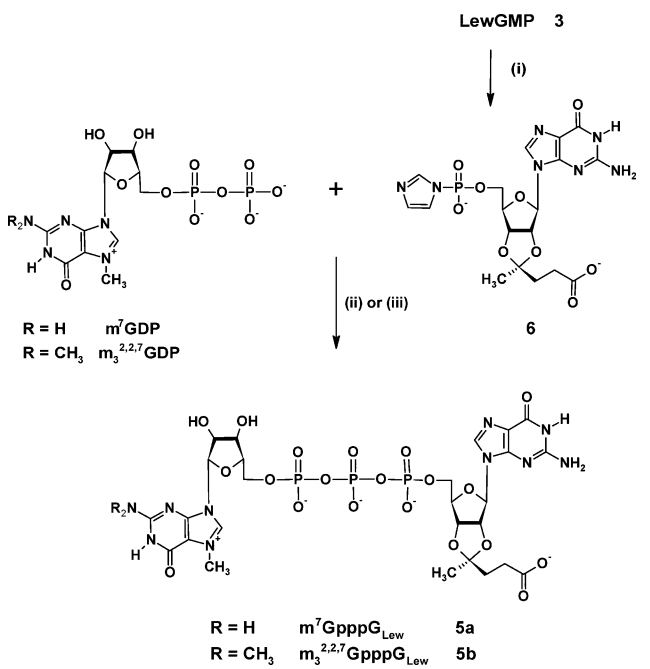
Synthesis of mono- and trimethylated dinucleotide cap analogues modified with levulinic acid (m^7GpppG_{Lew} and $m_3^{2,2,7}GpppG_{Lew}$)

The crucial step in the synthesis of mono- and trimethylated dinucleotide cap analogues is pyrophosphate bond formation. Several widely used chemical approaches have been developed so far but the most commonly used method is based on the strategy in which two mononucleotide units, one of which has been converted to a reactive imidazolide derivative, are coupled.[45] Following the aforementioned methodology, in the first step imidazolide derivatives **4a** and **4b**, were prepared from appropriate nucleoside 5′-diphosphates (m^7GDP and $m_3^{2,2,7}GDP$) *via* reaction with imidazole in the presence of 2,2′-dithiodipyridine and triphenylphosphine.[46] Subsequently, the resultant compounds were coupled with LewGMP (Scheme 3). Since the final coupling leading to the formation of the 5′,5′-triphosphate bridge could have been carried out either in aqueous[47] or anhydrous conditions,[48] both possibilities were examined. Exploration of reaction progress, monitored by HPLC, revealed that m^7GpppG_{Lew} synthesis proceeded similarly regardless of coupling conditions and led to 23% yield of **5a**. Unfortunately, synthesis of $m_3^{2,2,7}GpppG_{Lew}$ turned out to be more difficult and obtaining the



Scheme 3 Synthesis of the P^1-7-methylguanosine 5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate (**5a**, m^7GpppG_{Lew}) and P^1-N^2,N^2,7-trimethylguanosine 5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)-ethylidene]}guanosine 5′-triphosphate (**5b**, $m^{2,2,7}GpppG_{Lew}$)—Strategy 1; (i) imidazole, triphenylphosphine, triethylamine, 2,2′-dithiodipyridine, DMF, (ii) $ZnCl_2$, DMF, (iii) $MnCl_2$, 0.1 M NEM pH 7.

desired product in satisfactory yield was not possible. Despite the fact that, due to chemical structure of LewGMP, the reaction with imidazole may occur not only with phosphate but also the free carboxyl group, an alternative pathway, in which an imidazolide derivative of LewGMP was coupled with m^7GDP or $m_3^{2,2,7}GDP$ was explored (Scheme 4). HPLC and mass spectrometry analyses of the obtained imidazolide derivative revealed that two products were formed either with one or two imidazole residues. The level of substitution was highly connected with reaction time and increased with its extension. Nevertheless, usage of single or/and double activated substrates in reaction with m^7GDP or $m_3^{2,2,7}GDP$ gave the desired products **5a** and **5b** as determined by ^1H and ^{31}P NMR spectra. Similarly as in the first strategy, reaction conditions (anhydrous or aqueous) had no influence on the final yield of m^7GpppG_{Lew} synthesis (*ca.* 20%). In the case of $m_3^{2,2,7}GpppG_{Lew}$ synthesis, substantial difference between coupling in respective environments was observed. Anhydrous conditions turned out to be more effective than the reaction in aqueous solution and led to trimethylated cap **5b** in satisfying yield (24%). Summarizing, the results indicated that both pathways (Scheme 3 and 4) leading to m^7GpppG_{Lew} formation can be performed without harming the final yield using anhydrous or aqueous conditions. However, synthetic strategy and reaction conditions play crucial roles in $m_3^{2,2,7}GpppG_{Lew}$ synthesis. Our experiments have shown that the most efficient pathway to synthesize the trimethylated cap functionalized with levulinic acid is to couple the imidazolide derivative of LewGMP with $m_3^{2,2,7}GDP$ (Scheme 4) in anhydrous conditions. This strategy not only allows one to obtain $m_3^{2,2,7}GpppG_{Lew}$ with satisfying yield but also has an enormous advantage over the first strategy as activation conditions are worked out only once for LewGMP instead of their determination for each nucleotide independently.

