9578–9590 *Nucleic Acids Research, 2016, Vol. 44, No. 20* *Published online 7 October 2016*
doi: 10.1093/nar/gkw896

Cap analogs modified with 1,2-dithiodiphosphate moiety protect mRNA from decapping and enhance its translational potential

Malwina Strenkowska[1], Renata Grzela[2], Maciej Majewski[1], Katarzyna Wnek[1], Joanna Kowalska[1], Maciej Lukaszewicz[1], Joanna Zuberek[1], Edward Darzynkiewicz[1,2], Andreas N. Kuhn[3,4], Ugur Sahin[3,4] and Jacek Jemielity[2,*]

[1]Division of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw, Zwirki i Wigury 93 Warsaw 02-089, Poland, [2]Centre of New Technologies, University of Warsaw, S. Banacha 2c, 02-097 Warsaw, Poland, [3]BioNTech RNA Pharmaceuticals GmbH, An der Goldgrube 12, 55131 Mainz, Germany and [4]Translational Oncology (TRON), Freiligrathstraße 12, 55131 Mainz, Germany

Received June 23, 2016; Revised September 24, 2016; Accepted September 28, 2016

ABSTRACT

Along with a growing interest in mRNA-based gene therapies, efforts are increasingly focused on reaching the full translational potential of mRNA, as a major obstacle for *in vivo* applications is sufficient expression of exogenously delivered mRNA. One method to overcome this limitation is chemically modifying the 7-methylguanosine cap at the 5' end of mRNA (m^7Gppp-RNA). We report a novel class of cap analogs designed as reagents for mRNA modification. The analogs carry a 1,2-dithiodiphosphate moiety at various positions along a tri- or tetraphosphate bridge, and thus are termed 2S analogs. These 2S analogs have high affinities for translation initiation factor 4E, and some exhibit remarkable resistance against the SpDcp1/2 decapping complex when introduced into RNA. mRNAs capped with 2S analogs combining these two features exhibit high translation efficiency in cultured human immature dendritic cells. These properties demonstrate that 2S analogs are potentially beneficial for mRNA-based therapies such as anti-cancer immunization.

INTRODUCTION

Therapeutic mRNA applications are currently gaining increased attention from immunologists, molecular biologists and chemists due to the recently uncovered potential of mRNAs to serve as versatile protein delivery molecules in cancer immunotherapies, gene therapies and cell reprogramming (1–4). The advantages of using mRNA for this purpose over DNA include the fact that there is no risk of integration into the host genome and no need to cross the nuclear membrane and that expression can be transient. However, there are also pressing issues that need to be solved before this becomes a viable method, including the instability of mRNA under cellular conditions, insufficient translational yield and difficulties in large-scale mRNA production. Therefore, the preparation of stable and efficiently translated mRNAs is one of the main goals of therapeutically oriented RNA-related research.

A variety of modifications have been explored as RNA optimization strategies (5–13). One way to alter the properties of mRNA is through chemical modification of the mRNA cap, which can be achieved through the use of properly designed cap mimics. The cap structure is present at the 5' end of all eukaryotic mRNAs and consists of a 7-methylguanosine (m^7G) connected via a 5',5'-triphosphate bridge to the first transcribed RNA nucleotide. Through recognition by specific proteins and enzymes, the cap participates in mRNA expression and turnover, including initiation of translation and 5'-to-3' RNA decay. Synthetic cap analogs can be incorporated easily into RNA during transcription *in vitro*. Several previously reported cap analogs have been shown to have a large impact on the quality of *in vitro*-synthesized mRNA and its biochemical properties (14). This includes the so-called anti-reverse cap analogs (ARCAs), molecules that are modified within the ribose moiety of the m^7G, eliminating the problem of incorrect cap incorporation during mRNA synthesis *in vitro*, which generally causes up to 50% of transcripts to be dysfunctional (15–17). Another example is an analog that contains a single phosphorothioate (O-to-S) substitution in the triphosphate bridge, termed an S analog. ARCAs substituted with an S at the β position of the triphosphate bridge (β-S-ARCAs) significantly increase the half-life and translational efficiency of mRNA in cultured cells (18,19). β-S-ARCAs have also been shown to augment expression of antigen-

*To whom correspondence should be addressed. Tel: +48 22 55 43774; Fax: +48 22 55 40771; Email: jacekj@biogeo.uw.edu.pl

© The Author(s) 2016. Published by Oxford University Press on behalf of Nucleic Acids Research.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

encoding mRNAs in cultured dendritic cells and in mice (11) and are currently used in clinical trials of mRNA-based immunotherapeutic vaccines against melanoma.

The desirable properties of mRNAs capped with β-S-ARCAs result from two independent factors: high affinity of the cap for eukaryotic translation initiation factor 4E (eIF4E) (19) and decreased susceptibility to decapping complex Dcp1/2, (18), which is responsible for cleaving the cap between the α and β phosphates to release the m^7GDP molecule and expose RNA to 5'-to-3' degradation (20). The efficient interaction of the cap with eIF4E is essential for the translation of a majority of mRNAs, both *in vivo* and *in vitro* (21). Strong and specific binding of the cap to eIF4E is due to the cation-π stacking between the aromatic rings of positively charged m^7G and two Trp residues and to the electrostatic interactions of the negatively charged oligophosphate chain with positively charged amino acids (22). The affinity of cap analogs for eIF4E has been shown to correlate with their ability to inhibit cap-dependent translation *in vitro* (23). High affinity to eIF4E is also a prerequisite for high translational efficiency of capped mRNA (15,18–19). Increased resistance to decapping is also a valuable trait for mRNA, as the presence of the cap protects mRNA from degradation through the 5'-to-3' pathway, making it available for translation for a longer period of time. In a previous study, we found that the introduction of a phosphorothioate moiety at the β position of the triphosphate bridge significantly decreased the susceptibility of capped RNA to Dcp2 *in vitro* (18). The susceptibility of $m_2^{7,2'\text{-O}}GppSpG$-RNA to Dcp2 was also dependent on the configuration of the stereogenic phosphorus atom, as isomer D2 was less susceptible than D1, correlating with relative mRNA half-lives in mammalian cells of 257 ± 4 and 185 ± 20 min, respectively (versus 155 ± 9 min for $m_2^{7,2'\text{-O}}GpppG$-RNA).

Studies with β-S-ARCA indicate that mRNAs with cap structures that combine a high affinity for eIF4E and resistance to Dcp2 (the catalytic subunit of the Dcp1/2 complex) result in greater protein yields *in vitro* and *in vivo*. Therefore, we previously conducted a systematic search for cap analogs modified within the oligophosphate bridge to identify those with properties similar or superior to β-S-ARCA. Among these analogs is a group with a single O-to-S substitution within a tetraphosphate bridge (4P-S-ARCAs) (24). Due to the presence of an additional phosphate group, these analogs exhibit a higher affinity to eIF4E than 3P-S-ARCAs and RNAs capped with these 4P analogs are efficiently translated in rabbit reticulocyte lysates (RRLs). However, preliminary studies indicated that the single O-to-S substitution in the tetraphosphate bridge (either β or γ position) provides only partial protection against Dcp2 (24), which could limit *in vivo* applications.

Here, we have developed and evaluated a novel class of phosphate-modified cap analogs that are a logical continuation of our previous work toward designing cap analogs useful for building stable and efficiently translated mRNAs. We describe the synthesis and characterization of cap analogs that contain a 1,2-dithiodiphosphate moiety, i.e., a sulfur substitution at two neighboring phosphate moieties in either a tri- or tetraphosphate chain. These molecules are thus termed 2S analogs. Two neighboring O-to-S substitutions were introduced at the α,β positions of the triphosphate bridge or the α,β/β,γ positions of the tetraphosphate bridge, with the aim of identifying analogs that decrease the susceptibility of RNA to Dcp2 while maintaining or increasing affinity for eIF4E. Some of the 2S analogs (compounds **4** and **7**) were additionally modified by the presence of a 2'-*O*-methyl group to convert them into ARCAs. These 2S analogs each represent four diastereoisomeric forms, differing in physicochemical properties. The 2S analogs were then evaluated as reagents for RNA modification by determining their affinities for eIF4E. Subsequently, 2S-ARCAs were incorporated into transcripts and assessed for their susceptibility to Dcp2 and their translational properties in human immature dendritic cells (hiDCs). As a result, we have identified 2S analogs that exhibit properties for mRNA-based gene therapies that are superior to those of any previously published phosphate-modified cap analog used in clinical trials.

MATERIALS AND METHODS

Preparation of compounds 1–7 (for compounds 8–18 see Supplemental Experimental Procedures)

Detailed information about materials and methods such as equipment and reagents used, preparation of starting materials, purification methods, nuclear magnetic resonance (NMR) spectra, high-resolution mass spectra and HPLC profiles of compounds **1**–**7** is available in Supporting Information file.

*General procedure for the synthesis of **1–2**.* An appropriate nucleotide (1 eq, triethylammonium (TEA) salt) was placed in a 10 ml microwave tube and suspended in dimethylformamide (DMF) (final concentration: 0.1 M). Then, P-imidazolide of *S*-(2-cyanoethyl)phosphorothioate (3 eq.) and anhydrous $MgCl_2$ (8 eq.) were added to the suspension. The tube was heated for 10–30 min in the microwave oven using dynamic power mode (parameters: $P_{max} = 10$ W and $T_{max} = 45 \pm 1\,^{\circ}C$, 2.45 GHz). Then, the mixture was diluted with DMF and DBU (1 eq.) and dithiothreitol (DTT) (3 eq.) were added to the mixture to remove the 2-cyanoethyl group. The tube was again heated in the microwave oven using the same parameters as given above. The reaction was stopped by addition of water and washed with ethyl acetate. Products were purified on DEAE-Sephadex and isolated as TEA salts. Exact details, yields, reaction times and characterization of **1** and **2** (HPLC, HRMS, NMR spectra) are provided in Supplementary Material (Experimental procedures, Supplementary Tables S1–4).

*General procedure of synthesis **3–7**.* Dithiodiphosphate modified nucleotide (**1** or **2**, TEA salt, 1 eq.) was mixed with appropriate P-imidazolide (1.5 eq.) and anhydrous $MgCl_2$ (8–12 eq.) and placed in a 10 ml microwave tube and suspended in DMF (final concentration of nucleotides 0.2 M). The tube was sealed and heated for 30–90 min in the microwave oven using dynamic power mode (parameters: $P_{max} = 10$ W and $T_{max} = 45 \pm 1\,^{\circ}C$, 2.45 GHz). The reaction was diluted with 10-fold excess of deionized water. Products were purified on DEAE-Sephadex and isolated as TEA salts. Diastereoisomeres were later separated

on semi-preparative RP HPLC and after repeated freeze-drying, were isolated as ammonium salts. Exact details, yields, reaction times and characterization of **3**–**7** (HPLC, HRMS, NMR spectra) are provided in Supplementary Material (Experimental procedures, Supplementary Tables S1–4).

