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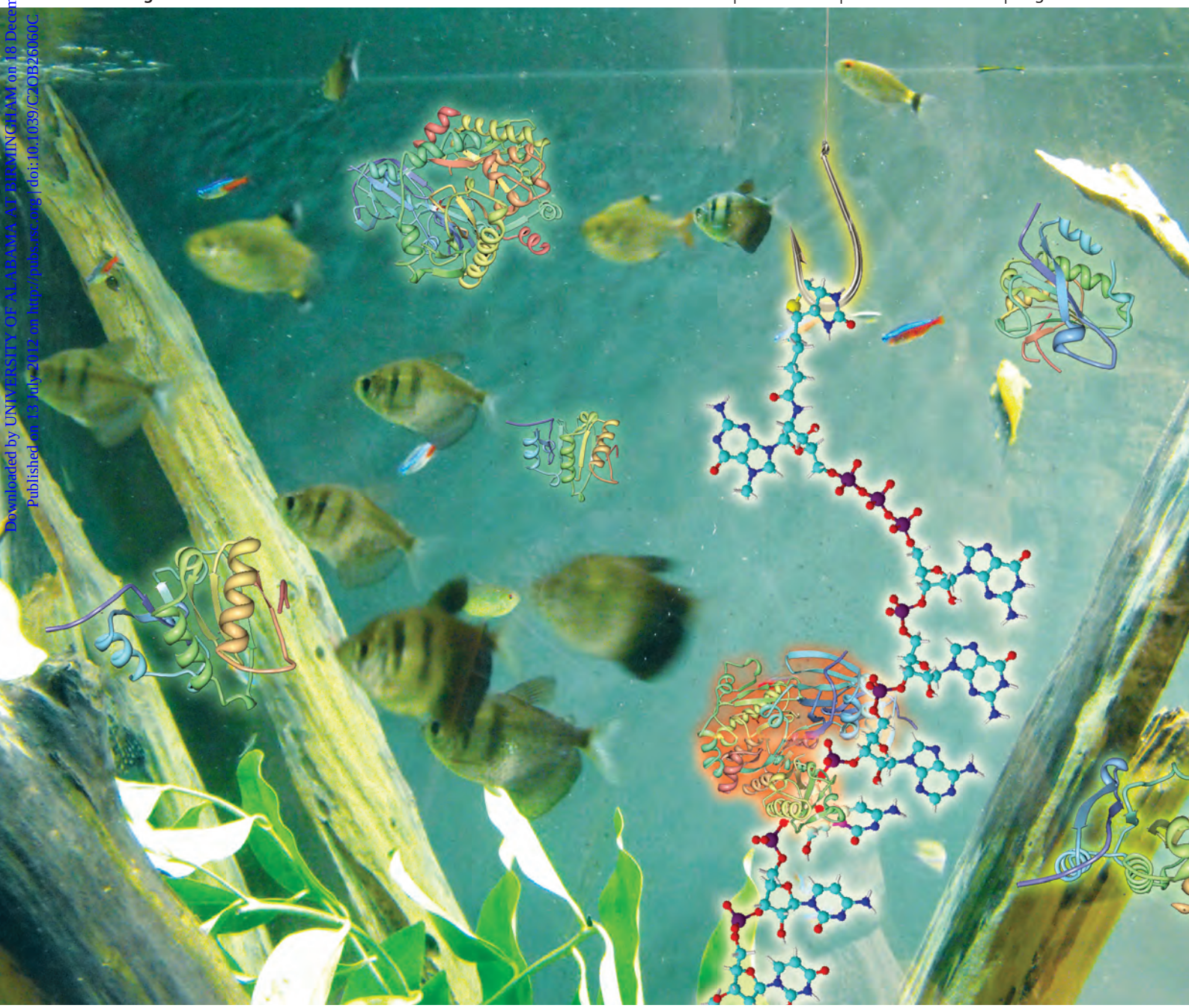

