Preparation and purification of 5′-amino-5′-deoxyguanosine-5′-*N*-phosphoramidate and its initiation properties with T7 RNA polymerase†

David Williamson and David R. W. Hodgson*

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5′-Amino-5′-deoxyguanosine-5′-*N*-phosphoramidate (GNHP) was synthesised in four steps from guanosine. Its identity was confirmed using spectroscopic and kinetic studies. A chromatographic method for the purification of this unstable compound was developed. GNHP was found to initiate T7 RNAP promoted transcriptions to afford 5′-O_3P-NH-RNA, which hydrolyses readily to yield 5′-H_2N-RNA that can be conjugated to activated esters.

Indroduction

RNA conjugates have been used widely in biological research to determine the nature of protein–nucleic acid interactions,[1,2] understand RNA folding problems[3] and for the *in vitro* selection of ribozymes.[4] Whilst automated synthesis can produce many of these conjugates, the technique is limited to the production of shorter oligonucleotides; thus for some applications, such as *in vitro* selection of ribozymes and the study of longer RNA sequences, one must rely on enzymatic preparation methods.

RNA molecules that are modified at their 5′-termini have been produced by enzymatic incorporation of modified initiator nucleotides, usually based on guanosine.[1,5–8] Unfortunately many of the synthetic routes towards these initiator molecules are laborious and act as a deterrent to the use of these systems. We aimed to develop a simple synthetic route towards an initiator molecule that would broaden the appeal of this approach to a wider research community.

The use of amine-based initiators has been investigated by Suga[9] and Jäschke.[5] Once incorporated, the amine functional group can be further modified in a post-transcriptional step using standard bioconjugation reagents such as reactive esters.[10] Suga's approach, namely the use of 5′-amino-5′-deoxyguanosine in transcriptions, whilst appealing owing to the ease of synthesis of the nucleoside, unfortunately afforded low yields of RNA where the RNA population contains only a small proportion (∼20%) of the amine functional group. The likely cause of the poor performance is the poor solubility of 5′-amino-5′-deoxyguanosine in aqueous solution. Jäschke and coworkers have prepared several more elaborate nucleotides that show good initiation characteristics, but unfortunately the syntheses of these materials are quite lengthy and thus limited to specialists. We hoped to develop methodology that provides a simple synthetic route towards a modified initiator molecule that is then effectively incorporated at the 5′-termini of RNA molecules in the transcription process.

With this goal in mind, we set about designing a simple strategy for the preparation and use of a new modified initiator molecule. Given that the use of 5′-amino-5′-deoxyguanosine by Suga *et al.* was limited by poor solubility, we decided to improve the solubility of this system by the introduction of a phosphoryl group on the 5′-amino functional group. The resulting phosphoramidate-based nucleotide, 5′-amino-5′-deoxyguanosine-5′-*N*-phosphoramidate (GNHP), should be soluble at pH 8.0 (optimum pH for T7 RNA polymerase activity[11]), but the phosphoramidate functional group is also hydrolytically labile at this pH with a predicted half-life on the order of several hours.[12] Thus we hoped that the phosphoramidate would be sufficiently stable to allow incorporation during transcription whilst being sufficiently labile to allow facile removal of the phosphoryl group in order to reveal the 5′-amino group.

We have recently communicated details of the preparation of GNHP from 5′-amino-5′-deoxyguanosine and usage of crude GNHP in transcription studies.[13] In this paper we shall discuss the development of the aqueous phosphorylation method that was used to prepare GNHP, the development of a purification strategy for GNHP, and the use of purified GNHP in transcription in comparison to the crude material.