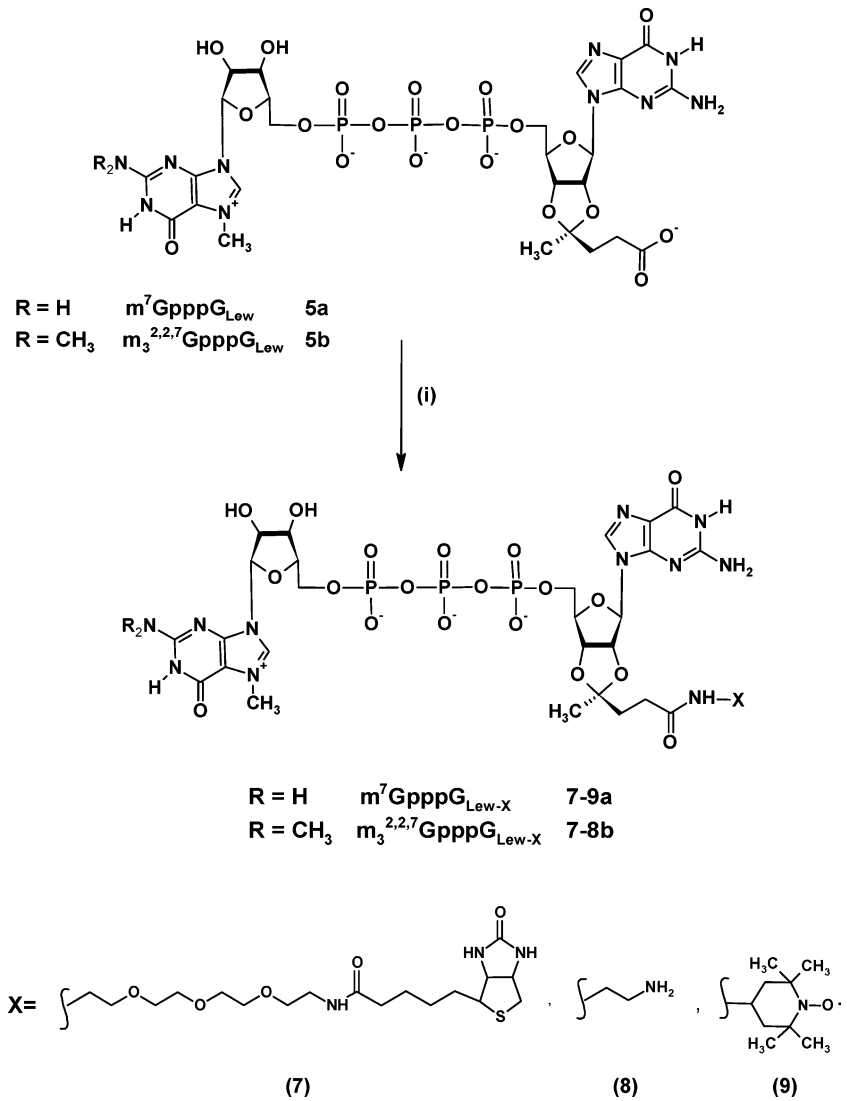


Scheme 4 Synthesis of the P^1-7-methylguanosine 5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]} guanosine 5′-triphosphate (**5a**, m^7GpppG_{Lew}) and P^1-N^2,N^2,7-trimethylguanosine 5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)-ethylidene]}guanosine 5′-triphosphate (**5b**, $m^{2,2,7}GpppG_{Lew}$)—Strategy 2; (i) imidazole, triphenylphosphine, triethylamine, 2,2′-dithiodipyridine, DMF, (ii) $ZnCl_2$, DMF, (iii) $MnCl_2$, 0.1 M NEM pH 7.

Synthesis of amine-PEG_3-biotin-, EDA- and 4-amino TEMPO derivatives of m^7GpppG_{Lew} and $m_3^{2,2,7}GpppG_{Lew}$

Numerous biophysical techniques have been established to study biomolecular binding events in the past years. Most commonly used are high-resolution multidimensional NMR spectroscopy and X-ray crystallography. Nevertheless, due to their limitations, they are often complemented with other increasingly popular biophysical techniques which allow direct detection of binding in solution (*e.g.* surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), mass spectrometry (MS)).[49–53]