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Jacek Jemielity *et al.*

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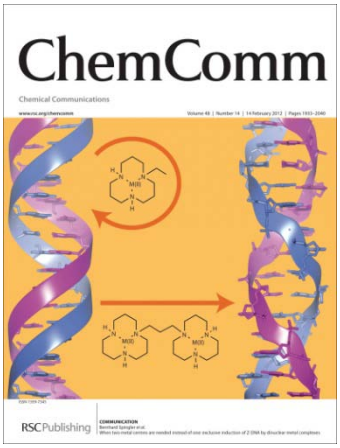

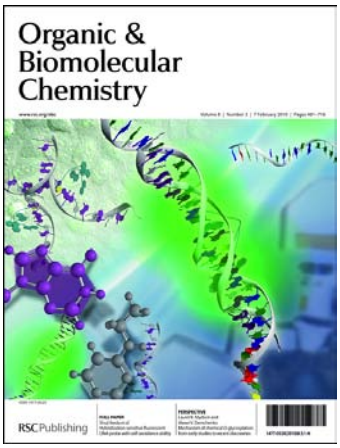

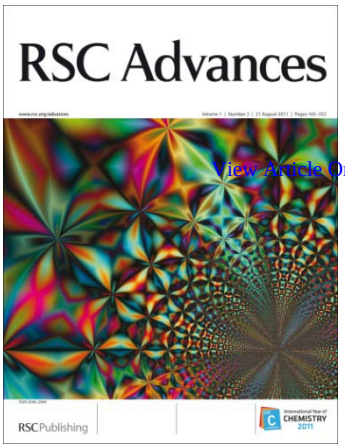

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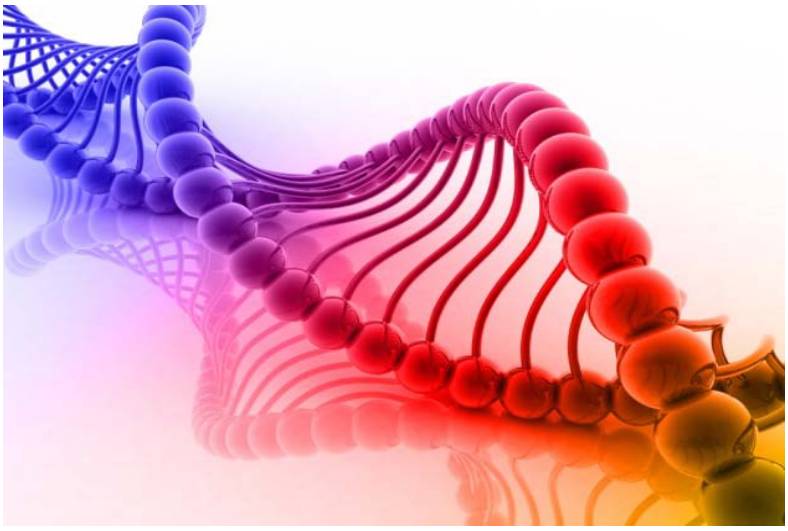

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COMMUNICATION

Synthesis of biotin labelled cap analogue – incorporable into mRNA transcripts and promoting cap-dependent translation†

Jacek Jemielity,* Maciej Lukaszewicz, Joanna Kowalska, Jakub Czarnecki, Joanna Zuberek and Edward Darzynkiewicz

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Analogues of the eukaryotic messenger RNA 5′ end (m^7G cap) are useful tools for studying mRNA fate and serve as reagents for *in vitro* preparation of 5′ capped mRNAs. We designed a biotin-labeled dinucleotide cap analogue that can be incorporated into transcripts to produce 5′-capped and biotinylated mRNAs which retain their biological functionality and may be employed for biotin–(strept)avidin technologies.