Preparation of recombinant proteins (eIF4E and SpDcp1/2)

Mouse eIF4E (residues 28–217) was expressed in *Escherichia coli* strain BL21(DE3) as inclusion bodies. Guanidinium-solubilized protein was refolded by one-step dialysis, and purified by ion-exchange chromatography on HiTrap SP column (GE Healthcare) without exposure to cap analogs. The concentration of eIF4E was determined spectrophotometrically (ϵ_{280} = 52 940 cm^{-1} M^{-1}). *Schizosaccharomyces pombe* Dcp1/2 complex was prepared as described previously (25).

Fluorescence assays (eIF4E)

The binding affinities of eIF4E and cap analogs **3**–**6** were determined using the fluorescence titration method (23). The measurements were carried out on an LS-55 spectrofluorometer (Perkin Elmer Co.) in a quartz cuvette (Hellma) with an optical path length of 4 mm for absorption and 10 mm for emission. The eIF4E titration measurements were performed in 50 mM HEPES/KOH (pH 7.2), 100 mM KCl, 0.5 mM ethylenediaminetetraacetic acid and 1 mM DTT at 20.0 ± 0.3°C. Aliquots of 1 μl increasing concentration of cap analog solutions were added to 1.4 ml of 0.1 μM eIF4E solutions. Fluorescence intensities (excitation at 280 nm with 2.5-nm bandwidth and detection at 340 nm with 4 nm bandwidth and 290 nm cutoff filter) were corrected for sample dilution and the inner filter effect. Equilibrium association constants (K_{AS}) were determined by fitting the theoretical dependence of the fluorescence intensity on the total concentration of cap analog to the experimental data points as described previously (23). The final K_{AS} was calculated as a weighted average of three to five independent titration assays. Numerical nonlinear least-squares regression analysis was performed using ORIGIN 9.1 (Microcal Software Inc., USA). The Gibbs free energy of binding was calculated from the K_{AS} value according to the standard equation $\Delta G^0 = -RT\ln K_{AS}$

Enzymatic assays (SpDcp2)

In vitro synthesis of short mRNAs. Short capped transcripts 34 nt in length were synthesized by *in vitro* transcription of pSP-luc+ vector linearized at EcoRI site. Reactions were performed with T7 polymerase in the presence of all four nucleoside triphosphates (final concentration 0.1 mM) and various cap dinucleotides (final concentration 0.5 mM) for 45 min at 37°C. Subsequently samples were treated with RQ1 RNase-Free DNase (Promega) and purified with RNA Clean & Concentrator™-5 columns (Zymo Research). The quality and quantity of transcripts were determined by Pico200 (Picodrop Ltd) and on 15% polyacrylamide sequencing gel with 7 M urea.

Decapping assays. A total of 20 ng of capped transcripts were subjected to digestion with 50 or 200 nM *S. pombe* Dcp1/2 complex in 10 mM Hepes pH 7.5, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol at 30°C for indicated time periods. Control reactions were performed without addition of enzyme. All reactions were stopped by cooling on ice and adding FORMAzol (Molecular Research Center) followed by denaturation for 3 min at 55°C. RNA sequencing gels (15% polyacrylamide, 7 M urea) were run at 400 V for about 4 to 5 h. The gels were stained with SYBR Gold Nucleic Acid Gel Stain (Life technologies) and visualized with ChemiDoc™ MP Imaging System (Bio-Rad). Quantity of material in individual bands was analyzed with Image Lab Software (Bio-Rad). The data represent the means ± SD from two experiments.

In vitro translation and inhibition of translation in the RRL system

mRNA synthesis. Capped, polyadenylated luciferase mRNAs (for studies in the RRL translation system) were synthesized *in vitro* on a dsDNA template (obtained by polymerase chain reaction reaction) using SP6 RNA polymerase, as described previously (Rydzik *et al.*, (46)). RNA transcripts, after removal of the DNA template with RQ1 DNase (Promega), were purified using NucleoSpin RNA clean-up columns (Machery-Nagel), checked on a non-denaturing 1% agarose gel and RNA concentrations were measured by ultraviolet absorption at 260 nm Pico200 (Picodrop Ltd).

Translation efficiency in RRL. The translation efficiency of differently capped luciferase transcripts was determined in Flexi RRL (Promega) as described previously (24). Briefly, the translation reactions were performed in 10 μl volume for 60 min at 30°C under conditions determined for cap-dependent translation. Reaction mixtures were preincubated (60 min at 30°C) before transcript addition. A typical reaction contained: 40% flexi RRL lysate (with 1.7 mM endogenous Mg^{2+} concentration), a mixture of amino acids (0.01 mM), magnesium acetate (1 mM), potassium acetate (190 mM) and 5′-capped mRNA. Four different concentrations of each analyzed transcript were tested. The activity of synthesized luciferase was measured in a luminometer (Glomax, Promega), and depicted as a function of the capped luciferase mRNA concentration. After linear fitting to the obtained data points (Excel software), the translation efficiency of tested transcripts was normalized to the m^7GpppG-capped luciferase mRNA.

Inhibition of translation. Inhibition of cap-dependent translation in RRL (Flexi RRL, Promega) by cap analogs, analysis of their stability in RRL and calculation of IC_{50} values were performed as described previously (26). Briefly, the *in vitro* translation reaction was performed in 12.5 μl volume for 60 min at 30°C, in conditions determined for cap dependent translation. The reaction mixture was preincubated for 60 min at 30°C prior to addition of dinucleotide cap analog (inhibitor) and $m_2^{7,3'\text{-}O}$GpppG-capped luciferase mRNA to start the translation reaction. Reactions were stopped by chilling on ice and the luciferase activity was measured in a luminometer (Glomax, Promega).

mRNA expression in dendritic cells

mRNA synthesis. Capped luciferase-encoding mRNAs were prepared by *in vitro* transcription with T7 RNA polymerase as described before (11). As a template for transcription pST1-2hBgUTR-A120 vector was used (11). Subsequent to enzymatic digestion of plasmid DNA, the RNA was purified using magnetic beads (Dynabeads MyOne carboxylic acid, Invitrogen, Carlsbad, CA, USA) and was finally stored in water (27). RNA concentrations were determined spectrophotometrically at 260 nm (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA). Homogenity of samples was verified by microfluidics-based electrophoreses (2100 Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA, USA) and no significant differences in RNA integrity were observed.

Translation in hiDCs (luciferase assay). Purification and cultivation of human immature dendritic cells (hiDCs), the transfer of mRNA into cells and the luciferase reporter gene assay were performed as previously (11). Briefly, differentially capped mRNAs were electroporated into hiDCs using single-pulse procedure (Gene-Pulser-II, Bio-Rad, Munich, Germany) with 300 V per 150 μF (t∼12 ms), diluted into culture medium and incubated in 37°C. Luciferase expression was measured at different time points (2, 4, 8, 24, 48 and 72 h) (Bright-Glo Luciferase Assay System, Promega, Madison, USA, Infinite M200, Tecan, Crailsheim, Germany). Luciferase activity was depicted as a function of time (Figure 3) and further analysis was performed by spline interpolation of the experimental data points as described previously (11), using the R software (R Development Core Team, 2008). Calculated parameters of translational efficiency (maximal slope), functional RNA stability (time of maximal protein expression) and total protein expression (integral of the curve) are set in Table 2.

RESULTS

Analogs synthesis

Cap analogs and other 5',5'-dinucleotides are conventionally synthesized by formation of a pyrophosphate bond between two mononucleotides. The bond is formed in a reaction of *P*-nucleophilic and *P*-electrophilic subunits (usually nucleotides 5'-substituted by an activating group (28–30), which is often mediated by divalent cations in an aprotic polar solvent (31,32). We previously implemented this general method to synthesize cap analogs using *P*-imidazolides as *P*-electrophiles, phosphate-modified or unmodified nucleotides as nucleophiles and $ZnCl_2$ or $MgCl_2$ as catalysts (33). However, modifications of the polyphosphate chain often alter the chemical properties of nucleotides, so in the case of the 2S analogs, the procedure had to be adjusted to suit the specific properties of the chosen modification.

The synthesis of 2S analogs (compounds **3**–**7**, see 'Materials and Methods' section) required obtaining compounds **1** and **2** (p_Sp_SG and $p_Sp_Sp_SpG$, respectively) as key *P*-nucleophilic cap precursors with terminal dithiodiphosphate moieties (Figure 1A). To achieve this, we employed our recently published procedure for the extension of 5'-*O*-polyphosphate chain-modified nucleotides by reacting them with the *P*-imidazolide derivative of either 2-cyanoethylphosphate (8) or *S*-2-cyanoethylthiophosphate (9), followed by 2-cyanoethyl group removal under basic conditions (34). Using this methodology, compounds **1** and **2** were synthesized with adequate yields of 30 and 24%, respectively (Figure 1B). The structures of both compounds were confirmed by HPLC, HRMS, ^{1}H NMR and ^{31}P NMR (see Supplementary Tables S1–4).