Here, we describe the synthesis of five mono- and trimethylated cap analogues connected with ligands: amine-PEG_3-biotin, EDA and 4-amino TEMPO that have been chosen due to their versatility as tools in biophysical methods. The avidin–biotin and streptavidin–biotin complexes are very useful and universal mediators in a wide range of bioanalytical applications and for years they have been proven to be versatile tools with extremely broad applications. EDA introduced to the compound of interest can be either a precursor for coupling of other functional ligands or the resultant compound can be used by itself, for example in SPR experiments for immobilization on the sensor chip surface. 4-Amine TEMPO is a well-known, stable nitroxide radical that when linked to the biologically active compound of interest, due to its ESR activity, is used to determine the relative location of molecules or residues in large molecular systems.[54,55] All derivatives were synthesized from **5a** or **5b** based on a well known procedure in which a carboxyl-containing molecule is activated with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC)[56] and coupled with an appropriate amine (Scheme 5). Since EDC reacts with a carboxyl group of the molecule forming a reactive intermediate susceptible to hydrolysis in aqueous solution, we carried out all of the reactions with addition of *N*-hydroxysuccinimide (NHS) which stabilizes the amine reactive intermediate.[57] The optimal pH and reaction time were selected based on comparison of reaction progress monitored by HPLC (see the ESI†). The best results were achieved by carrying out reactions for 4.5 h in 2-(*N*-morpholino)ethanesulfonic buffer pH 6. Final products were isolated from reaction mixtures on semi-preparative reversed-phase (**7a**,**7b** and **9a**) or ion-exchange (**8a** and **8b**) HPLC columns (see the ESI†). However, for $m^7GpppG_{Lew\text{-}EDA}$ it was also checked that there is a possibility to scale up the reactions if needed and purify the final products on DEAE-Sephadex (see the ESI†). The structure and homogeneity of the final products were confirmed by re-chromatography on LC-18-T HPLC, MS, ^1H NMR and ^{31}P NMR. ^1H NMR spectra of the final products did not contain signals from a methyl group with *exo* orientation coupled to acetal quaternary carbon which showed that only the desired diastereoisomers were obtained. Probably, due to steric hindrance, only m^7GpppG_{Lew} and $m_3^{2,2,7}GpppG_{Lew}$ with *exo* oriented side chain containing a free carboxyl group, were subjected to reaction with the appropriate amine. NMR spectra of **9a** were not obtained. Although the first NMR spectrum of a TEMPO-containing paramagnetic compound has been recently reported,[39] acquiring such spectra is difficult and time-consuming due to the strong paramagnetic broadening of resonance signals. Alternatively, ESR spectroscopy



Scheme 5 Synthesis of amine-PEG_3-biotin, EDA and 4-amino TEMPO derivatives of m^7GpppG_{Lew} and $m_3{}^{2,2,7}GpppG_{Lew}$; (i) EDC, NHS, 0.1 M MES pH 6.

was used to check whether the nitroxide radical TEMPO had been incorporated into the final product **9a**. As shown in Fig. 2, the ESR spectrum of **9a** reveals a characteristic signal broadening due to a partially hampered rotation of the nitroxide radical covalently bound to the dinucleotide cap analogue. To increase the usefulness of the obtained compounds for biophysical studies, their stability was explored. Cap analogues turned out to be stable in aqueous solution at room temperature over a couple of days (see the ESI†).

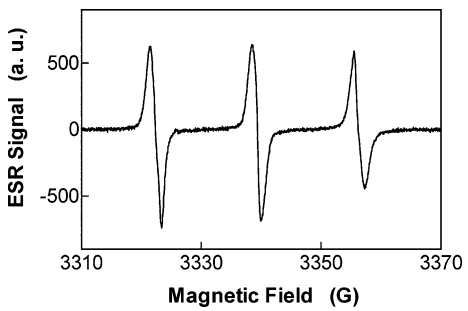


Fig. 2 ESR spectra of a spin-labeled cap analogue, $m^7GpppG_{Lew\text{-}TEMPO}$, in water.

Conclusions

In this work, we have described the synthesis of the first mono- and trimethylated dinucleotide cap analogues with different introduced molecular probes (biotin, EDA and TEMPO) designed for studies involving cap-binding proteins. Functionalization of the second nucleotide in the cap structure was achieved by derivatization of the 2′,3′-*cis* diol of the ribose moiety with levulinic acid. This modification allows introduction of not only proposed probes but also other ligands which might be useful in biophysical methods not mentioned above (*e.g.*, fluorescence probes). $m^7GpppG_{Lew\text{-}EDA}$ has already been used in studies on interaction between CBC and the cap.[58] Kinetic measurements of binding of the CBC and its mutants to surface immobilized m^7GpppG derivative by means of SPR were performed. Based on those studies association/dissociation constants of CBC binding to the cap were determined and

provided important clues for a kinetic model of the CBC–cap association.[58] Other prepared analogues are currently used in studies on interaction of *Ascaris* eIF4E isoforms with mono- and trimethylated caps and the results will be published soon.

Materials and methods

All used reagents were purchased in the highest available purity from Sigma-Aldrich Chemical Co. and were used without any further treatment. Amine-PEO_3-biotin was purchased from Pierce (Thermo Fischer Scientific). Triethylammonium bicarbonate (TEAB) buffer was prepared by bubbling CO_2 through an ice-cold aqueous solution of redistilled triethylamine.