All eukaryotic messenger RNAs possess a cap at their 5′ end, consisting of 7-methylguanosine (m^7G) linked by a 5′,5′-triphosphate bridge to the first transcribed nucleotide.[1] This structure, distinct from typical nucleic acids, serves as an intrinsic mRNA 5′ end tag, which enables selective binding to proteins involved in mRNA translation and turnover. These include cytoplasmic translation initiation factor 4E (eIF4E), the nuclear cap binding complex (CBC) or decapping enzymes (Dcp2, hNUDT16, DcpS).[2–9]

Not surprisingly then, synthetic cap analogues have been used for studying numerous mRNA-related processes, *e.g.* translation, translational repression or mRNA decay pathways.[10] In order to further facilitate studies on gene expression by means of cap-derived molecular tools, in this work we propose a simple method for obtaining mRNA molecules site-specifically biotinylated within the 5′ cap. We describe an efficient chemical synthesis of a biotin-labelled dinucleotide cap analogue, which can be incorporated into transcripts by enzymatic transcription *in vitro*. We also demonstrate that biotinylated transcripts obtained in this manner undergo cap-dependent translation and are bound by streptavidin. Hence, these transcripts retain their biological activity, coincidently being applicable for biotin–(strept)avidin interaction based experiments.

The biotin tag is commonly used for purification, detection and visualization of proteins and nucleic acids *via* anti-biotin antibodies or avidin/streptavidin tagged detectors, such as horseradish peroxidase or fluorescent dyes, as well as for labelling molecules for imaging or delivery of therapeutics.[11–13] Different approaches have been developed for nucleic acid labelling with biotin. RNA has been labelled both at the intrastrand positions and at the 5′ or 3′ ends.[14–20] Such labelled RNAs are useful for many applications; however, since the cap is involved in crucial processes of mRNA metabolism, particularly initiation of translation, their use for studying eukaryotic mRNA function may be limited. In the case of our studies we aimed at developing a biotin-labelled analogue of the mRNA cap that would fulfil the following three conditions: (1) specifically interacts with avidin/streptavidin. (2) enables synthesis of 5′-biotinylated capped full-length mRNA by transcription *in vitro*, *i.e.* serves as a substrate for bacteriophage RNA polymerases during initiation of transcription. (3) bears the biotin label at a position that introduced the smallest possible disturbance of cap–eIF4E interaction, thus enabling efficient translation of the biotinylated mRNA.

Such biotin-labelled mRNAs could be useful *e.g.* for determining cellular localization and following the fate of capped mRNAs, for pull-down assays, ELISA tests, western blots and other immuno-analytical methods.

To enable attachment of biotin through its carboxylic group, we designed a dinucleotide cap analogue **1** bearing an aliphatic amino group at the 2′-position of 7-methylguanosine. The analogue was converted into its biotinylated derivative **2** by coupling with NHS-activated biotin (Fig. 1). The biotin NHS-ester was conveniently generated *in situ* using *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU).‡ The coupling reaction of **1** with NHS-biotin was highly selective and efficient. The HPLC analysis revealed a complete conversion of **1** into **2** (Fig. 2) within 60 min. No by-products derived from the coupling of nucleobase exocyclic amino groups or ribose hydroxyl groups have been detected. After RP-HPLC purification the triammonium salt of compound **2** was isolated with a 60% yield.

The synthetic route for cap analogue **1** is depicted in Fig. 3. The synthesis has been designed to minimize the number of steps employing 2′-amino-2′-deoxyguanosine. It has been found that the 2′-amino group does not require protection, neither at the 5′-phosphorylation nor during the N^7-methylation step, which made the synthesis relatively straightforward. The procedure

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†Electronic supplementary information (ESI) available: Fig. S1–S6, Tables S1 and S2, detailed experimental procedures, HRMS, ^1H NMR and ^{31}P NMR spectra of compounds **1** and **2**. See DOI: 10.1039/c2ob26060c