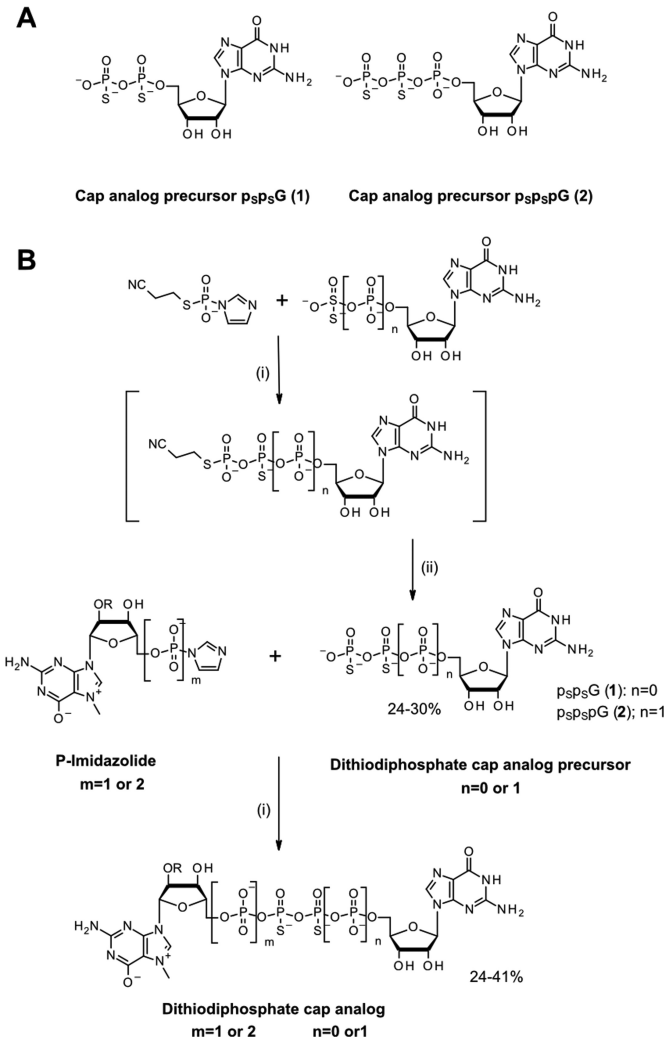

Figure 1. Synthesis of dithiodiphosphate cap analogs. (**A**) Structures of dithiodiphosphate cap precursors synthesized in this study. (**B**) Scheme of dithiodiphosphate cap synthesis. Reagents included (i) $MgCl_2$, microwave radiation (MW), dimethylformamide (DMF); and (ii) 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), dithiothreitol (DTT), MW, DMF. R = H, CH_3. Combinations of substrates used in final coupling included combination 1: m = 1, n = 0; combination 2: m = 1, n = 1; and combination 3: m = 2, n = 0.

The next step in analog synthesis was the coupling of an appropriate *P*-imidazolide with compound **1** or **2** (Figure 1B). The *P*-imidazolides were prepared by reacting an appropriate nucleotide (m^7GMP (**10**), $m_2^{7,2'\text{-O}}$GMP (**11**), m^7GDP (**12**), or $m_2^{7,2'\text{-O}}$GDP (**13**)) with imidazole in the presence of a 2,2'-dithiodipyridine/triphenylphosphine activation system (35). Depending on the length of the oligophosphate chain and the desired localization of the

modification, three types of starting material combinations were used (Figure 1B). The coupling reaction conditions were optimized for the reaction of either **1** or **2** with m^7GMP-Im (**14**), leading to cap analogs **3** and **6**, respectively. As a starting point, we used coupling conditions that were previously used for the synthesis of cap analogs with a single O-to-S substitution, i.e. excess $ZnCl_2$ in DMF at room temperature (19,24). Surprisingly, these conditions resulted in very low yields of compounds **3** and **6**. In addition, the formation of the desired products under these conditions was slow (not exceeding 5% yield after 24 h) and excessive hydrolysis of **1** and **2** (to GMPS and GDPβS, respectively) was observed as a competing reaction, likely due to the acidification of the reaction mixture preceded by hydrolysis of $ZnCl_2$ in the presence of moisture. Degradation occurred even if high-grade anhydrous solvents and dried reagents were used, indicating that dithiodiphosphate nucleotides **1** and **2** are extremely unstable under such conditions. To avoid this problem, we tried $MgCl_2$ as a less acidic reaction mediator (34), but no reaction products were observed, even after several days. Finally, we tried to accelerate the reaction using microwave irradiation, as we have recently shown that many pyrophosphate bond formation reactions may be facilitated by microwaves (34). We found that in the presence of mild microwave radiation at constant temperature (dynamic mode, $P_{max} = 10$ W, $T_{max} = 45 \pm 1°C$, 2.45 GHz) and $MgCl_2$ in DMF, the desired coupling reactions were completed within 0.5–1.5 h.

We therefore used the above conditions to synthesize cap analogs **3**–**7** (Figure 2A). Reaction progress was monitored using RP-HPLC. In each case, the products of coupling were formed within 30–90 min in the presence of microwave heating at 45 ± 1°C. Importantly, conventional heating at 45°C did not yield similar results and degradation of starting materials was observed at higher temperatures. The reactions were quenched by addition of water, and the products were isolated with satisfying yields (24–41%) as diastereoisomeric mixtures by ion-exchange chromatography on diethylaminoethyl (DEAE) Sephadex resin (see 'Materials and Methods' section). In contrast to the extremely labile precursors **1** and **2**, cap analogs **3**–**7** were chemically stable, similar to their unmodified counterparts. For example, they are fairly stable in aqueous solutions and can be stored at −20°C for several months.

Due to the presence of four different substituents around the phosphorus atoms in the 1,2-dithiodiphosphate moiety, these atoms are stereogenic centers. Therefore, each of the 2S analogs contains two *P*-stereogenic centers and consequently exists as a mixture of four diastereoisomers. These were resolved into single diastereoisomers or pairs of diastereoisomers by RP-HPLC and termed D1–D4 according to the elution order (Figure 2B). The $m^7Gpp_Sp_SG$ diastereoisomers (**3a–d**) were fully resolved, and the compound could therefore be subjected to further analysis as four separate samples. In other cases, at least one pair of diastereoisomers co-eluted from the RP HPLC column (as D1–D2 and/or D3–D4 mixtures). Therefore, for these analogs, some stereoisomers were subjected to further study as pure samples and some as mixtures, as shown in Figure 2B. The structures and purities of compounds were confirmed by HRMS, ^{1}H NMR and ^{31}P NMR (Supplementary Tables S1–4). The presence of the 1,2-dithiodiphosphate moiety is manifested by an approximately 50-ppm downfield shift of the phosphorus resonances in ^{31}P NMR spectra vs. the corresponding resonances of the unmodified parent compounds (Figure 2C).

Affinity for eIF4E

The protein eIF4E is responsible for mRNA cap binding and initiation of translation in the cytoplasm. High affinity of cap for eIF4E is essential to enable efficient translation of capped mRNA. We therefore evaluated the affinities of the 2S analogs to eIF4E. Because compounds **3a–d**, **5a–b** and **6a–b** are representative of all 2S analogs, as it has been previously shown that the presence of a 2'-*O*-methyl group does not influence binding affinity (Jemielity *et al.*, (15)), these analogs were assayed to provide an understanding of how the binding affinity is influenced by the location of the dithiodiphosphate group and the length of the oligophosphate chain. The equilibrium association constants (K_{AS}) for the cap analog–eIF4E complexes were determined using the fluorescence quenching titration method (23).

The results (summarized in Table 1) indicate that the presence of the 1,2-dithiodiphosphate moiety generally stabilizes the cap–eIF4E complex. For example, the K_{AS} values for **3a–d,** the cap analogs containing the α,β-dithiodiphosphate modification in the triphosphate chain (3P-2S series), range from 34.8 ± 1.7 μM^{-1} to 54.5 ± 2.2 μM^{-1}; this is three to five times higher than that of the unmodified 3P cap analogs, m^7GpppG and $m_2{}^{7,2'\text{-O}}$GpppG (9.4 ± 0.4 and 10.8 ± 0.3 μM^{-1}, respectively). In agreement with previous studies, extension of the polyphosphate chain increased the K_{AS} values. The K_{AS} values for 2S analogs containing a modification in the tetraphosphate chain (4P-2S series) range from 180.6 ± 5.2 μM^{-1} (**6a**) to 918.5 ± 36.6 μM^{-1} (**5b**), compared to 99.8 ± 6.0 μM^{-1} for the unmodified 4P cap analog, $m_2{}^{7,2'\text{-O}}$GpppG. The stabilization was higher for analogs modified in the β,γ positions than in the α,β positions.

Susceptibility to decapping enzyme SpDcp1/2

In addition to high affinity for eIF4E, the resistance of capped RNA to enzymatic decapping by Dcp1/2 pyrophosphatase is another factor required for high overall protein yield of mRNAs *in vivo*. Therefore, in the next step of our study, we investigated the susceptibility of RNA transcripts capped with 2S analogs to decapping by Dcp1/2. To assess Dcp1/2 susceptibility, cap analogs **4a–c**, **5a–b** and **7a–b** were incorporated into short (34 nt) RNAs by *in vitro* transcription and the capped RNAs were subsequently incubated with recombinant *S. pombe* Dcp1/2 complex. Six previously reported cap analogs were also included in the assay, including those previously identified as human (h) Dcp2-susceptible ($m_2{}^{7,2'\text{-O}}$GpppG) or Dcp2-resistant or partially resistant ($m_2{}^{7,2'\text{-O}}Gpp_SpG$ D1 and D2) and those for which Dcp2 susceptibility had not yet been reported or studied in detail ($m_2{}^{7,2'\text{-O}}$GppppG, $m_2{}^{7,2'\text{-O}}Gppp_SpG$ D1D2 and $m_2{}^{7,2'\text{-O}}Gpp_SppG$ D1D2). Decapping reactions were stopped at different time points (5,15 and 30 min); the substrates and products were then separated on an RNA sequencing gel, stained with fluorescent dye and visualized on