N^2,N^2-dimethylguanosine was prepared using a modified procedure described by Eritja *et al.*[59] Other used nucleotides, *i.e.* 7-methylguanosine-5′-diphosphate and N^2,N^2,7-trimethylguanosine-5′-diphosphate, were prepared as previously reported.[10,60]

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, evaporated under reduced pressure with several additions of ethanol and isolated as triethylammonium salts (TEA salts). Final dinucleotide cap analogues were separated from reaction mixtures on reversed-phase or ion exchange HPLC columns. Semi-preparative HPLC was performed on a Knauer instrument, using Waters Spherisorb SAX column (10 × 250 mm, flow rate 3.0 mL min^{-1}) with a linear gradient of 0.006 M KH_2PO_4 containing 0.01 M acetic acid (pH 4) to 0.6 M KH_2PO_4 (pH 5) or Supelcosil LC-18-DB column (10 × 250 mm, flow rate 3.0 mL min^{-1}) with a linear gradient of methanol from 0 to 50% (v/v) in 0.05 M ammonium acetate (pH 5.9). HPLC analytical analyses were performed on Supelcosil LC-18-T RP column (4.6 × 250 mm, flow rate 1.0 mL min^{-1}) with a linear gradient of methanol from 0 to 50% (v/v) in 0.05 M ammonium acetate (pH 5.9) or Supelcosil LC-SAX1 column (4.6 × 250 mm, flow rate 1.0 mL min^{-1}) with a linear gradient of 0.006 M KH_2PO_4 containing 0.01 M acetic acid (pH 4) to 0.6 M KH_2PO_4 (pH 5). UV detection was performed at 254 nm. Retention times given in the Experimental are based on HPLC analysis on Supelcosil LC-18-T RP column. MS spectra were acquired using a Waters Micromass Q-TOF Premier spectrometer with positive electrospray ionization source. ^{13}C NMR spectra were obtained with a Varian UnityPlus 200 MHz spectrometer. ^1H NMR spectra were recorded on a Varian UnityPlus 200 MHz, a Varian INOVA 400 MHz, a Varian INOVA 500 MHz, or Varian NMR System 700 MHz spectrometer. ^{31}P NMR spectra were recorded on a Varian INOVA 500 MHz. *J* values are given in Hz. ESR spectra of the spin-labelled cap analogue was obtained with an X-band EPR spectrometer, EMX from Bruker (USA).

Experimental

2′,3′-*O*-[1-[2-(Ethoxycarbonyl)ethyl]ethylidene]guanosine (1)

Triethyl orthoformate (1.30 mL, 7.8 mmol) was added dropwise to a suspension of 566 mg (2 mmol) guanosine and 428 mg (2.25 mmol) *p*-toluenesulfonic acid in 7.65 mL (54 mmol) of ethyl levulinate at room temperature. After 6 h the reaction mixture was neutralized with 25% aqueous ammonia. The induced cream-colored precipitate was collected and washed several times with diethyl ether. Filtration afforded 719 mg (1.76 mmol, 88%) of **1** as a fine white powder. ^1H NMR (D_2O, 200 MHz): δ 7.91 (s, 1H, H-8), 6.07 (d,1H, $J_{1',2'}$ = 2.6, H-1′), 5.46 (dd, 1H, $J_{2',3'}$ = 6.6, H-2′), 5.09 (m, 1H, $J_{3',4'}$ = 2.6, H-3′), 4.40 (m, 1H, $J_{4',5'}$ = 3.7, H-4′), 3.73 (m, 2H; H-5′), 2.75 (t, 2H, *J* = 6.5, CH_2, levulinic acid), 2.58 (t, 2H, CH_2, levulinic acid), 2.37 (m, 2H, *J* = 7.0, CH_2, OEt), 1.41 (s, 3H, CH_3, levulinic acid), 1.24 (t, 3H, CH_3, OEt); ^{13}C NMR (DMSO, 200 MHz): δ 171.8 (C═O), 156.0 (C-6), 152.9 (C-2), 149.9 (C-4), 135.22 (C-8), 116.06 (C-5), 112.9 (acetal C), 87.5 (C-1′), 86.20 (C-4′), 82.9 (C-3′) 80.4 (C-2′), 60.8 (C-5′), 59.2 (CH_2, OEt), 33.3 (CH_2CO), 28.5 (C_{acetal} -CH_2), 23.1 (C_{acetal} -CH_3), 13.3 (CH_3, OEt); MS-ESI: *m*/*z*: calcd for $C_{17}H_{23}N_5O_7$: 410.1670 $[M + H]^+$, found: 410.1698; HPLC t_R 23.1 min.