Results and discussion

1. Optimisation of the aminophosphorylation procedure

Schattka and Jastorff attempted the synthesis of GNHP using 2′,3′-isopropylidene-protected 5′-amino-5′-deoxyguanosine as their substrate and ester-protected phosphorylating agents.[14] Unfortunately they were unable to remove the isopropylidene protecting group without also removing the phosphoramidate group. Thus when designing our synthetic route towards GNHP we limited ourselves to strategies that avoided protecting groups and where we could use 5′-amino-5′-deoxyguanosine as our starting material, Scheme 1. A recent report from Dean allowed us to produce 5′-amino-5′-deoxyguanosine *via* a convenient three-step procedure.[15]

We attempted aminophosphorylation using phosphorus oxychloride in acetonitrile, as employed by Hampton and co-workers in their synthesis of the inosine analogue of GNHP,[16] and we found that a solution of 5′-amino-5′-deoxyguanosine could not be obtained. The use of phosphorus oxychloride with trialkyl phosphate as the solvent[17,18] led to a bis-phosphorylation product

Department of Chemistry, University Science Laboratories, South Road, Durham, DH1 3LE, England. E-mail: d.r.w.hodgson@durham.ac.uk

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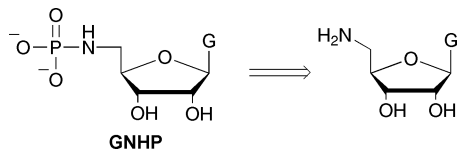

Scheme 1 Synthetic strategy towards GNHP.

which we tentatively identified as 5′-amino-5′-deoxyguanosine-5′-*N*-phosphoramidate-2′,3′-cyclic phosphate based on ^{31}P NMR and ES-MS analyses.

At this point we investigated a convenient water-based procedure from the Drueckhammer laboratory that had been used for the preparation of analogues of dihydroxyacetone phosphate.[19] The procedure used an aqueous alkaline solution of the substrate, where alkali was added at the same time as phosphorus oxychloride in order to both hydrolyse P–Cl bonds in the amino phosphorodichloridate intermediate and at the same time maintain a high pH, at which the phosphoramidate product is more stable.[12] Unfortunately, hydroxide ion also competes against the amine substrate as a reactant for phosphorus oxychloride, and thus a balance between maintaining high pH and minimising hydroxide ion concentration is needed. We were already aware that 5′-amino-5′-deoxyguanosine showed poor solubility in water, and thus we began our investigations using dimethyl sulfoxide as a cosolvent. An initial experiment where the 5′-amino-5′-deoxyguanosine substrate was dissolved in DMSO and phosphorus oxychloride in THF, and aqueous sodium hydroxide solution was added simultaneously by pipette, gave a crude reaction mixture that contained material giving rise to a triplet signal at *ca.* 8.5 ppm in the ^{1}H-coupled ^{31}P NMR spectrum, which corresponds to the expected signal for the desired phosphoramidate.[20] With this preliminary result in hand, we proceeded to systematically study the effect of the DMSO–water ratio and the effect of the amount of sodium hydroxide on reaction outcome.

In order to maintain consistency between experiments with different ratios of DMSO to water, an excess of sodium hydroxide was added to each experiment at the outset rather than during the addition of the phosphorus oxychloride solution, so that the co-addition rate was no longer a variable. The resulting crude reaction mixtures were then analysed by ^{31}P NMR spectroscopy. The DMSO content was varied between 0 and 50%, and for the experiment that did not contain DMSO we found that 5′-amino-5′-deoxyguanosine was actually soluble in aqueous alkali (rather than pure water). This improved solubility probably results from deprotonation of the guanine to provide the water-soluble guanine anion (pK_a of *N*-1 of guanosine is *ca.* 9.4[21]). The results from this series of experiments are summarised in Table 1. In addition to the desired phosphoramidate, the ^{31}P NMR spectra showed signals corresponding to by-products that we assigned to inorganic phosphate and 5′-amino-5′-deoxyguanosine-2′- and 3′-monophosphates. We found that the experiment that did not contain DMSO cosolvent led to the highest conversion to the desired product. There are several factors that may have given rise to this outcome, the most likely being a mixture of increased nucleophilicity of hydroxide ion towards $POCl_3$ owing to reduced solvation at higher DMSO concentrations, and the increased hydrolytic activity of phosphoramidates (even at higher pHs) in mixed aqueous–organic systems.[22] Together these factors would both lead to increased production of inorganic phosphate ion and consequent reduction in the conversion of $POCl_3$ to GNHP.