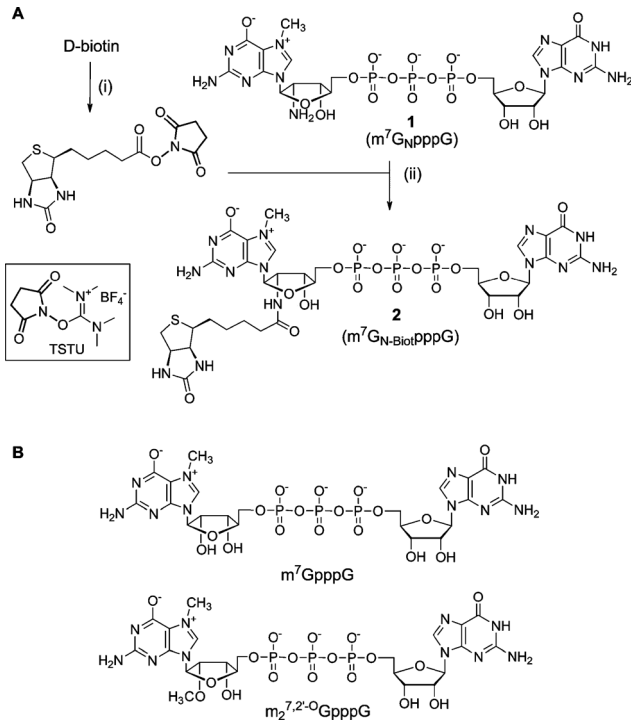

Fig. 1 (A) Conversion of 2′-NH_2-substituted cap analogue (**1**) into 2′-biotinylated cap analogue (**2**) *via* coupling with an *in situ* generated NHS-biotin active ester. Reaction conditions: (i) *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU), TEA, DMSO, 30 min; (ii) 0.5 M aqueous borane buffer pH 8.5, 1 h. (B) Structure of cap analogues used in this work as reference compounds: standard cap analogue, m^7GpppG, and anti-reverse cap analogue, $m_2^{7,2'\text{-O}}$GpppG.

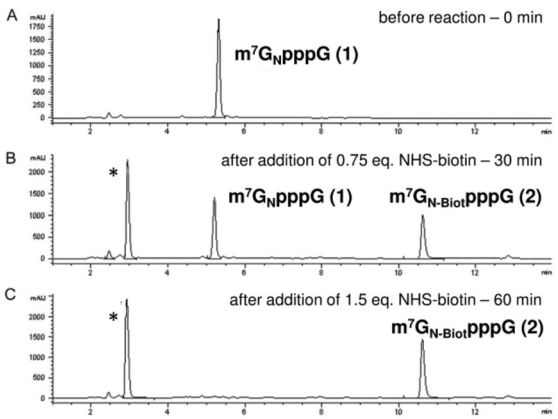

Fig. 2 RP HPLC profiles from the reaction of 2′-NH_2 substituted cap analogue **1** with NHS-activated biotin at different time points: (A) 0 min (before addition of NHS-biotin); (B) after 30 min (5 additions of 0.15 equiv. of NHS-biotin); (C) after 1 h (10 additions of 0.15 equiv. of NHS-biotin). The peak designated with an asterisk (*) with $R_t \sim 3$ min is not a nucleotide derivative, but is associated with the presence of DMSO and TSTU in the reaction mixture.

encompassed obtaining appropriate mononucleotide subunits **4** and **6**, their subsequent coupling to the dinucleotide **1**. To obtain **4**, the starting material, 2′-amino-2′-deoxyguanosine, was first phosphorylated with $POCl_3$ in Yoshikawa conditions to yield the corresponding 5′-monophosphate (**3**).[21] As monitored by RP