2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]guanosine 5′-monophosphate (3)

Phosphorus trichloride oxide (562 μL, 6 mmol) in 12 mL (0.1 mmol) trimethyl phosphate at 4 °C was added to dried 2′,3′-*O*-[1-[2-(ethoxycarbonyl)ethyl]ethylidene]guanosine **1** (695 mg, 1.7 mmol) and kept under vigorous stirring for 4 h. The solution was allowed to warm to RT and 1 M aqueous TEAB was added to maintain pH as neutral. The solution containing compound **2** was treated with 1 M NaOH and kept for 5 h at RT. The reaction mixture was neutralized with 1 M HCl and purified on DEAE–Sephadex using a linear 0–1.0 M gradient of TEAB. Compound **3** was obtained as colourless crystals (796 mg, 1.2 mmol, 72%, TEA salt). ^1H NMR (D_2O, 500 MHz): δ 8.01 (s, 1H, H-8), 6.10 (d, 1H, $J_{1',2'}$ = 2.5, H-1′), 5.40 (dd, 1H, $J_{2',3'}$ = 6.5, H-2′), 5.20 (dd, 1H, $J_{3',4'}$ = 2.6, H-3′), 4.60 (br s, 1H, H-4′), 4.02–4.08 (m, 2H, H-5′, H-5″), 2.46 (t, 2H, *J* = 7.5, CH_2, levulinic acid), 2.20 (t, 2H, CH_2, levulinic acid), 1.43 (s, 3H, CH_3, levulinic acid); MS-ESI: *m*/*z*: calcd for $C_{15}H_{20}N_5O_{10}P_1$: 462.1020 $[M + H]^+$, found: 462.1017; HPLC t_R 10.9 min.

P^1-7-methylguanosine-5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate (5a) and P^1-N^2,N^2,7-trimethylguanosine 5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate (5b)

Strategy 1. Activation of m^7GDP and coupling of obtained imidazolide **4a** with **3**

7-Methylguanosine 5′-diphosphate imidazolide 4a. 7-Methylguanosine 5′-diphosphate (117 mg, 0.178 mmol, TEA salt), imidazole (242 mg, 3.6 mmol), 2,2′-dithiodipyridine (80 mg, 0.36 mmol) and triethylamine (25 μL) were mixed in anhydrous DMF (0.9 mL). After 20 min triphenylphosphine (95 mg, 0.36 mmol) was added and the mixture was stirred for 6–8 h at RT. The product was precipitated from the reaction mixture with a solution of anhydrous sodium perchlorate (87 mg, 0.71 mmol) in dry acetone (1.3 mL). After cooling at 4 °C, the precipitate was filtered, washed repeatedly with cold, dry acetone, and dried overnight under vacuum over P_4O_{10}. Compound **4a** was obtained as a white powder.

P^1-7-methylguanosine-5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate (anhydrous conditions) 5a. 7-Methylguanosine 5′-diphosphate imidazolide **4a** (111 mg, 0.2 mmol, Na salt) and $ZnCl_2$ (270 mg, 2 mmol) were

stirred in anhydrous DMF (4 mL) with 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate **3** (113 mg, 0.2 mmol, TEA salt) at room temperature for 24 h. The reaction mixture was poured into a solution of EDTA (95 g, 0.25 mmol) in water (1.5 mL) and neutralized to pH 7 by addition of 1 M TEAB. The product was separated from the reaction mixture by chromatography on DEAE–Sephadex using a 0–1.0 M gradient of TEAB. Compound **5a** was obtained as colourless crystals (52 mg, 0.04 mmol, 20%, TEA salt).

P^1-7-methylguanosine-5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate (aqueous conditions) 5a. 7-Methylguanosine 5′-diphosphate imidazolide **4a** (83 mg, 0.15 mmol, Na salt) and $MnCl_2$ (30 mg, 0.15 mmol) were stirred in anhydrous 0.1 M NEM pH 6 (3 mL) with 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate **3** (85 mg, 0.15 mmol, TEA salt) at room temperature for 24 h. The reaction mixture was poured into a solution of EDTA (76 mg, 0.2 mmol) in water (1.2 mL) and neutralized to pH 7 by addition of 1 M TEAB. The product was separated from the reaction mixture by chromatography on DEAE–Sephadex using a 0–1.0 M gradient of TEAB. Compound **5a** was obtained as colourless crystals (46 mg, 0.035 mmol, 23%, TEA salt).

Strategy 2. Activation of 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate **3** and coupling of the obtained imidazolide **6** with 7-methylguanosine-5′-diphosphate or N^2,N^2,7-trimethylguanosine-5′-diphosphate

2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]guanosine 5′-monophosphate imidazolide 6. 2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]-guanosine 5′-monophosphate **3** (118 mg, 0.178 mmol, TEA salt), imidazole (60 mg, 0.89 mmol), 2,2′-dithiodipyridine (78 mg, 0.356 mmol) and triethylamine (25 μL) were mixed in anhydrous DMF (0.9 mL). After 20 min triphenylphosphine (93 mg, 0.356 mmol) was added and the mixture was stirred for 6–8 h in RT. The product was precipitated from the reaction mixture with a solution of anhydrous sodium perchlorate (87 mg, 0.71 mmol) in dry acetone (1.3 mL). After cooling to 4 °C, the precipitate was filtered, washed repeatedly with cold, dry acetone, and dried overnight under vacuum over P_4O_{10}. Compound **6** was obtained as a white powder.

