Synthesis of 5′-amino-5′-deoxyguanosine-5′-*N*-phosphoramidate and its enzymatic incorporation at the 5′-termini of RNA molecules

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Received (in Cambridge, UK) 6th August 2007, Accepted 19th September 2007
First published as an Advance Article on the web 11th October 2007
DOI: 10.1039/b712066d

5′-Amino-5′-deoxyguanosine-5′-*N*-phosphoramidate (GNHP) was synthesized in four steps from guanosine and was found to initiate T7 RNAP-promoted transcriptions to afford 5′-H_2N-RNA that can be conjugated to activated esters.

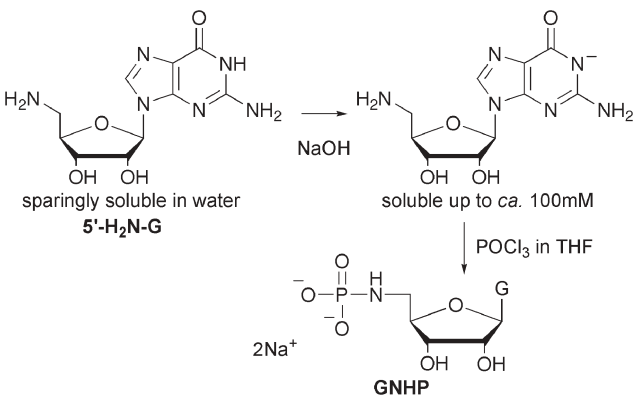
Bioconjugates of the 5′-termini of RNA molecules are often essential for *in vitro* selection, fluorophore labelling and photo-crosslinking experiments.[1,2] Several methods that allow selective chemical modification of enzymatically produced RNAs have been devised,[3] but by far the most widely used method relies on the incorporation of guanosine monophosphorothioate (GMPS) as the initiating nucleotide in T7 RNAP-promoted transcription reactions.[1] Once incorporated into an RNA transcript the 5′-thiophosphoryl group of GMPS may be chemoselectively modified using α-haloacetyl derivatives or other thiol-reactive agents.[4] Whilst the use of GMPS has proven effective, the synthesis of GMPS is often a major deterrent to its use. Although improved, simplified syntheses have been reported[5] these are still technically challenging.

We have devised a simple method for the incorporation of a 5′-amino-group into RNA transcripts. Once incorporated into an RNA molecule the amino group can be chemoselectively modified using standard coupling systems (*e.g. N*-hydroxy succinimide esters) to give amide-linked bioconjugates.

A previous report of the use of 5′-amino-5′-deoxyguanosine (5′-H_2N-G) in *in vitro* transcription gave low yields of RNA where only 20% of the RNA transcripts appeared to contain the 5′-H_2N-group.[6] The low incorporation level likely results from the poor solubility of 5′-H_2N-G at the optimal pH for T7 RNAP-promoted transcriptions (pH 8.0) so Suga *et al.*[6] attempted to overcome this problem by increasing the pH of their transcription reactions to pH 9.0. However, even with this increase in pH the solubility of 5′-H_2N-G was limited and the performance of the transcription reaction in terms of incorporation of the 5′-amino and in terms of the overall yield of RNA was severely affected. Recently, Schlatterer and Jäschke[7] have reported the use of a 5′-H_2N-oligoethylene glycol-guanosine monophosphate initiator nucleotide. The initiator was incorporated at levels of up to 65%. Whilst this approach does include a flexible linker which may prove useful in some applications the chemical synthesis involves time-consuming HPLC steps.

In order to overcome the poor solubility of 5′-H_2N-G we chose to phosphorylate its 5′-amino group to give the 5′-phosphoramidate (GNHP). Alkyl phosphoramidates are hydrolytically labile at low pH, but stable at high pH.[8] Around neutral pH the rate of hydrolysis shows a pH-independent plateau where the half-life for hydrolysis is on the order of a few hours.[8,9] Thus we hoped that the phosphoramidate would be sufficiently stable to withstand transcription, but labile enough to allow facile removal. Previous unsuccessful attempts towards the synthesis of GNHP have focused on the use of protecting groups and organic solvents,[10] however given the lability of the phosphoramidate group and the poor solubility characteristics of guanosine derivatives in many solvents we chose to investigate the use of an aqueous solvent system and to avoid the use of protecting groups. Our synthetic method relies on the fact that 5′-H_2N-G is soluble in alkaline aqueous solution (Scheme 1).† Despite its anionic nature the deprotonated guanine group appears to be a poor nucleophile, which probably results from a mixture of delocalisation and solvation effects. The 5′-amino group, on the other hand, is not heavily solvated and thus is able to react selectively with $POCl_3$.[9] The other competing nucleophiles within the aqueous reaction mixture are water and hydroxide. By using five equivalents of hydroxide ion we were able to minimise the potential conflict between nucleophilic attack of hydroxide ion upon $POCl_3$ and the need to maintain alkaline pH in order to avoid hydrolysis of the phosphoramidate group once it has been formed.