Table 1 Formation of GNHP as a function of solvent composition

DMSO (%)	GNHP (%)[a]
0	69
10	61
20	48
30	62
40	55
50	48

[a] Determined by ^{31}P NMR spectroscopy on the crude reaction mixture.

We now turned our attention to the number of equivalents of sodium hydroxide in the initial reaction mixture using a wholly aqueous system. A series of experiments was run where the initial number of equivalents of sodium hydroxide with respect to substrate was varied. Phosphorus oxychloride was then added to each aqueous substrate mixture as a solution in THF. The results from this series of experiments are summarised in Fig. 1.

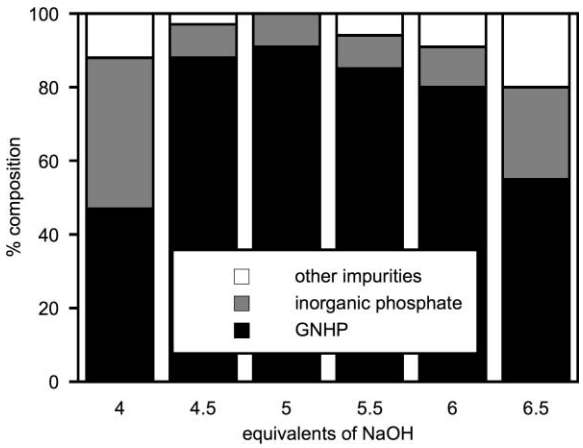

Fig. 1 Optimisation of NaOH. The number of equivalents of NaOH was varied and the effect on product distribution was assessed by ^{31}P NMR spectroscopy.

An optimum of five equivalents was found to give the largest conversion of phosphorus oxychloride to GNHP with the lowest level of by-products. This number of equivalents likely represents a balance between the need to ionise the guanine ring in order to bring the substrate into solution, ionisation of the 2′,3′-*cis* diol system, reaction of hydroxide ion with $POCl_3$ and hydrolysis of the phosphoramidate product once it has been formed. With this crude material in hand, we began kinetic studies on its hydrolysis in order to confirm that our material shows the expected pH-dependent hydrolysis properties, and to determine conditions under which the material may be purified and isolated.

2. Kinetic evidence for the phosphoramidate

Crude GNHP was subjected to a hydrolysis study over a range of pHs in order to demonstrate that the product material displayed similar kinetic properties to known alkyl phosphoramidates.[12,16]

We used ^{31}P NMR spectroscopy to monitor the disappearance of the phosphoramidate signal in a series of buffers of varying pHs. A comparison of our data (obtained at 37 °C) with Benkovic's[12]

(obtained at 55 °C) shows that both profiles are very similar in form, with the observed rate constants for Benkovic's study being higher in value owing to the elevated temperature used in their experiments, Fig. 2. We chose 37 °C as the temperature for our study because this is the temperature used for T7 RNA polymerase-catalysed transcription reactions. The half-life for hydrolysis of GNHP on the pH-independent plateau was 1.8 h, and at pH 8.0, which is the optimal pH for T7-catalysed transcription, the half-life was 2.5 h. Thus the phosphoramidate appeared to be sufficiently stable to allow incorporation into RNA during transcription (we use 2.5 h incubations for transcription reactions), but sufficiently labile to allow facile removal of the phosphoryl group after (and during) the transcription process.