P^1-7-methylguanosine-5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate 5a and P^1-N^2,N^2,7-trimethylguanosine 5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate 5b (anhydrous conditions). 7-Methylguanosine-5′-diphosphate or N^2,N^2,7-trimethylguanosine-5′-diphosphate (0.2 mmol, TEA salt) and $ZnCl_2$ (270 mg, 2 mmol) were stirred in anhydrous DMF (4 mL) with 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate imidazolide **6** (106 mg, 0.2 mmol, Na salt) at room temperature for 24 h. The reaction mixture was poured into a solution of EDTA (95 mg, 0.25 mmol) in water (1.5 mL) and neutralized to pH 7 by addition of 1 M TEAB. The product was separated from the reaction mixture by chromatography on DEAE–Sephadex using a 0–1.0 M gradient of TEAB. Compounds **5a** and **5b** were obtained as colourless crystals (50 mg, 0.038 mmol, 19% for **5a** and 64 mg, 0.048 mmol, 24% for **5b**).

P^1-7-methylguanosine-5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate 5a and P^1-N^2,N^2,7-trimethylguanosine 5′-P^3-{2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]}guanosine 5′-triphosphate 5b (aqueous conditions). 7-Methylguanosine-5′-diphosphate or N^2,N^2,7-trimethylguanosine-5′-diphosphate (0.2 mmol, TEA salt) and $MnCl_2$ (40 mg, 0.2 mmol) were stirred in anhydrous 0.1 N EM pH 6 (4.0 mL) with 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]guanosine 5′-monophosphate imidazolide **6** (111 mg, 0.2 mmol, Na salt) at room temperature for 24 h. The reaction mixture was poured into a solution of EDTA (95 mg, 0,25 mmol) in water (1.5 mL) and neutralized to pH 7 by addition of 1 M TEAB. The product was separated from the reaction mixture by chromatography on DEAE–Sephadex using a 0–1.0 M gradient of TEAB. Compounds **5a** and **5b** were obtained as colorless crystals (52 mg, 0.04 mmol, 20% for **5a** and 24 mg, 0,018 mmol, 9% for **5b**).

m^{7}GpppG$_{Lew}$; ^{1}H NMR (D_2O, 400 MHz) m^{7}G–δ 6.00 (d,1H, $J_{1',2'}$ = 3.2, H-1′), 4.56 (dd, 1H, $J_{2',3'}$ = 5.2, H-2′), 4.50 (m, 1H, $J_{3',4'}$ = 4.9, H-3′), 4.42 (m, 1H, H-4′), 4.30 (m, 1H, H-5′), 4.21 (m, 1H, H-5″), 4.06 (s, 3H, N^7-CH_3); G–7.92 (s, 1H, H-8), 5.88 (d, 1H, $J_{1',2'}$ = 3.6, H-1′), 5.30 (dd, 1H, $J_{2',3'}$ = 6.0, H-2′), 5.20 (m, 1H, $J_{3',4'}$ = 2.8, H-3′), 4.33 (m, 1H, H-4′), 4.26 (m, 1H, H-5′), 4.20 (m, 1H, H-5″), 2.39 (m, 2H, J = 7.5, CH_2, levulinic acid), 2.15 (m, 2H, CH_2, levulinic acid), 1.39 (s, 3H, CH_3, levulinic acid); ^{31}P NMR (D_2O, 400 MHz) δ –12.23 (2P, $P_{\alpha,\gamma}$), –23.73 (1P, P_β); MS-ESI: *m*/*z*: calcd for $C_{26}H_{36}N_{10}O_{20}P_3$: 901.1319, found: 901.1321; HPLC t_R 9.8 min.

m$_3^{2,2,7}$GpppG$_{Lew}$; ^{1}H NMR (D_2O, 500 MHz) m$_3^{2,2,7}$G–δ 8.44 (s, 1H, H-8), 5.95 (d,1H, $J_{1',2'}$ = 3.5, H-1′), 4.55 - 4.53 (m, 2H^a, H-2′), 4.41 (m, 1H, $J_{3',2'}$ = 5.1, $J_{3',4'}$ = 4.9, H-3′), 4.35–4.32 (m, 2H^b, H-4′), 4.29–4.20 (m, 2H^c; H-5′), 4.19 - 4.14 (m, 1H, H-5″), 4.04 (s, 3H, N^7-CH_3), 3.09 (s, 6H, N^2(CH_3)$_2$); G–7.86 (s, 1H, H-8), 5.92 (d, 1H, $J_{1',2'}$ = 4.0, H-1′), 5.23 (dd, 1H, $J_{2',3'}$ = 6.0, H-2′), 5.15 (dd, 1H, $J_{3',4'}$ = 3.5, H-3′), 4.55 - 4.53 (m, 2H^a, H-4′), 4.35–4.32 (m, 2H^b, H-5′), 4.29–4.20 (m, 2H^c, H-5″), 2.36 (m, 2H, J = 7.5, CH_2, levulinic acid), 2.12 (m, 2H, CH_2, levulinic acid), 1.37 (s, 3H, CH_3, levulinic acid); ^{31}P NMR (D_2O, 500 MHz) δ –12.93 (2P, $P_{\alpha,\gamma}$), –22.44 (1P, P_β); MS-ESI: *m*/*z*: calcd for $C_{28}H_{40}N_{10}O_{20}P_3$: 929.1632, found: 929.1631; HPLC t_R 11.7 min.