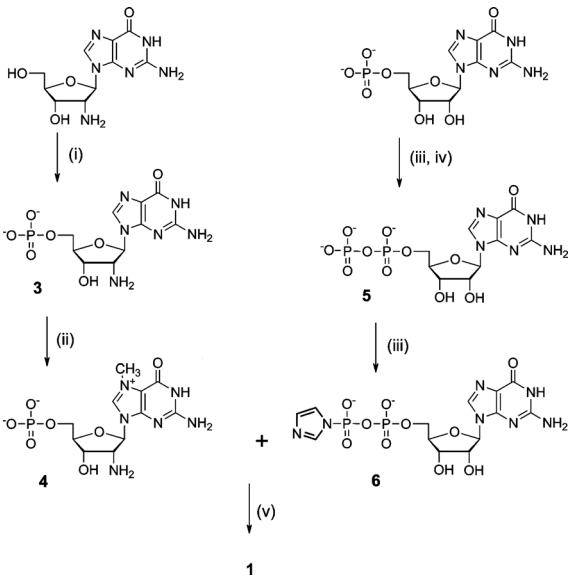

Fig. 3 Synthesis of cap analogue **1**. Reaction conditions: (i) 1. $POCl_3$ (3 equiv.), $(MeO)_3PO$, 0 °C, 8 h; 2. H_2O, $NaHCO_3$; (ii) CH_3I (8 equiv.), DMSO, 4 h; (iii) 1. Imidazole (10 equiv.), 2,2′-dithiodipyridine (3 equiv.), triphenylphosphine (3 equiv.), TEA, DMF, 8 h; 2. $NaClO_4$, acetone; (iv) 4 equiv. PO_4^{3-}/TEA salt, 8 equiv. $ZnCl_2$, DMF, 3 h; (v) 8 equiv. $ZnCl_2$, DMF, 6 h.

HPLC, the reaction proceeded slowly. The conversion of the starting material into the desired 5′-phosphorylated product reached 95% after 8 h, whereas in the case of guanosine an almost quantitative conversion under the same conditions requires *ca.* 2 h. Compound **3** was isolated after ion-exchange purification with an 80% yield. Subsequently, **3** was treated with methyl iodide in DMSO to produce 2′-amino-2′-deoxy-7-methylguanosine 5′-monophosphate (**4**). The methylation proceeded smoothly and occurred at the expected N^7 position of guanine, and not at the 2′-amino group of ribose, yielding **4** with a 62% isolated yield. Compound **4** was then efficiently coupled with GDP-imidazolide (**6**) in DMF in the presence of excess $ZnCl_2$ to produce cap analogue **1** (58% isolated yield).

The 2′-position of 7-methylguanosine in the dinucleotide cap has been selected as a site for NH_2 substitution and subsequent biotin attachment, on the basis of the three factors listed above. It has been well documented in the literature that 2′-*O*-methyl substitution within the 7-methylguanosine of the dinucleotide cap, similar to that at the 3′-*O*-methyl one, produces so-called anti-reverse cap analogues (ARCA) that are incorporated into the mRNA chain during transcription *in vitro* exclusively in the correct orientation.[22–24] In the X-ray structures of cap–eIF4E complexes both 2′- and 3′-hydroxyls of 7-methylguanosine are exposed to the solution and do not significantly contribute to complex stabilization.[25–27] Consequently, introduction of methyl groups at either of these positions, as well as other, more bulky substituents, was shown to have only a minor or moderate influence on cap interaction with eIF4E and the translational properties of capped-mRNA.[22,23,28]

Hence, we presumed that even the presence of a bulky biotin residue at either the 2′- or 3′-position of cap 7-methylguanosine

Table 1 Binding affinity of cap analogues **1** and **2** for eukaryotic translation initiation factor 4E

Cap analogue	K_{AS} [a] [μM^{-1}]
m^7GpppG	9.4 ± 0.4[b]
$m_2^{7,2'\text{-O}}GpppG$	10.2 ± 0.4[b]
m^7G_NpppG (**1**)	5.6 ± 0.6
$m^7G_{N\text{-Biot}}pppG$ (**2**)	3.8 ± 0.1

[a] Determined in 50 mM Hepes–KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA, 2 mM DTT at 20 ± 0.2 °C. [b] Data from ref. 22.