After removal of the THF and water the resulting crude material was analysed by ^{31}P NMR and ES-MS. ^{31}P NMR showed 90% conversion of the added $POCl_3$ to the desired GNHP. The other components within the crude mixture appeared to be made up of inorganic salts and small amounts (< 2%) of 2′- and 3′-phosphate esters of 5′-H_2N-G.



Scheme 1 5′-H_2N-G can be dissolved readily in sodium hydroxide solution.

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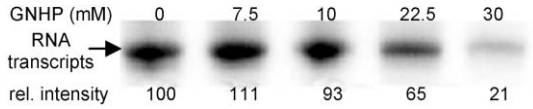

Fig. 1 Phosphorimage of a purification gel of transcription reactions containing increasing concentrations of GNHP.

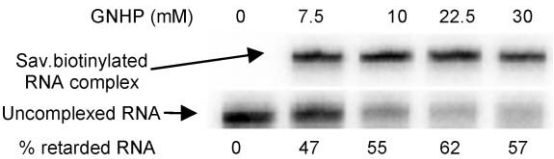

Fig. 2 Phosphorimage of biotinylated transcription products from transcription reactions containing increasing concentrations of 5′-GNHP (blank central area of the gel has been removed).

Given that the aim of this work was to produce a straightforward method towards an initiator molecule, preferably avoiding time-consuming chromatography steps, we chose to perform transcription reactions using this unpurified material.

Transcriptions were carried out under standard conditions using over-expressed T7 RNA polymerase.[11] A series of experiments were performed where the concentration of GNHP was systematically increased. The overall level of transcription was assessed by phosphorimaging of the purification gel.

As the concentration of GNHP was increased the overall yield of RNA was seen to increase slightly before going down again at higher concentrations (Fig. 1).

The RNA transcripts were then isolated from the gel and were passively eluted overnight. During this time period we believed that the phosphoramidate groups that were incorporated into transcripts would hydrolyse to reveal the desired 5′-amino RNA. In order to assess the level of incorporation of the 5′-amino group into the RNA transcripts we used a biotinylation assay followed by a streptavidin dependent gel shift experiment (Fig. 2).

A systematic increase in the proportion of 5′-amino group within the RNA transcripts was seen as the concentration of GNHP was increased. Up to 60% incorporation of the amino label is seen at higher concentrations of GNHP whilst overall RNA yield remains reasonable (22.5 mM). The results described in Figs. 1 and 2 are summarised in Fig. 3.

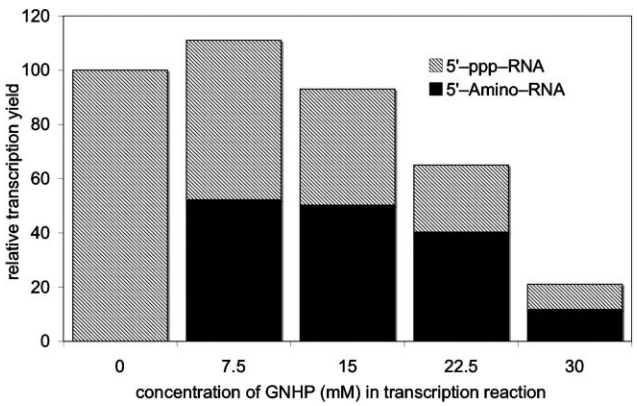

Fig. 3 Combined data from Figs. 1 and 2.

In conclusion, we have devised a simple and effective method for the enzymatic incorporation of a 5′-amino group into RNA molecules. The synthetic approach avoids chromatography steps and thus offers significant advantages over established methods. In addition we have also developed a convenient aqueous method for the solubilisation and phosphorylation of 5′-H_2N-G. We shall explore the use of other aqueous chemistry on guanosine nucleosides using this aqueous alkali solubilisation technique.