[a,b,c]–overlapped signals

General procedure for synthesis 7-monomethyl- and N^2,N^2,7-trimethyl-dinucleotide cap analogues functionalized through carboxylic group of levulinic acid (7–9a and 7–8b)

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (1.5 mg, 8 μmol), *N*-hydroxysuccinimide (NHS) (0.23 mg, 2 μmol) and compound **5a** or **5b** (0.006 mmol, TEA salt) were dissolved in 20 μL of 0.1 M MES buffer pH 6 and stirred at RT. After 20 min the appropriate amine (0.008 mmol) was added and reaction mixture was stirred additional 4.5 h.

The products were separated from the reaction mixtures on semi-preparative reversed-phase (**7a**,**7b** and **9a**) or ion-exchange (**8a** and **8b**) HPLC columns (more details in the ESI†). Yields based on integration of HPLC peaks are as follows; **7a**: 26%, **7b**:12%. **8a**: 20%, **8b**: 15%, **9a**:10%.

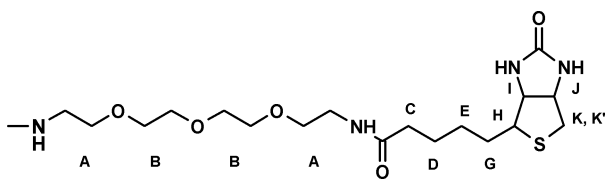
m^{7}GpppG$_{Lew\text{-}biotin}$ (**7a**) ^{1}H NMR (D_2O, 700 MHz), m^{7}G–δ 8.90 (s, 1H, H-8), 5.84 (d, 1H, $J_{1',2'}$ = 3.0, H-1′), 4.44–4.41 (m, 2H^a; H-2′), 4.30 (t, 1H, $J_{3',2'}$ = 5.2, $J_{3',4'}$ = 4.8, H-3′), 4.24–4.20 (m, 3H^b; H-4′), 4.12–4.08 (m, 1H, H-5′), 4.05–4.01 (m, 1H, H-5″), 3.90 (s, 3H, N^7-CH_3); G–7.80 (s, 1H, H-8), 5.76 (d, 1H, $J_{1',2'}$ = 3.0, H-1′), 5.17 (dd, 1H, $J_{2',3'}$ = 7.0, H-2′), 5.05 (dd, 1H, $J_{3',4'}$ = 3.0,

H-3′), 4.40 (m, 1H, H-4′), 4.24–4.20 (m, 3H^b, H-5′), 4.15–4.12 (m, 1H, H-5″), 2.34 (m, 2H, J = 7.5, CH_2, levulinic acid), 2.04 (m, 2H, CH_2, levulinic acid), 1.26 (s, 3H, CH_3–levulinic acid); PEO_3-biotin (for designation of hydrogen atoms see picture below)—δ 4.44–4.41 (m, 2H^a; H_J-biotin), 4.24–4.20 (m, 3H^b, H_I-biotin), 3.53–3.50 (m, 8H, H_B), 3.48–3.46 (m, 4H, H_A), 3.28–3.21 (m, 4H, H_A), 3.11 (m, 1H, H_H-biotin), 3.07–3.04 (m, 2H, $H_{K,K'}$-biotin), 2.09 (t, 2H, H_C-biotin), 1.80 (m, 2H, H_G-biotin), 1.56–1.35 (m, 4H, $H_{D,E}$-biotin); ^{31}P NMR (D_2O, 700 MHz) δ –11.68 (2P, $P_{\alpha,\gamma}$), –23.14 (1P, P_β); MS-ESI: m/z: calcd for $C_{44}H_{68}N_{14}O_{24}P_3S_1$: 1301.3464, found: 1301.3429; HPLC t_R 17.1 min.