Table 2 Translation efficiency and translational inhibition of mRNAs encoding firefly luciferase and capped with cap analogues **1** and **2** in rabbit reticulocyte lysate

Cap analogue at the mRNA 5′ end	Relative translation efficiency[a]	IC_{50} for m^7GTP[a] [μM]
m^7GpppG	1	12.9 ± 3.9
$m_2^{7,2'\text{-O}}GpppG$	1.4 ± 0.2	9.1 ± 1.1
m^7G_NpppG (**1**)	0.9 ± 0.1	—
$m^7G_{N\text{-Biot}}pppG$ (**2**)	1.0 ± 0.1	2.45 ± 0.60
ApppG	0.16 ± 0.03	n.d.[b]

[a] Determined in the rabbit reticulocyte lysate as described in ESI.† [b] The value was not determined because m^7GTP did not inhibit the translation of this mRNA at the tested concentration range (1–100 μM).

would not completely disturb its interaction with eIF4E. When comparing the 2′- and 3′-positions as potential sites of modification, the former appeared to be more economical from the synthetic point of view, since chemically synthesized 2′-modified nucleosides are easier to prepare, and commercial ones less expensive, than their 3′ isomers.

To evaluate whether selection of the 2′-position as a modification site was correct, analogues **1** and **2** were tested as ligands for the eIF4E protein by means of fluorescent quenching titration experiments.[27] The K_{AS} values for cap–eIF4E were lower than the value for corresponding unmodified analogues (Table 1). Nonetheless, all the values were in the same order of magnitude, which suggested that the analogues are bound by eIF4E in a specific manner and, thus, may at least partially retain their biological functionality. In the case of analogue **2**, the lower affinity is likely a result of the presence of bulky biotin residue, whereas in the case of analogue **1**, it could rather result from an electrostatic repulsion between the protonated 2′-amino group and basic amino acid side chains in the eIF4E–cap binding pocket.

The cap analogues **1** and **2** were then tested in the RNA polymerase catalyzed transcription reaction under conditions routinely used for obtaining capped RNAs.[24] First, we tested whether the new cap analogues could be incorporated into short, 5-nt transcripts by SP6 RNA polymerase. We found that **1** and **2** are both incorporated into transcripts with efficiencies comparable to m^7GpppG and $m_2^{7,2'\text{-O}}GpppG$ and that the presence of the biotin label could be indirectly observed by a slower migration compared to other RNAs (ESI, Fig. S1†). Next, **1** and **2** were used as reagents for the preparation of full-length mRNAs encoding firefly luciferase. A typical reaction mixture contained dsDNA template, ATP, CTP, UTP at 0.5 mM concentration, GTP at 0.1 mM concentration, SP6 RNA polymerase and either compound **1** or **2** at 0.5 mM. Transcripts were then purified and analyzed by a non-denaturating 1% agarose gel electrophoresis (ESI, Fig. S2†). The yields and homogeneities of all transcripts were again comparable to those obtained by means of reference cap analogues, m^7GpppG and $m_2^{7,2'\text{-O}}GpppG$.

The synthesized capped, full-length Luc-mRNAs were then used to program the rabbit reticulocyte lysate system optimized for cap-dependent translation to test their translational efficiencies. We have found that the Luc-mRNA capped with biotinylated cap **2** ($m^7G_{N\text{-Biot}}pppG$-Luc-mRNA) is translated with an efficiency lower than $m_2^{7,2'\text{-O}}GpppG$-Luc-mRNA (Table 2). This suggests that the biotin substituent, presumably due to its bulkiness compared to the 2′-*O*-methyl substituent, has reduced the ability of cap **2** to compete for translation machinery. Nonetheless, $m^7G_{N\text{-Biot}}pppG$-Luc-mRNAs was translated with an efficiency comparable to m^7GpppG-Luc-mRNA, which is more than 6-fold higher than the translation of mRNA bearing a non-functional cap (ApppG) under these conditions. This strongly suggests that $m^7G_{N\text{-Biot}}pppG$-Luc-mRNA is recruited for the translational machinery by a cap dependent mechanism, *i.e.* as in the majority of natural mRNAs. The translation efficiency of mRNA capped with analogue **1**, which bears a non-bulky 2′-amino substituent, was also diminished compared to $m_2^{7,2'\text{-O}}GpppG$-Luc-mRNA (Table 2), which is in agreement with the determined K_{AS} value for the cap **1**–eIF4E complex (Table 1).