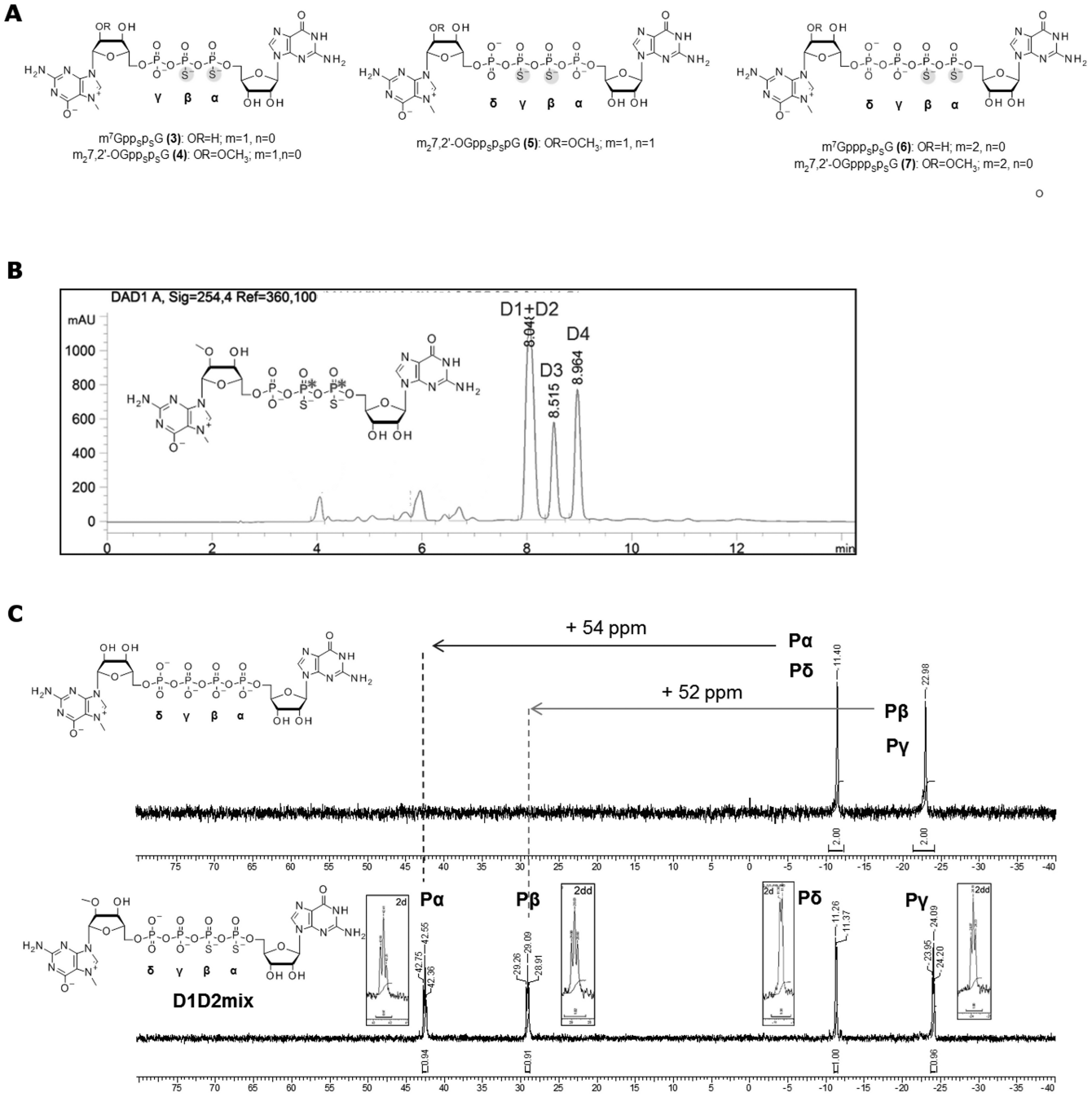

Figure 2. Dithiodiphosphate cap analogs and their selected characteristic properties. (**A**) Structures of dithiodiphosphate cap analogs synthesized in this study. (**B**) During synthesis, two new P-chiral centers (indicated by *) are generated, leading to four diastereoisomeric forms of each cap analog (D1–D4). Diastereoisomers were separated by semi-preparative RP-HPLC, resulting in the following final compounds/mixtures: **m^{7}Gpp$_S$p$_S$G (3)**: D1 (**3a**), D2 (**3b**), D3 (**3c**), D4 (**3d**); **m$_2^{7,2'\text{-O}}$Gpp$_S$p$_S$G (4)**: D1D2 (**4a**), D3 (**4b**), D4 (**4c**); **m$_2^{7,2'\text{-O}}$Gpp$_S$p$_S$pG (5)**: D1D2 (**5a**), D3D4 (**5b**); **m^{7}Gppp$_S$p$_S$G (6)**: D1D2 (**6a**), D3D4 (**6b**); **m$_2^{7,2'\text{-O}}$Gppp$_S$p$_S$G (7)**: D1D2 (**7a**), D3D4 (**7b**). (**C**) An example ^{31}P NMR spectrum of compound **7a** displays the characteristic shifts of P-signals as the result of sulfur substitutions. In the spectrum, signals of P atoms of two diastereoisomers (D1+D2) are present and partially overlap. Dual signals from diastereoisomeric forms are marked here with a preceding '2'; for example, '2d' means that two doublet signals of the same P nucleus from both diastereoisomers overlap.

Table 1. Equilibrium association constants (K_{AS}) for complexes of cap analogs with mouse eIF4E obtained from analysis of fluorescence titrations at 20°C

Entry	Cap analog	Series	K_{AS} (cap–eIF4E) [μM^{-1}]	ΔG° (kcal·mol^{-1})
1	m^7GpppG	3P	9.4 ± 0.4[a]	−9.35 ± 0.03[a]
2	$m_2^{7,3'\text{-O}}$GpppG	3P	10.2 ± 0.3[a]	−9.40 ± 0.02[a]
3	$m_2^{7,2'\text{-O}}$GpppG	3P	10.8 ± 0.3[b]	−9.42 ± 0.02[b]
4	$m_2^{7,2'\text{-O}}$GppppG	4P	99.8 ± 6.0[b]	−10.73 ± 0.04[b]
5	m^7Gp_5G	5P	543 ± 55[b]	−11.71 ± 0.06[b]
6	$m_2^{7,2'\text{-O}}$GppspG D1	3P-S	43.1 ± 1.4[a]	−10.23 ± 0.02[a]
7	$m_2^{7,2'\text{-O}}$GppspG D2	3P-S	19.3 ± 2.2[a]	−9.77 ± 0.07[a]
8	$m_2^{7,2'\text{-O}}$GpppspG D1D2	4P-S	158 ± 2[c]	−10.99 ± 0.01[c]
9	$m_2^{7,2'\text{-O}}$GppsppG D1D2	4P-S	282 ± 12[c]	−11.33 ± 0.02[c]
10	m^7GppspsG D1 (**3a**)	3P-2S	42.1 ± 1.6	−10.22 ± 0.02
11	m^7GppspsG D2 (**3b**)	3P-2S	54.5 ± 2.2	−10.37 ± 0.02
12	m^7GppspsG D3 (**3c**)	3P-2S	49.7 ± 7.0	−10.32 ± 0.08
13	m^7GppspsG D4 (**3d**)	3P-2S	34.8 ± 1.7	−10.11 ± 0.03
14	$m_2^{7,2'\text{-O}}$GppspspG D1D2 (**5a**)	4P-2S	505 ± 69	−11.67 ± 0.08
15	$m_2^{7,2'\text{-O}}$GppspspG D3D4 (**5b**)	4P-2S	918 ± 37	−12.02 ± 0.02
16	m^7GpppspsG D1D2 (**6a**)	4P-2S	180.6 ± 5.2	−11.07 ± 0.02
17	m^7GpppspsG D3D4 (**6b**)	4P-2S	227.1 ± 7.1	−11.20 ± 0.02

[a]Ref. (19).
[b]Ref. (15).
[c]Ref (24).

a transilluminator (Supplementary Figure S1). The amount of capped (substrate) and decapped (product) RNA was determined by densitometry, and the decapping percentage was calculated as the ratio of decapped to total mRNA, as described in 'Materials and Methods' section.

The results of the decapping assay are shown in Supplementary Figure S1 and summarized in Table 2. Capped mRNAs migrate more slowly than decapped mRNAs. For RNA capped with the unmodified cap analog $m_2^{7,2'\text{-O}}$GpppG, the intensity of the more slowly migrating band decreased rapidly over time, reflecting decapping of ∼70 and 75% after 5 and 15 min, respectively (Supplementary Figure S1). The small fraction of quickly migrating RNA at time point 0 min likely corresponds to uncapped (GTP-initiated) RNA or transcript N-1 (33 nt), as RNA polymerases have a tendency to fall off the template before reaching the end of the DNA matrix (36). Both β-S-ARCA analogs, $m_2^{7,2'\text{-O}}$GppspG D1 and $m_2^{7,2'\text{-O}}$GppspG D2 (Supplementary Figure S1, lanes *41–48*), became decapped more slowly than the unmodified analogs. Transcripts with the unmodified tetraphosphate analog $m_2^{7,2'\text{-O}}$GppppG were as susceptible to decapping by SpDcp1/2 (Supplementary Figure S1 A, lanes *1–4*,*17–20*) as those capped with $m_2^{7,2'\text{-O}}$GpppG (18).

Interestingly, tetraphosphate cap analogs (4P-S) containing a single phosphorothioate substitution—$m_2^{7,2'\text{-O}}$GpppspG D1D2, $m_2^{7,2'\text{-O}}$GppspppG and 4P-2S analog **7a**—did not stabilize RNAs against Dcp1/2 in our assay. The 2S analogs **4b, 5b** and **7b** stabilized RNA against SpDcp1/2 as much as or slightly more than $m_2^{7,2'\text{-O}}$GppspG D1 and $m_2^{7,2'\text{-O}}$GppspG D2. In contrast, RNAs capped with the 2S analogs **4a, 4c** and **5a** ($m_2^{7,2'\text{-O}}$GppspspG D1D2-RNA) were notably less susceptible to SpDcp1/2 than $m_2^{7,2'\text{-O}}$GppspG D2-RNA, as they were decapped by only 10–30% after 5–15 min. To confirm these results, the most resistant transcripts were subjected to incubation at a 5-fold higher SpDcp1/2 concentration. Under these harsher conditions, mRNAs capped with **4a, 4c** and **5a** still exhibited increased resistance to the SpDcp1/2 complex (Supplementary Figure S1 B). The cap analog least susceptible to degradation was $m_2^{7,2'\text{-O}}$GppspsG D4 (**4c**), with mRNAs decapped by less than 20% after 30 min.

Translational properties

Cap analogs that combine high affinity for eIF4E and resistance to decapping may confer enhanced translational potential to mRNA (18,37). Consequently, we next evaluated the translational potential of mRNAs containing the representative dithiodiphosphate cap analogs **4a–c**, **5a–b** and **7a–b**. After an initial experiment in RRL that confirmed the functionality of all cap analogs and transcripts in the translation process (Table 3), we assessed the translational behavior of new transcripts in mammalian cells. As a model system, we used human immature dendritic cells (hiDCs). Efficient expression of proteins in hiDCs is of great interest for mRNA-based immunotherapies against cancer, since hiDCs are the main group of antigen-presenting cells in the immune system. Luciferase-mRNAs were synthesized *in vitro* with various cap analogs at the 5'-end and 120-nt poly(A) tails at the 3'-end. The mRNAs were introduced into hiDCs by electroporation and the cells were incubated for up to 72 h. Luciferase mRNA levels were then measured. Independent experiments were performed for tri- and tetraphosphate cap analogs.