This work was supported by a studentship from EPSRC (D. W.) and Durham University.

Notes and references

† 5′-H_2N-G was prepared using Dean's approach.[12] 5′-H_2N-G (500 mg, 1.77 mmol) was dissolved in a mixture of NaOH (8.85 mL of 1 M, 8.85 mmol) and water (1.15 mL) then cooled to 0 °C. $POCl_3$ (165 μL, 1.77 mmol) in anhydrous THF (5 mL) was then added dropwise to the solution over 10 min. After addition was complete the solvents were removed *in vacuo* to give crude product as a white solid (746 mg). ^{31}P [^{1}H coupled] NMR, δ_P(121 MHz, H_2O): 7.61 (t, O*P*(O)$_2$–NH–CH_2), 3.82 (s, inorganic OPO_3), 2.60, 2.20 (d, 2′/3′-phosphoester by-products). *m/z* (LC-ES$^-$) 361 ([GNHP monoanion]). ^{31}P NMR revealed 90% conversion of $POCl_3$ to GNHP. The crude product was used in transcription studies without further purification, however, a small amount of material was purified *via* ion exchange and gel filtration chromatography (method will be published elsewhere) and subjected to detailed spectroscopic analysis. The material that was obtained was always found to contain a small amount of the hydrolysis product 5′-H_2N-G. Spectroscopic data for GNHP: ^{1}H NMR, δ_H(500 MHz, D_2O; Me_4Si): 2.86 (1 H, *ABX* system, J_{AB} 13.5, J_{AXP} 6.5, 5′-CH_AH$_B$), 2.93 (1 H, *ABX* system, J_{AB} 13.5, J_{BXP} 5.5, 5′-CH$_A$$H_B$), 4.04–4.02 (1H, m, 4′-$H_X$), 4.20 (1H, t, *J* 4, 3′-H), 4.61 (1H, t, *J* 5.5, 2′-H), 5.66 (2H, d, *J* 6.5, 1′-H), 7.74 (1H, s, 8-H); δ_C(100.6 MHz, D_2O; Me_4Si): 44.4 (5′-*C*), 71.5 (3′-*C*), 73.1 (2′-*C*), 85.4 (d, J_{CP} 10.1, 4′-*C*), 86.9 (1′-*C*), 117.9 (5-*C*), 136.2 (8-*C*), 151.8 (4-*C*), 161.1 (2-*C*), 167.8 (6-*C*); FT-MS *m/z* (ES$^-$): found 361.06661 at a FWHH resolving power of 10^6; $C_{10}H_{14}N_6O_7P^-$ requires 361.06671. Transcription reactions contained 40 mM Tris (pH 7.9), 10 mM DTT, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 350–500 nM dsDNA template (ATRib^{TL} template was used),[13] 1.25 mM of each NTP and 83 nM [α-^{32}P]-UTP. Crude GNHP was added to each reaction mixture as a 100 mM stock where this concentration calculation was based on the assumption that complete conversion of 5′-H_2N-G to GNHP had occurred. 420 nM T7 RNA Polymerase was then added to each mixture[11] and the mixtures were incubated at 37 °C for 2.5 h. DNA template was digested by adding RQ1 RNase-free DNase (1U, Promega) followed by incubation at 37 °C for a further 0.5 h. Unincorporated GNHP was removed *via* gel filtration using a spin-column (ZEBA Desalt 0.5 mL, Pierce). Eluted material was then purified *via* urea PAGE. The relative amounts of RNA produced in each transcription reaction were determined *via* phosphorimaging. The RNA bands were then located by UV shadowing, the bands were excised and the RNA was then extracted from the gel by overnight passive elution into 0.3 M NaCl. Gel pieces were removed using a filter spin-column and the RNA was precipitated by addition of 3 volumes of ethanol. Biotinylation reactions contained 67 mM HEPES (pH 8.1), 3.3 mM sulfo-NHS-Biotin and approx. 1 μM RNA. Reaction mixtures were incubated at room temperature for 2 h. Reactions were stopped *via* the addition of 3 volumes of ethanol. The RNA was diluted 1 : 4 with streptavidin loading buffer (a 1 : 1 mixture of streptavidin solution [2 mg mL^{-1}] : 8 M urea loading buffer) or control buffer without streptavidin and analysed *via* urea PAGE and phosphorimaging.

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