To gain more insight into how the biotin label influences the functionality of the cap in the translation initiation process, we tested if $m^7G_{N\text{-Biot}}pppG$-RNA is susceptible to inhibition by the mononucleotide cap analogue, m^7GTP. This compound is known to be a potent inhibitor of cap-dependent translation, acting by competitive blocking of mRNA cap binding to eIF4E.[27,29]

Translation of all tested mRNAs capped with a functional (m^7G) cap was strongly inhibited by m^7GTP (Table 2, ESI, Fig. S3†). Interestingly, $m^7G_{N\text{-Biot}}pppG$-RNA had an IC_{50} value 3–5-fold lower than m^7GpppG-RNA and $m_2^{7,2'\text{-O}}GpppG$-RNA. We believe that this confirms our hypothesis that cap **2** promotes cap-dependent translation, but is less competitive for the translation machinery than *e.g.* $m_2^{7,2'\text{-O}}GpppG$, and hence mRNAs bearing this cap are more prone to inhibition by a free cap analogue (m^7GTP in this case).

Another set of experiments was conducted to confirm the functionality of the biotin label introduced into RNA by capping with analogue **2**. An electrophoretic mobility shift assay in a non-denaturating 1.4% agarose gel was used to follow the interaction of 140 nt $m^7G_{N\text{-Biot}}pppG$-RNA transcripts with recombinant tetrameric streptavidin (Promega) (Fig. 4). In the presence of streptavidin a fraction of the RNA pool migrated distinctly slower than in its absence and the intensity of the slower-migrating band was streptavidin concentration dependent. Somehow unexpectedly, when a higher-resolution, automated RNA separation system (Experion automated station, semi-denaturating conditions) was used to analyze the transcripts, we were able to distinguish two different more slowly migrating bands, whose relative ratio depended on the concentration of streptavidin added to the RNA portion (ESI, Fig. S4†). We assume these

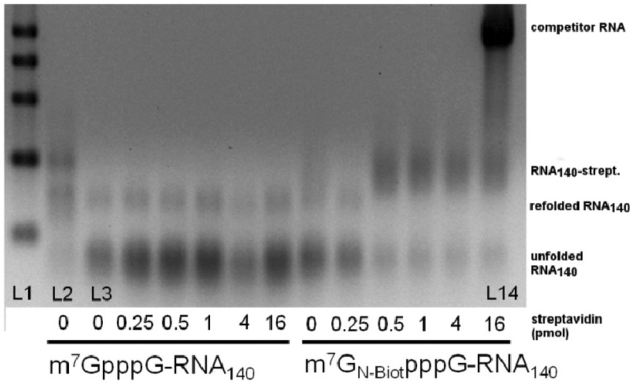

Fig. 4 Electrophoretic mobility of transcripts capped *in vitro* with m^7GpppG or $m^7Gp_{N\text{-}Biot}$ppG (2) in the presence of streptavidin (RNA-EMSA). Capped 140 nt RNA transcripts (2 pmol) were incubated with increasing concentrations of tetrameric streptavidin (Promega). Unbound RNA_{140} and formed RNA_{140}–streptavidin complexes were analyzed in 1.4% agarose gel in non-denaturating conditions as described in Experimental procedures (for details see ESI† available online): L1 – RNA size marker (from the bottom: 200, 500, 1000, 1500, 2000 nt). L2 – non-denaturated m^7GpppG-RNA_{140} transcript, where higher order bands of RNA_{140} are visible. L3 – thermally-denaturated m^7GpppG-RNA_{140}. L4–L8 – binding reactions of m^7GpppG-RNA_{140} with increasing concentrations of streptavidin. L9 – thermally-denaturated $m^7Gp_{N\text{-}Biot}$ppG-RNA_{140}. L10–L13 – binding reactions of $m^7Gp_{N\text{-}Biot}$ppG-RNA_{140} with increasing concentrations of streptavidin. L14 – binding reaction of $m^7Gp_{N\text{-}Biot}$ppG-RNA_{140} with streptavidin in the presence of competitive RNA (1750 nt luciferase mRNA). In all cases, except L2, thermally-denaturated transcripts were used, which migrate below the 200 nt size marker. Additionally, a less intense, slower-migrating band corresponding to the refolded form of RNA_{140} was observed (compare to L2). The complexes of $m^7Gp_{N\text{-}Biot}$ppG-RNA_{140} with streptavidin (L11–L14) migrate slightly slower than the refolded RNA. In L12–L14, the portion of unbound RNA, observed even in the presence of high excess streptavidin, corresponds to the non-capped (GTP initiated) RNA, whose presence is typical for the synthesis of capped RNAs *via* co-transcriptional capping (usually its content ranges from 10 to 20%).