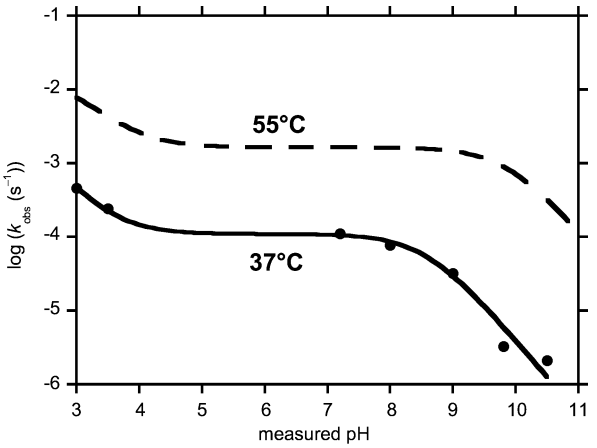

Fig. 2 Kinetic data from ^{31}P NMR studies on the hydrolysis of GNHP at different pHs. Experimental data points (●) were fitted to eqn (1) (see experimental) with the fitting being represented by the solid line. A plot of the pH hydrolysis behaviour of butyl-*N*-phosphoramidate (obtained at 55 °C) is shown by the dotted line (data from Benkovic and Sampson).[12]

3. Chromatographic purification of GNHP

Ideally we wished to avoid the use of time-consuming chromatographic steps, and we have seen, from the viewpoint of T7-promoted transcription, that unpurified material gives satisfactory yields of RNA, with a reasonable proportion of the RNA containing the 5′-amino group.[13] In order to assess the effect of GNHP purity on transcription performance, however, we needed to develop a method for the purification of this hydrolytically labile material. Hampton and co-workers were able to effect a series of selective precipitation steps in order to gain the inosine analogue of GNHP in a form that was homogeneous by paper electrophoresis and thin layer chromatography. The nature of our synthetic approach towards GNHP unfortunately leads to the formation of some by-products and probably some unconverted starting material (although ^{31}P NMR does not give any information on this). Owing to the lower hydrolysis rate of GNHP and the improved solubility of 5′-amino-5′-deoxyguanosine at high pH, we explored the use of strong anion exchange chromatography using a gradient of increasing concentration of sodium hydroxide solution as the eluent. This system provides the necessary increase in salt concentration that allows elution of the desired product whilst maintaining a high pH. A chromatographic profile of the crude mixture shows good separation of four major components, Fig. 3.

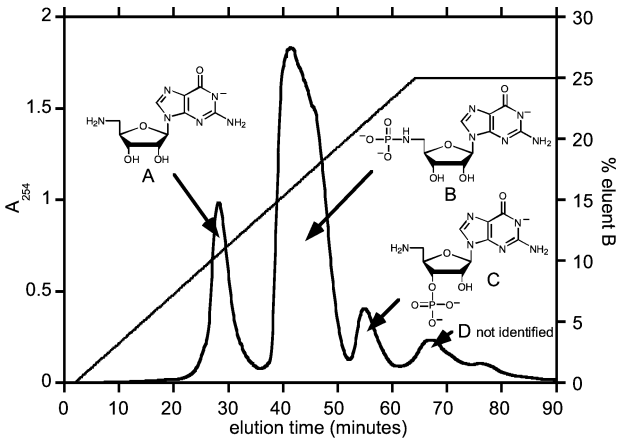

Fig. 3 Capto Q anion exchange chromatography of crude GNHP using a gradient of 0.1 M (eluent A) to 1 M NaOH (eluent B).