The results of the translational assay are summarized in Figure 3 and Table 4. All results are relative to the so-called 'gold standard' of transcripts capped with $m_2^{7,2'\text{-O}}$GppspG D1, which is the best expression-enhancing cap analog identified so far and is currently being used in clinical trials for melanoma (11). For all transcripts capped with 2S analogs, translation efficiency was higher than that ob-

Table 2. Susceptibility of analog-capped transcripts to decapping by SpDcp1/2 complex

Cap analog at 5' end of mRNA	Series	Decapping %		
		5 min	15 min	30 min
$m_2^{7,2'\text{-}O}$GpppG	3P	68.9 ± 0.4	78.6 ± 2.4	79.7 ± 7.5
$m_2^{7,2'\text{-}O}$GppppG	4P	73.4 ± 7.6	78.4 ± 9.4	82.9 ± 11.3
$m_2^{7,2'\text{-}O}$GppspG D1	3P-S	47.5 ± 9.0	56.1 ± 11.5	60.9 ± 12.1
$m_2^{7,2'\text{-}O}$GppspG D2	3P-S	47.3 ± 10.6	51.4 ± 15.6	57.4 ± 10.7
$m_2^{7,2'\text{-}O}$GppspsG D1D2 (**4a**)	3P-2S	29.7 ± 2.8	32.2 ± 1.5	36.7 ± 3.9
$m_2^{7,2'\text{-}O}$GppspsG D3 (**4b**)	3P-2S	44.7 ± 3.4	51.5 ± 0.7	59.8 ± 4.8
$m_2^{7,2'\text{-}O}$GppspsG D4 (**4c**)	3P-2S	13.4 ± 0.7	16.7 ± 2.0	19.8 ± 2.5
$m_2^{7,2'\text{-}O}$GpppspsG D1D2 (**5a**)	4P-2S	13.2 ± 1.2	19.7 ± 0.6	17.5 ± 1.5
$m_2^{7,2'\text{-}O}$GpppspsG D3D4 (**5b**)	4P-2S	63.4 ± 6.7	76.4 ± 10.6	68.7 ± 2.5
$m_2^{7,2'\text{-}O}$GppppspsG D1D2 (**7a**)	4P-2S	69.6 ± 7.9	79.8 ± 2.4	80.1 ± 2.2
$m_2^{7,2'\text{-}O}$GppppspsG D3D4 (**7b**)	4P-2S	55.2 ± 5.6	64.7 ± 12.5	65 ± 15
$m_2^{7,2'\text{-}O}$GppppspG D1D2	4P-S	72.7 ± 1.9	78.3 ± 1.0	79.5 ± 3.5
$m_2^{7,2'\text{-}O}$GppspppG D1D2	4P-S	57.9 ± 14.6	65.6 ± 18.0	71.6 ± 17.2

Quantitative analysis of gels presented in Supplementary Figure S1. Decapping percentage was calculated as the percent loss in the upper band normalized by the total quantity in the upper and lower bands.

Table 3. Translational properties of mRNAs 5′-capped with 2S analogs measured in RRL along with eight previously reported cap analogs included into the assay for comparison

		Translational properties in RRL	
cap analog	series	relative translation efficiency[a]	relative cap-dependent translation efficiency[b]
m^7GpppG	3P	1	1
ApppG		0.16 ± 0.04	0.00
$m_2^{7,3'\text{-}O}$GpppG	3P	1.83 ± 0.64	1.99 ± 0.81
$m_2^{7,2'\text{-}O}$GpppG	3P	1.81 ± 0.32	1.96 ± 0.43
$m_2^{7,2'\text{-}O}$GppppG	4P	1.79 ± 0.43	1.94 ± 0.56
$m_2^{7,2'\text{-}O}$GppspG D1	3P-S	2.07 ± 0.18	2.27 ± 0.10
$m_2^{7,2'\text{-}O}$GppspG D2	3P-S	2.54 ± 0.66	2.83 ± 0.26
$m_2^{7,2'\text{-}O}$GppspppG D1D2mix	4P-S	2.51 ± 0.59	2.80 ± 0.83
$m_2^{7,2'\text{-}O}$GppspsG D1D2mix (**4a**)	3P-2S	3.16 ± 0.76	3.57 ± 0.95
$m_2^{7,2'\text{-}O}$GppspsG D3 (**4b**)	3P-2S	1.82 ± 0.78	1.98 ± 0.98
$m_2^{7,2'\text{-}O}$GppspsG D4 (**4c**)	3P-2S	2.77 ± 0.95	3.11 ± 1.18
$m_2^{7,2'\text{-}O}$GpppspspG D1D2mix (**5a**)	4P-2S	2.52 ± 0.51	2.81 ± 0.65
$m_2^{7,2'\text{-}O}$GpppspspG D3D4mix (**5b**)	4P-2S	2.63 ± 0.79	2.94 ± 0.99
$m_2^{7,2'\text{-}O}$GppppspsG D1D2mix (**7a**)	4P-2S	2.83 ± 0.64	3.18 ± 0.81
$m_2^{7,2'\text{-}O}$GppppspsG D3D4mix (**7b**)	4P-2S	2.20 ± 0.33	2.43 ± 0.44

[a]The mean values were calculated from 2–3 assay repetitions for each of two independent mRNA syntheses.
[b]Calculated by correcting the translation of each mRNA in a particular experiment by subtracting the value of translation of mRNA capped with ApppG (a non-functional cap structure) before normalization to m^7GpppG.
The translation experiment was performed under conditions of linear production of luciferase with respect to both mRNA concentration and time of incubation (16).

served for $m_2^{7,2'\text{-}O}$GppspG D1 mRNA, with a top value of 3.97 ± 0.25 for $m_2^{7,2'\text{-}O}$GppspsG D3D4 (**7b**)-mRNA. Interestingly, with the exception of $m_2^{7,2'\text{-}O}$GppspspG D3D4, the time of maximal expression (t_{max}) was much longer for 2S analogs, with a maximum value of 25.3 ± 0.54 h for $m_2^{7,2'\text{-}O}$GppspspsG D3D4, indicating higher functional stability of the transcripts in hiDCs. A similar trend was observed for total protein expression. mRNAs capped with the 2S analogs **4a**, **4b**, **4c**, **5a**, **7a** and **7b** exhibited increased overall translation in hiDCs, with relative values of 1.56 ± 0.23 for $m_2^{7,2'\text{-}O}$GppspspG D1D2, 1.57 ± 0.34 for $m_2^{7,2'\text{-}O}$GppspspsG D3D4, 1.60 ± 0.01 for $m_2^{7,2'\text{-}O}$GppspsG and a maximum value of 1.79 ± 0.24 for $m_2^{7,2'\text{-}O}$GppspspsG D1D2.

We also determined the ability of 2S analogs to inhibit cap-dependent translation in RRLs. In this experiment, translation efficiency for $m_2^{7,3'\text{-}O}$GpppG-capped luciferase mRNA in the presence of increasing concentrations of a given cap analog was measured to determine the IC_{50} value. The results are shown in Table 5. All 2S-analogs turned out to have better inhibitory properties than unmodified cap m^7GpppG, The values of IC_{50} for 2S analogs ranged between 1.4 ± 0.2 and 7.0 ± 0.8 μM. The lowest value of IC_{50} was determined for analogs **5b** and **7b**, (respectively 1.4 ± 0.2 and 1.4 ± 0.2 μM) being around 10-times lower than that for m^7GpppG.

Table 4. Translational properties of capped mRNA relative to that of 5'-$m_2^{7,2'\text{-O}}$GppSpG D1-mRNA in human immature dendritic cells (hiDCs)

	Translational properties in hiDCs[a]			
Cap analog	Series	Relative translation efficiency	t_{max} (h)	Relative total expression
$m_2^{7,2'\text{-O}}$GppppG	4P	1.25 ± 0.15	5.3 ± 0.05	0.11 ± 0.03
$m_2^{7,2'\text{-O}}$GppSpG D1	3P-S	1.00 ± 0.03	11.5 ± 0.33	1.00 ± 0.02
$m_2^{7,2'\text{-O}}$GppSpG D2	3P-S	1.33 ± 0.02	15.5 ± 0.20	0.64 ± 0.04
$m_2^{7,2'\text{-O}}$GppSppG D1D2	4P-S	0.53 ± 0.17	10.5 ± 0.05	0.20 ± 0.02
$m_2^{7,2'\text{-O}}$GpppSpG D1D2	4P-S	0.82 ± 0.12	8.5 ± 0.05	0.28 ± 0.01
$m_2^{7,2'\text{-O}}$GppppSG D1	4P-S	0.63 ± 0.05	15.5 ± 0.20	0.62 ± 0.03
$m_2^{7,2'\text{-O}}$GppppSG D2	4P-S	0.77 ± 0.01	14.5 ± 0.21	0.61 ± 0.01
$m_2^{7,2'\text{-O}}$GppSpSG D1D2 (**4a**)	3P-2S	1.21 ± 0.15	17.75 ± 0.05	1.60 ± 0.01
$m_2^{7,2'\text{-O}}$GppSpSG D3 (**4b**)	3P-2S	1.13 ± 0.18	16.0 ± 0.03	1.20 ± 0.04
$m_2^{7,2'\text{-O}}$GppSpSG D4 (**4c**)	3P-2S	1.57 ± 0.21	14.50 ± 0.35	1.50 ± 0.10
$m_2^{7,2'\text{-O}}$GppSpSpG D1D2 (**5a**)	4P-2S	1.37 ± 0.40	15.0 ± 0.40	1.56 ± 0.23
$m_2^{7,2'\text{-O}}$GppSpSpG D3D4 (**5b**)	4P-2S	1.19 ± 0.04	6.0 ± 0.04	0.40 ± 0.02
$m_2^{7,2'\text{-O}}$GpppSpSG D1D2 (**7a**)	4P-2S	2.11 ± 0.22	14.8 ± 0.31	1.79 ± 0.24
$m_2^{7,2'\text{-O}}$GpppSpSG D3D4 (**7b**)	4P-2S	3.97 ± 0.25	25.3 ± 0.54	1.57 ± 0.34

[a]Curves presented in Figure 3 were analyzed by mathematical modeling to determine translational efficiency (slope of the curve), time-point of maximal protein amount as indicator of functional RNA stability and total amount of protein (integral of the curve) in hiDCs. Values shown are averages ±s.d.