bands correspond to RNA complexes with different oligomeric forms of streptavidin, since the dissociation of streptavidin tetramers in denaturating conditions is a known issue of the biotin–streptavidin technology.[30–32]

We also tested whether the biotin label is recognized by streptavidin in a more complex biological system, in which streptavidin would be competing with other proteins, including those capable of m^7G cap binding. Different concentrations of streptavidin were added to the rabbit reticulocyte lysate programmed with either $m_2^{7,2'\text{-}O}$GpppG-Luc-mRNA or $m^7G_{N\text{-}Biot}$pppG-Luc-mRNA. The translation of $m_2^{7,2'\text{-}O}$GpppG-mRNA remained unaffected by streptavidin at all of the tested concentrations, whereas translation of $m^7G_{N\text{-}Biot}$pppG-RNA was not inhibited by streptavidin concentrations up to 8 nM, but was diminished 2–3-fold in the presence of 20 nM tetrameric streptavidin, which corresponds to a *ca.* 1 : 2 RNA : streptavidin ratio (see ESI, Tables S1 and S2 and Fig. S5 and S6†). We attribute this to the formation of an $m^7G_{N\text{-}Biot}$pppG-RNA–streptavidin complex which is formed in the RRL and impedes the translation initiation process.

Conclusions

We developed a chemoenzymatic method for obtaining RNAs that are specifically labelled with biotin at the 5′ m^7G cap moiety. The method encompasses co-transcriptional incorporation into the RNA transcript of a biotinylated, dinucleotide cap analogue **2**, obtained *via* chemical synthesis. The procedure is applicable for labelling short RNA transcripts as well as full-length mRNAs. We confirmed that mRNAs capped with the biotinylated cap analogue **2** both undergo cap-dependent translation and retain functionality of the biotin.

To introduce the biotin label at the 2′-amino group of the cap's 7-methylguanosine, we developed a simple and efficient chemical procedure based on *in situ* generated NHS-biotin. The high selectivity and efficiency of this reaction makes it a beneficial option for introducing other tags bearing NHS-activatable carboxyl groups, *e.g.* fluorescent ones.

The biotin labelled RNAs obtained by our method may be applied to a variety of biological experiments based on biotin–(strept)avidin technology or by means of biotin specific antibodies, including protein affinity purification, pull-down assays, *in vivo* visualization and many others. Particularly beneficial could be their application for following eukaryotic mRNA cellular fate.

Finally, it is worth mentioning that cap analogue **1**, bearing a reactive 2′-amino group, once incorporated into transcripts creates the possibility of post-transcriptional RNA labelling, which is currently under investigation by our group.

Acknowledgements

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Notes and references

‡ A brief description of the activation procedure (for more experimental details see the ESI†): To a solution of D-biotin (1.5 equiv., ~0.25 M) in DMSO were added TEA (2 equiv.) and TSTU (1.5 equiv.) and the mixture was shaken at RT for 30 min. The resultant solution was added portion-wise (10 additions over a period of 1 h) to a solution of m^7G_NpppG (compound **1**) (1 equiv., ~0.1 M) in 0.5 M aqueous borane buffer, pH 8.5. After each addition the pH was re-adjusted to 8.5 with aqueous NaOH if necessary. The reaction progress was monitored by RP HPLC. The reaction was quenched by dilution with water (~4×) and neutralization with a few drops of 50% acetic acid. The product was purified by semi-preparative RP HPLC. Yield 60%.

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