[a,b]–overlapped signals

$m_3^{2,2,7}$GpppG$_{Lew\text{-}biotin}$; (**7b**) ^{1}H NMR (D_2O, 500 MHz) $m_3^{2,2,7}$G–δ 9.01 (s, 1H, H-8), 5.98–5.94 (m, 2H^a, H-1′), 4.63–4.58 (m, 2H^b, H-2′), 4.60 (m, 1H, H-3′), 4.42–4.39 (m, 3H^c, H-4′), 4.34–4.22 (m, 3H^d; H-5′, H-5″), 4.09 (s, 3H, N^7-CH_3) 3.21 (s, 6H, $N^2(CH_3)_2$); G–7.95 (s, 1H, H-8), 5.98–5.94 (m, 2H^a, H-1′), 5.29 (dd, 1H, $J_{2',1'}$ = 3.0, $J_{2',3'}$ = 6.0, H-2′), 5.20 (dd, 1H, $J_{3',4'}$ = 3.5, H-3′), 4.56 (m, 1H, H-4′), 4.42–4.39 (m, 3H^c, H-5′), 4.34–4.22 (m, 3H^d, H-5″), 2.51 (m, 2H, CH_2, levulinic acid), 2.23 (m, 2H, CH_2, levulinic acid), 1.99 (s, 3H, CH_3, levulinic acid); PEO_3-biotin (for designation of hydrogen atoms see picture below)—δ 4.63–4.58 (m, 2H^b, H_J-biotin), 4.42–4.39 (m, 3H^c, H_I-biotin), 3.70–3.68 (m, 8H, H_B), 3.66–3.62 (m, 4H, H_A), 3.46–3.39 (m, 4H, H_A), 3.29 (m,1H, H_H-biotin), 3.23–3.21 (m, 2H, $H_{K,K'}$-biotin), 2.25 (t, 2H, H_C-biotin), 2.01 (m, 2H, H_G-biotin), 1.73–1.52 (m, 4H, $H_{D,E}$-biotin);^{31}P NMR (D_2O, 500 MHz) δ –12.05 (2P, $P_{\alpha,\gamma}$), –23.62 (1P, P_β); MS-ESI: m/z: calcd for $C_{46}H_{72}N_{14}O_{24}P_3S_1$: 1329.3777, found: 1329.3780; HPLC t_R 18.6 min.

[a,b,c,d]–overlapped signals



m^7GpppG$_{Lew\text{-}EDA}$; (**8a**) ^{1}H NMR (D_2O, 500 MHz), m^7G–δ 6.07 (d, 1H, $J_{1',2'}$ = 2.7, H-1′), 4.82 (m, 1H, $J_{2',3'}$ = 4.9, H-2′), 4.62 (m, 1H, $J_{3',4'}$ = 4.7, H-3′), 4.42 (m, 2H, H-4′), 4.36–4.24 (m, 2H^a, H-5′), 4.19–4.10 (m, 2H^b, H-5″), 4.01 (s, 3H, N^7-CH_3); G–7.90 (s, 1H, H-8), 5.98 (d, 1H, $J_{1',2'}$ = 4.0, H-1′), 5.32 (m, 1H, $J_{2',3'}$ = 6.5, H-2′), 5.25 (m, 1H, $J_{3',4'}$ = 3.0, H-3′), 4.58 (m, 1H, H-4′), 4.36–4.24 (m, 2H^a,H-5′), 4.19–4.10 (m, 2H^b, H-5″), 3.53 (t, 2H, J = 6.2, CH_2-EDA), 3.28 (t, 2H, CH_2-EDA), 2.40 (m, 2H, J = 6.5, CH_2, levulinic acid), 2.19 (m, 2H, CH_2, levulinic acid), 1.44 (s, 3H, CH_3, levulinic acid);^{31}P NMR (D_2O, 500 MHz) δ –12.22 (2P, $P_{\alpha,\gamma}$), –23.80 (1P, P_β); MS-ESI: m/z: calcd for $C_{28}H_{42}N_{12}O_{19}P_3$: 943.1901, found: 943.1914; HPLC t_R 12.5 min.

[a,b]–overlapped signals

$m_3^{2,2,7}$GpppG$_{Lew\text{-}EDA}$; (**8b**) ^{1}H NMR (D_2O, 500 MHz), $m_3^{2,2,7}$G–δ 5,91 (d,1H, $J_{1',2'}$ = 3.5, H-1′), 4.52 (m, 1H, $J_{2',3'}$ = 5.5, H-2′), 4.49 (m, 1H, $J_{3',4'}$ = 3.0, H-3′), 4.38–4.32 (m, 4H^a; H-4′ and H-5′), 4.18–4.14 (m, 2H^b, H-5″), 3.99 (s, 3H, N^7-CH_3), 3.16 (s, 6H, $N^2(CH_3)_2$); G–7.83 (s, 1H, H-8), 5.86 (d, 1H, $J_{1',2'}$ = 3.5, H-1′), 5.16 (dd, 1H, $J_{2',3'}$ = 6.5, H-2′), 5.08 (dd, 1H, $J_{3',4'}$ = 4.0, H-3′), 4.38–4.32 (m, 4H^a; H-4′ and H-5′), 4.18–4.14 (m, 2H^b, H-5″), 3.34 (t, 2H, J = 6.3, CH_2-EDA), 3.10 (t, 2H, CH_2-EDA), 2.36 (m, 2H, J = 6.2, CH_2, levulinic acid), 2.11 (m, 2H, CH_2, levulinic acid), 1.36 (s, 3H, CH_3, levulinic acid);^{31}P NMR (D_2O, 500 MHz) δ –11.84 (2P, $P_{\alpha,\gamma}$), –23.32 (1P, P_β); MS-ESI: m/z: calcd for $C_{30}H_{46}N_{12}O_{19}P_3$: 971.2214, found: 971.2212; HPLC t_R 13.9 min.

[a,b]–overlapped signals

m^7GpppG$_{Lew\text{-}TEMPO}$; (**9a**) MS-ESI: m/z: calcd for $C_{35}H_{53}N_{12}O_{20}P_3$: 1054.2711, found: 1054.2709; HPLC t_R 17.3 min.

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