Table 5. Inhibition of $m_2^{7,2'\text{-O}}$GpppG-capped-luciferase mRNA translation in RRL lysate by novel 2S- cap analogs

Entry	Cap analog	Series	IC_{50} [μM]
1	m^7GpppG	3P	13.9 ± 0.6
6	$m_2^{7,2'\text{-O}}$GppSpG D1	3P-S	9.8 ± 1.0
7	$m_2^{7,2'\text{-O}}$GppSpG D2	3P-S	3.7 ± 0.3
8	$m_2^{7,2'\text{-O}}$GpppSpG D1D2	4P-S	3.3 ± 0.3
9	$m_2^{7,2'\text{-O}}$GppSppG D1D2	4P-S	2.6 ± 0.3
10	$m_2^{7,2'\text{-O}}$GppSpSG D1D2 (**4a**)	3P-2S	2.3 ± 0.2
11	$m_2^{7,2'\text{-O}}$GppSpSG D3 (**4b**)	3P-2S	7.0 ± 0.8
12	$m_2^{7,2'\text{-O}}$GppSpSG D4 (**4c**)	3P-2S	2.8 ± 0.2
14	$m_2^{7,2'\text{-O}}$GppSpSpG D1D2 (**5a**)	4P-2S	2.2 ± 0.3
15	$m_2^{7,2'\text{-O}}$GppSpSpG D3D4 (**5b**)	4P-2S	1.4 ± 0.2
16	$m_2^{7,2'\text{-O}}$GpppSpSG D1D2 (**7a**)	4P-2S	2.3 ± 0.2
17	$m_2^{7,2'\text{-O}}$GpppSpSG D3D4 (**7b**)	4P-2S	1.4 ± 0.2

DISCUSSION

This work features the chemical synthesis and biochemical characterization of novel class of RNA cap analogs (termed 2S analogs) that contain a 1,2-dithiophosphate moiety, i.e. two O-to-S substitutions each positioned within two neighbouring phosphate moieties. While there are numerous reported syntheses of nucleotide analogs containing a single O-to-S substitution within the oligophosphate bridge (19,38–41), synthesis and properties of compounds containing two neighbouring O-to-S substitutions have hardly been described. Ludwig and Eckstein achieved the synthesis and purification of guanosine 5′-O-(1,2-dithiotriphosphates) (GTPαSβS) diastereomers via chemoenzymatic method and studied their interaction with Cd^{2+} ions.(42) However, to the best of our knowledge, such moiety has never been introduced into dinucleoside 5′,5′-oligophosphates nor its influence on the interaction with nucleotide-binding proteins has been studied. We have previously shown that introduction of a single O-to-S substitution into triphosphate bridge of mRNA cap can increase affinity to eIF4E protein and decrease susceptibility to Dcp2. Both these features are beneficial in terms of mRNA stability and expression efficiency. However, these effects were strongly position-dependent, with analogs substituted at the β position of triphosphate combining most favorable properties. We envisaged that introduction of a second O-to-S substitution may additionally alter charge localization and geometry of the tri- or tetraphosphate bridge and, at least in some cases, lead to further improvements in biochemical properties of cap analogs. Therefore, we undertook a challenging task of developing a chemical methodology enabling a straightforward and efficient synthesis of 2S-analogs. The chemical synthesis of 2S-analogs was achieved via phosphorimidazolide chemistry combined with metal-ion catalysis in polar aprotic solvent, as previously described for many other cap analogs. However, 2S-analogs were not formed under conditions previously applied for S-analogs or BH_3-analogs (19,24,43) The reason for that may be relatively lower nucleophilicity and higher chemical lability under reaction conditions of precursors containing the 1,2-dithiodiphosphate moiety. Nonetheless, we found conditions affording the target compounds in reasonable yields due to application of microwave irradiation as a key improvement. Importantly, the proposed synthetic methodology is universal and may

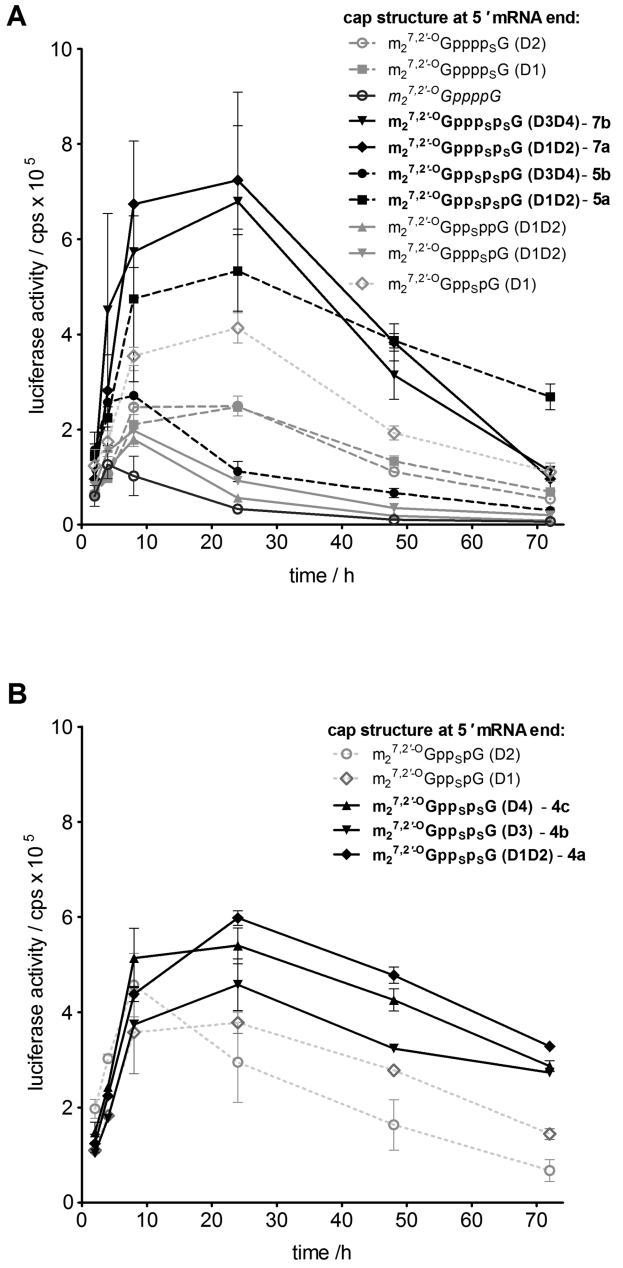

Figure 3. Expression of analog-capped mRNAs in human immature dendritic cells (hiDCs). Luciferase activity was measured for 72 h following electroporation of mRNA containing the respective tetraphosphate (**A**) or triphosphate (**B**) 5′-cap into hiDCs. In each experiment, $m_2^{7,2'\text{-O}}GppSpG$ (D1) was used as a reference. Duplicate measurements were performed and the average bioluminescence signal (±s.d.) is shown as a function of time.

give access to 2S-analogs of other biologically relevant nucleoside and dinucleoside oligophosphates, such as Ap_3A, NAD, etc. which have not been reported before.

We then characterized influence of 1,2-dithiodiphosphate moiety on several important aspects of the functionality of the cap. For a cap analog to be able to synthesize mRNA with increased stability and translational capability under cellular conditions, the analog must favorably interact with two structurally distinct cellular proteins, eIF4E and Dcp2, and must also be incorporated into mRNA by T7 RNA polymerase (14,33).

In terms of the first property, eIF4E binding affinity, comparison of the determined K_{AS} values with the ones previously reported for tri- and tetraphosphate analogs with a single O-to-S substitution (3P-S analogs and 4P-S analogs) revealed that each O-to-S substitution in the 2S analogs contributed to cap–eIF4E complex stabilization. For example, in the 3P-S series, the compound with the strongest eIF4E binding affinity was modified at the β-position ($m_2^{7,2'\text{-O}}GppSpG$ D1, K_{AS} 43.1 ± 1.4), whereas in the 3P-2S series, the D2 isomer of an α,β-modified compound (**3b**) was the strongest binder with K_{AS} of 54.5 ± 2.2. In the 4P-S series, the highest affinity was exhibited by a compound with a modification at the γ position, followed by the β position. Consequently, a combination of modifications at the β and γ positions yielded the 2S analog with the highest reported K_{AS} so far: $m_2^{7,2'\text{-O}}GppSpSpG$ D3D4 (**5b**). Interestingly, we observed up to a twofold difference in K_{AS} values for different diastereoisomers of **3**, **5** and **6**, suggesting distinct preferences in the geometry of the interaction. However, for a deeper understanding of this phenomenon, the absolute configurations of compounds and co-crystal structures with the eIF4E protein should be determined. Although the K_{AS} values were determined only for selected compounds (**3a-d**, **5a-b** and **6a-b**), the affinities of compounds **4** and **7,** which are 2′-O-methylated derivatives of compounds **3** and **6**, respectively, can also be well assessed based on those data. As previous studies have shown, the 2′-OH and 3′-OH groups in N7-methylguanosine do not contribute to eF4E binding significantly and, consequently, methylation of either of those positions has little influence on K_{AS} value (15) (see Supplementary Table S5 for representative examples from previous works showing that K_{AS} values for m^7G 2′-O methyl compounds and their parent analogs are very similar). As such, in the further parts of the discussion we will occasionally refer to affinities of diastereomers of compounds **4** and **7** for eIF4E, assuming they are similar to the affinities of corresponding diastereomers of compounds **3** and **6,** respectively**.**

To additionally confirm that 2S analogs are tight binders of eIF4E we determined their ability inhibit cap-dependent translation in RRL. Typically, cap analogs characterized by high affinities for eIF4E are potent inhibitors of translation (14,19,26,33,44). We found that all 2S analogs were more potent inhibitor of translation than standard cap analog, m^7GpppG. Moreover, we observed a typical qualitative inverse correlation between IC_{50} and K_{AS} (the higher K_{AS}, the lower IC_{50}), as previously reported for many different cap analogs (26,43–47). This indicates that indeed 2S analogs with highest affinities for eFI4E compete more effectively for eIF4E's cap binding site in a biological medium.

As previous experiments demonstrated that increases in K_{AS} over a certain level did not lead to further improvements in translation (15), we turned our attention toward the stability of the cap analogs. We found that both β-S-ARCA analogs, $m_2^{7,2'\text{-O}}GppSpG$ D1 and $m_2^{7,2'\text{-O}}GppSpG$ D2, became decapped more slowly than the unmodified analogs, which is in qualitative agreement with previous studies on recombinant hDcp2 (37). However, in contrast to previous observations in which isomer D2 was almost

completely resistant to decapping, both diastereoisomers were degraded to a significant extent under our assay conditions (18). This difference may result from the fact that the Dcp1/2 complex was used in our assay instead of Dcp2 alone, or it may be due to different enzyme sources (*S. pombe* SpDcp1/2 versus hDcp2). Our decapping analysis revealed increased stability of some of the 2S analogs. Taking $m_2^{7,2'\text{-O}}GppSpG$ D2 as a point of reference, the 2S analogs **4c**, **4a** and **5a** show significantly better stability toward Dcp1/2. Varying degrees of stability was observed among the other 2S analogs. Analogs **4b** and **7b** are characterized by comparable percent of degradation by Dcp1/2 in our experiment as $m_2^{7,2'\text{-O}}GppSpG$ D2. Analogs **5b** and **7a** are significantly less stable toward decapping. We assume that the reason for the differences is related to the alignment of cap analogs in SpDcp1/2 catalytic site. Dcp2, the catalytic subunit of the complex, is a NUDIX family pyrophosphatase that utilizes three glutamate residues and corresponding Mg^{2+} cations to carry out catalytic hydrolysis of the α,β-pyrophosphate bond (48). Previous studies, including crystallographic data and an experiment with S-ARCAs and hDcp2 (18,25), suggest that the reaction may occur by nucleophilic attack of a water molecule at the β-phosphate. As also suggested previously, the replacement of a non-bridging P-O bond by a P-S bond may act in two ways: (i) disruption of the enzyme-substrate complex geometry and (ii) reduction of the affinity of the substrate to Mg^{2+} ions (18). In our assay, none of the analogs containing a single O-to-S substitution were fully protected against SpDcp1/2, with the slowest decapping rates observed for $m_2^{7,2'\text{-O}}GppSpG$ D1 and D2. This indicates that such modifications may reduce but do not fully eliminate RNA decapping. In contrast, neither of the studied 4P-S analogs ($m_2^{7,2'\text{-O}}GpppSpG$ and $m_2^{7,2'\text{-O}}GppSpp$) exhibited decreased susceptibility to decapping (Table 2). This may be due to greater flexibility of the tetraphosphate chain compared to that of the triphosphate chain, which may enable the enzyme to optimize the phosphate chain conformation and perform catalysis via attack at either the β or γ phosphorus atoms. We envisage that combination of two O-to-S substitutions in a tri- or tetraphosphate chain may further disrupt the enzyme-substrate geometry and decrease the affinity for Mg^{2+}. This hypothesis is supported by the fact that two 3P-2S analogs (**4a** and **4c,** modified at the α,β positions) and one 4P-2S analog (**5a**, modified at the β,γ positions) are highly resistant to SpDcp1/2. Interestingly, $m_2^{7,2'\text{-O}}GppSpSpG$ D3D4 (**5b**), which only differs from **5a** by diastereoisomer composition, was much more susceptible to hydrolysis by Dcp1/2, emphasizing the role of phosphate stereochemistry in catalysis. In fact **5a** is the first tetraphosphate cap analog with non-bridging modification exhibiting significant resistance to Dcp2, making it a useful tool for further studies. Recently, Ziemniak *et al*. identified a two-headed tetraphosphate cap analog $m^7GpSppSm^7G$ as a first small molecule inhibitor of Dcp2 (49). Similarly, 2S-analogs resistant to cleavage by Dcp2 are good candidates for molecular probes in biophysical, biochemical and crystallographic studies aimed at unraveling the detailed mechanism of binding and Dcp2 activity, allowing the design of more efficient and therapeutically relevant decapping inhibitors.

By introducing a dithiodiphosphate modification into cap structure, we significantly improved the translation of capped mRNAs, for 4P-2S analogs **5a** and **7a-b** achieving more than a 14-fold greater increase in total translation compared to that of $m_2^{7,2'\text{-O}}GppppG$-mRNA and for 2S analogs **4a-c**, **5a** and **7a-b** more than a 1.2-fold increase in total translation compared to that of the control, $m_2^{7,2'\text{-O}}GppSpG$ D1-mRNA. Comparing the translation results of the capped transcripts with their susceptibility to decapping (Table 2), in several cases we can observe the dependence of RNA properties on susceptibility to Dcp2. This is most clear when comparing cap structures that are structurally closely related, especially diastereomers of the same compound. For example for analogs **4a**, **4b** and **4c**, the analog providing the lowest overall protein expression is the one most susceptible to Dcp2 (**4b**). Similarly, when comparing **5a** and **5b** pair or **7a** and **7b**, the less susceptible analog in each pair produces also RNAs with higher overall expression. Somehow surprising is a result of high translation for **7a,** especially when comparing to analog **5a**. In this pair, **5a** is more resistant to decapping to Dcp1/2 and having higher affinity to eIF4E than **7a** (as mentioned before, we approximate here the K_{AS} value of **7a** based on value of **6a** assuming that methylation of 2′OH does not influence the binding). Nonetheless, RNA capped with **7a** exhibits better translational properties reflected in slightly higher overall protein yield (1.79 ± 0.24 versus 1.56 ± 0.34 for **5a** and **7b,** respectively).

Our study was limited to evaluation of translational potential of mRNAs capped with 2S analogs only in one cell type—hiDCs. Translational properties of mRNAs in hiDCs are of great interest for immunologist, especially ones who are focused on developing successful mRNA-based immunotherapies. However the same mRNAs capped with 2S analogs studied in different cell types might show different translational potential. Uncovering differences in translational properties of capped mRNA in other cell types might be one possible direction of future research leading to new potential therapeutic applications of mRNAs.

CONCLUSION

The work presented here addresses the problem of translational efficiency of mRNA *in vivo*, which is limited by the poor stability of mRNA transcripts under physiological conditions. Insufficient protein production from mRNA is problematic, as mRNA encoding a therapeutic agent must live long enough in the cytoplasm to reach optimal expression of the desired protein. Therefore mRNA-based therapies require simple methods for enhancing mRNA stability and translational properties. One approach toward optimization of the mRNA molecule is the engineering of a 5'-end that provides better protection against decapping enzymes and 5'-to-3' RNA degradation while simultaneously improving the interaction with the translational machinery.

In this work, we obtained a new class of cap analogs, termed 2S analogs, that combine a dithiodiphosphate modification, ARCA and (optionally) extended polyphosphate chain. 2S-analogs were synthesized for the first time and exhibited some interesting biological properties. Individual cap analogs were characterized by high affinities for

eIF4E and low susceptibility to Dcp2. Such properties make them interesting tools for investigation of interactions and molecular mechanisms of both eIF4E and Dcp2 but also are desired qualities when it comes to enhancing translation of mRNA. We reported that mRNAs capped with dithiodiphosphate cap analogs **4a**, **4b**, **4c**, **5a**, **7a** and **7b**, exhibited elevated overall translation in hiDCs relative to the current transcripts capped with $m_2^{7,2'\text{-O}}GppSpG$ D1, which is currently a 'gold standard' used in clinical trials for melanoma. Because the synthesis of mRNA that can be efficiently translated in hiDCs *in vitro* represents a promising tool for cancer immunotherapies, dithiodiphosphate cap analogs offer an alternative to the ARCA and β-S-ARCA cap analogs previously used for enhancing the translational capabilities of mRNA *in vivo*. As 2S analogs are simply and efficiently incorporated into mRNA during *in vitro* transcription, they represent a simple and practical method for enhancing mRNA stability and translational properties *in vivo*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors are grateful to Jacek Oledzki from the Laboratory of Mass Spectrometry (IBB PAS, Warsaw) for recording HRMS spectra.

FUNDING

Polish National Science Centre [UMO/2012/05/E/ST5/03 893, UMO/2013/08/A/NZ1/00866]; Foundation for Polish Science International PhD Projects Programme Scholarship co-financed by the EU European Regional Development (to M.S.).

Conflict of interest statement. A.N.K. and U.S. are employees of BioNTech RNA Pharmaceuticals GmbH, a company that develops mRNA-based therapeutics.

REFERENCES

1. Quabius,E. and Krupp,G. (2014) Synthetic mRNAs for manipulating cellular phenotypes: an overview. *N. Biotechnol.*, **32**, 229–235.
2. Deering,R. and Kommareddy,S. (2014) Nucleic acid vaccines: prospects for non-viral delivery of mRNA vaccines. *Expert Opin. Drug Deliv.*, **11**, 885–899.
3. Saunders,A. and Wang,J. (2014) Export and expression: mRNAs deliver new messages for controlling pluripotency. *Cell Stem Cell*, **14**, 549–550.
4. Sahin,U., Karikó,K. and Türeci,Ö. (2014) mRNA-based therapeutics—developing a new class of drugs. *Nat. Rev. Drug Discov.*, **13**, 759–780.
5. Astakhova,I.K. and Wengel,J. (2014) Scaffolding along nucleic acid duplexes using 2′-amino-locked nucleic acids. *Acc. Chem. Res.*, **47**, 1768–1777.
6. Niemeyer,C.M. (2001) Nanoparticles, proteins, and nucleic acids: biotechnology meets materials science. *Angew. Chem. Int. Ed.*, **40**, 4128–4158.
7. Cutler,J.I., Auyeung,E. and Mirkin,C.A. (2012) Spherical nucleic acids. *J. Am. Chem. Soc.*, **134**, 1376–1391.
8. Schade,M., Berti,D., Huster,D., Herrmann,A. and Arbuzova,A. (2014) Lipophilic nucleic acids–a flexible construction kit for organization and functionalization of surfaces. *Adv. Colloid Interface Sci.*, **208**, 235–251.
9. Karikó,K., Muramatsu,H., Welsh,F.A., Ludwig,J., Kato,H., Akira,S. and Weissman,D. (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol. Ther.*, **16**, 1833–1840.
10. Thess,A., Grund,S., Mui,B.L., Hope,M.J., Baumhof,P., Fotin-Mleczek,M. and Schlake,T. (2015) Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. *Mol. Ther.*, **23**, 1456–1464.
11. Kuhn,A.N., Diken,M., Kreiter,S., Selmi,A., Kowalska,J., Jemielity,J., Darzynkiewicz,E., Huber,C., Türeci,O. and Sahin,U. (2010) Phosphorothioate cap analogs increase stability and translational efficiency of RNA vaccines in immature dendritic cells and induce superior immune responses in vivo. *Gene Ther.*, **17**, 961–971.
12. Lee,K. and Cohen,S.N. (2001) Effects of 3′ terminus modifications on mRNA functional decay during in vitro protein synthesis. *J. Biol. Chem.*, **276**, 23268–23274.
13. Russell,J.E. and Liebhaber,S.A. (1996) The stability of human beta-globin mRNA is dependent on structural determinants positioned within its 3′ untranslated region. *Blood*, **87**, 5314–5323.
14. Ziemniak,M., Strenkowska,M., Kowalska,J. and Jemielity,J. (2013) Potential therapeutic applications of RNA cap analogs. *Futur. Med. Chem.*, **5**, 1141–1172.
15. Jemielity,J., Fowler,T., Zuberek,J., Stepinski,J., Lewdorowicz,M., Niedzwiecka,A., Stolarski,R., Darzynkiewicz,E. and Rhoads,R.E. (2003) Novel 'anti-reverse' cap analogs with superior translational properties. *RNA*, **9**, 1108–1122.
16. Stepinski,J., Waddell,C., Stolarski,R., Darzynkiewicz,E. and Rhoads,R.E. (2001) Synthesis and properties of mRNAs containing the novel 'anti-reverse' cap analogs 7-methyl(3′-O-methyl)GpppG and 7-methyl (3′-deoxy)GpppG. *RNA*, **7**, 1486–1495.
17. Grudzien-Nogalska,E., Stepinski,J., Jemielity,J., Zuberek,J., Stolarski,R., Rhoads,R.E. and Darzynkiewicz,E. (2007) Synthesis of anti-reverse cap analogs (ARCAs) and their applications in mRNA translation and stability. *Methods Enzymol.*, **431**, 203–227.
18. Grudzien-Nogalska,E., Jemielity,J., Kowalska,J., Darzynkiewicz,E. and Rhoads,R.E. (2007) Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells. *RNA*, **13**, 1745–1755.
19. Kowalska,J., Lewdorowicz,M., Zuberek,J., Grudzien-Nogalska,E., Bojarska,E., Stepinski,J., Rhoads,R.E., Darzynkiewicz,E., Davis,R.E. and Jemielity,J. (2008) Synthesis and characterization of mRNA cap analogs containing phosphorothioate substitutions that bind tightly to eIF4E and are resistant to the decapping pyrophosphatase DcpS. *RNA*, **14**, 1119–1131.
20. Van Dijk,E. (2002) Human Dcp2: a catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. *EMBO J.*, **21**, 6915–6924.
21. Rhoads,R.E. (2009) eIF4E: new family members, new binding partners, new roles. *J. Biol. Chem.*, **284**, 16711–16715.
22. Tomoo,K., Shen,X., Okabe,K., Nozoe,Y., Fukuhara,S., Morino,S., Ishida,T., Tanigushi,T., Hasegawa,H., Terashima,A. *et al.* (2002) Crystal structures of 7-methylguanosine 5′-triphosphate (m7GTP)- and P1-7-methylguanosine-P3-adenosine-5′, 5′-triphosphate (m7GpppA)-bound human full-length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region. *Biochem. J.*, **362**, 539–544.
23. Niedzwiecka,A., Marcotrigiano,J., Stepinski,J., Jankowska-Anyszka,M., Wyslouch-Cieszynska,A., Dadlez,M., Gingras,A.-C., Mak,P., Darzynkiewicz,E., Sonenberg,N. *et al.* (2002) Biophysical studies of eIF4E cap-binding protein: recognition of mRNA 5′ cap structure and synthetic fragments of eIF4G and 4E-BP1 proteins. *J. Mol. Biol.*, **319**, 615–635.
24. Strenkowska,M., Kowalska,J., Lukaszewicz,M., Zuberek,J., Su,W., Rhoads,R.E., Darzynkiewicz,E. and Jemielity,J. (2010) Towards mRNA with superior translational activity: synthesis and properties of ARCA tetraphosphates with single phosphorothioate modifications. *New J. Chem.*, **34**, 993–1007.
25. Floor,S.N., Jones,B.N., Hernandez,G.A. and Gross,J.D. (2010) A split active site couples cap recognition by Dcp2 to activation. *Nat. Struct. Mol. Biol.*, **17**, 1096–1101.
26. Kowalska,J., Lukaszewicz,M., Zuberek,J., Ziemniak,M., Darzynkiewicz,E. and Jemielity,J. (2009) Phosphorothioate analogs of m7GTP are enzymatically stable inhibitors of cap-dependent translation. *Bioorg. Med. Chem. Lett.*, **19**, 1921–1925.

27. DeAngelis,M.M., Wang,D.G. and Hawkins,T.L. (1995) Solid-phase reversible immobilization for the isolation of PCR products. *Nucleic Acids Res.*, **23**, 4742–4743.
28. Marlow,A.L. and Kiessling,L.L. (2001) Improved chemical synthesis of UDP-galactofuranose. *Org. Lett.*, **3**, 2517–2519.
29. Mohamady,S., Desoky,A. and Taylor,S.D. (2012) Sulfonyl imidazolium salts as reagents for the rapid and efficient synthesis of nucleoside polyphosphates and their conjugates. *Org. Lett.*, **14**, 402–405.
30. Sun,Q., Gong,S., Sun,J., Liu,S., Xiao,Q. and Pu,S. (2013) A P(V)-N activation strategy for the synthesis of nucleoside polyphosphates. *J. Org. Chem.*, **78**, 8417–8426.
31. Kadokura,M., Wada,T., Urashima,C. and Sekine,M. (1997) Efficient synthesis of γ-methyl-capped guanosine 5′-triphosphate as a 5′-terminal unique structure of U6 RNA via a new triphosphate bond formation involving activation of methyl phosphorimidazolidate using ZnCl2 as a catalyst in DMF under anhydrous condit. *Tetrahedron Lett.*, **38**, 8359–8362.
32. Yanachkov,I.B., Dix,E.J., Yanachkova,M.I. and Wright,G.E. (2011) P1, P2-diimidazolyl derivatives of pyrophosphate and bis-phosphonates–synthesis, properties, and use in preparation of dinucleoside tetraphosphates and analogs. *Org. Biomol. Chem.*, **9**, 730–738.
33. Jemielity,J., Kowalska,J., Rydzik,A.M. and Darzynkiewicz,E. (2010) Synthetic mRNA cap analogs with a modified triphosphate bridge—synthesis, applications and prospects. *New J. Chem.*, **34**, 829–844.
34. Strenkowska,M., Wanat,P., Ziemniak,M., Jemielity,J. and Kowalska,J. (2012) Preparation of synthetically challenging nucleotides using cyanoethyl P-imidazolides and microwaves. *Org. Lett.*, **14**, 4782–4785.
35. Mukaiyama,T. and Hashimoto,M. (1971) Phosphorylation by oxidation-reduction condensation. Preparation of active phosphorylating reagents. *Bull. Chem. Soc. Jpn.*, **44**, 2284–2284.
36. Milligan,J.F. and Uhlenbeck,O.C. (1989) Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol.*, **180**, 51–62.
37. Su,W., Slepenkov,S., Grudzien-Nogalska,E., Kowalska,J., Kulis,M., Zuberek,J., Lukaszewicz,M., Darzynkiewicz,E., Jemielity,J. and Rhoads,R.E. (2011) Translation, stability, and resistance to decapping of mRNAs containing caps substituted in the triphosphate chain with BH3, Se, and NH. *RNA*, **17**, 978–988.
38. Ludwig,J. and Eckstein,F. (1989) Rapid and efficient synthesis of nucleoside 5′-0-(1-thiotriphosphates), 5′-triphosphates and 2′, 3′-cyclophosphorothioates using 2-chloro-4H-1, 3, 2-benzodioxaphosphorin-4-one. *J. Org. Chem.*, **54**, 631–635.
39. Misiura,K., Szymanowicz,D. and Stec,W.J. (2005) Synthesis of nucleoside alpha-thiotriphosphates via an oxathiaphospholane approach. *Org. Lett.*, **7**, 2217–2220.
40. Li,P., Xu,Z., Liu,H., Wennefors,C.K., Dobrikov,M.I., Ludwig,J. and Shaw,B.R. (2005) Synthesis of alpha-P-modified nucleoside diphosphates with ethylenediamine. *J. Am. Chem. Soc.*, **127**, 16782–16783.
41. Hevroni,B.L., Sayer,A.H., Blum,E. and Fischer,B. (2014) Nucleoside-2′, 3′/3′, 5′-bis(thio)phosphate analogues are promising antioxidants acting mainly via Cu+/Fe2+ ion chelation. *Inorg. Chem.*, **53**, 1594–1605.
42. Ludwig,J. and Eckstein,F. (1991) Stereospecific synthesis of guanosine 5′-O-(1, 2-dithiotriphosphates). *J. Org. Chem.*, **56**, 5860–5865.
43. Kowalska,J., Wypijewska del Nogal,A., Darzynkiewicz,Z.M., Buck,J., Nicola,C., Kuhn,A.N., Lukaszewicz,M., Zuberek,J., Strenkowska,M., Ziemniak,M. *et al.* (2014) Synthesis, properties, and biological activity of boranophosphate analogs of the mRNA cap: versatile tools for manipulation of therapeutically relevant cap-dependent processes. *Nucleic Acids Res.*, **42**, 10245–10264.
44. Ziemniak,M., Kowalska,J., Lukaszewicz,M., Zuberek,J., Wnek,K., Darzynkiewicz,E. and Jemielity,J. (2015) Phosphate-modified analogues of m7GTP and m7Gppppm7G—Synthesis and biochemical properties. *Bioorg. Med. Chem.*, **23**, 5369–5381.
45. Rydzik,A.M., Kulis,M., Lukaszewicz,M., Kowalska,J., Zuberek,J., Darzynkiewicz,Z.M., Darzynkiewicz,E. and Jemielity,J. (2012) Synthesis and properties of mRNA cap analogs containing imidodiphosphate moiety–fairly mimicking natural cap structure, yet resistant to enzymatic hydrolysis. *Bioorg. Med. Chem.*, **20**, 1699–1710.
46. Rydzik,A.M., Lukaszewicz,M., Zuberek,J., Kowalska,J., Darzynkiewicz,Z.M., Darzynkiewicz,E., Jemielity,J., Furuichi,Y., Shatkin,A.J., Mathonnet,G. *et al.* (2009) Synthetic dinucleotide mRNA cap analogs with tetraphosphate 5′, 5′ bridge containing methylenebis(phosphonate) modification. *Org. Biomol. Chem.*, **7**, 4763–4776.
47. Kalek,M., Jemielity,J., Grudzien,E., Zuberek,J., Bojarska,E., Cohen,L.S., Stepinski,J., Stolarski,R., Davis,R.E., Rhoads,R.E. *et al.* (2005) Synthesis and biochemical properties of novel mRNA 5′ cap analogs resistant to enzymatic hydrolysis. *Nucleosides Nucleotides Nucleic Acids*, **24**, 615–621.
48. She,M., Decker,C.J., Chen,N., Tumati,S., Parker,R. and Song,H. (2006) Crystal structure and functional analysis of Dcp2p from Schizosaccharomyces pombe. *Nat. Struct. Mol. Biol.*, **13**, 63–70.
49. Ziemniak,M., Mugridge,J.S., Kowalska,J., Rhoads,R.E., Gross,J.D. and Jemielity,J. (2016) Two-headed tetraphosphate cap analogs are inhibitors of the Dcp1/2 RNA decapping complex. *RNA*, **22**, 518–529.