The identities of the components corresponding to the peaks in the UV trace were deduced on the basis of an authentic standard in the case of peak **A** and the basis of ^{31}P NMR spectroscopy in the cases of peaks **B** and **C**. We were unable to identify the nature of the component that gave rise to peak **D**. Integration of the UV trace (assuming that the components giving rise to each peak have the same extinction coefficient) gives a product distribution of 18% **A**, 64% **B**, 8% **C** and 8% **D**. In comparison with earlier ^{31}P NMR studies on the crude material, this chromatographic method shows that the crude GNHP was less pure than we had previously estimated. Whilst this clearly did not prevent efficient transcription using the crude material, it makes it all the more important to study the effect of purity on transcription properties. After elution from the ion exchange system, the material was lyophilised and then subjected to gel filtration chromatography on Sephadex G10 media in order to reduce the salt content. Water was used as the eluent for this process, and thus a small amount of hydrolysis of the GNHP occurred as the material passed through the chromatography column (transit time approximately 20 minutes). The eluted material was immediately frozen and then lyophilised. We investigated other strategies for the desalting of the eluted GNHP, such as ethanol precipitation and reduction of the pH to the isoelectric point (*ca.* pH 5, see Section 2); however, these proved less effective than gel filtration. Gel filtration using a mildly basic eluent (*e.g.* 2 mM K_2CO_3[23] or 1 mM NaOH), whilst effective, did leave the eluted material contaminated with salt, which may have proven detrimental to the transcription process. A small amount of the freeze-dried material was then repeatedly lyophilised from D_2O before being subjected to ^{1}H NMR spectroscopy.

4. Spectroscopic evidence for the phosphoramidate

Given the number of potential phosphorylation sites that were available on the unprotected aminonucleoside, we were eager to confirm that phosphorylation had indeed taken place on the amino group of 5′-amino-5′-deoxyguanosine rather than one of the guanine functional groups or the ribose hydroxyls. We have already discussed the use of ^{31}P NMR spectroscopy, the significance of the triplet signal and its chemical shift, which we believe correspond to the phosphoramidate group. Kinetic data on the hydrolysis of the product are also in agreement with similar

compounds in the literature. ^{1}H NMR spectroscopy gave further confirmation of our assignment in the form of a complicated multiplet corresponding to the 5′-CH_2 group, Fig. 4. In addition to the ABX system that results from coupling to the 4′-proton and geminal coupling between the protons of the methylene group, the signals corresponding to the 5′-CH_2 group of the phosphoramidate also show coupling to the phosphorus nucleus within the phosphoramidate. ^{13}C NMR spectroscopy reveals ^{3}J coupling between the phosphorus centre and the 4′-C atom, but ^{2}J coupling to the 5′-C was not observed.

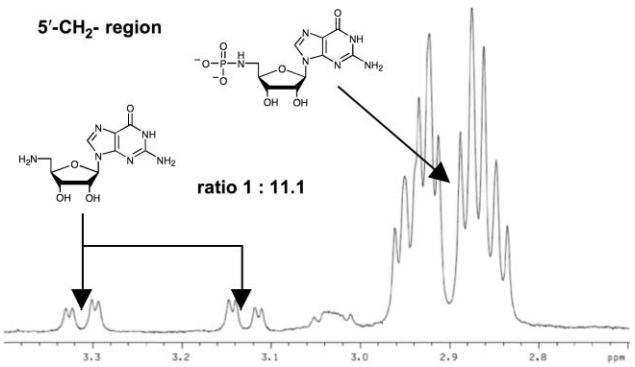

Fig. 4 The 5′-CH_2 region of the ^{1}H NMR spectrum of purified GNHP. A complex multiplet occurs at 2.9 ppm that corresponds to geminal and vicinal coupling to adjacent protons and vicinal coupling to phosphorus. The sample is contaminated with a small amount of 5′-amino-5′-deoxyguanosine owing to hydrolysis of GNHP.

5. Transcription studies

We have already reported our results on the use of crude GNHP in transcriptions,[13] and they are summarised in Fig. 8A.

We have seen from our chromatography studies that the crude GNHP contained 18% 5′-amino-5′-deoxyguanosine and 64% GNHP *i.e.* amine and phosphoramidate in a ratio of 1 : 3.6. The crude material that was used for transcriptions studies was originally estimated to contain approximately 90% GNHP on the basis of ^{31}P NMR spectroscopy, which, of course gave no direct information on the level of 5′-amino-5′-deoxyguanosine. In addition, given the labile nature of the phosphoramidate, the balance between amine and phosphoramidate is continuously changing. After chromatographic purification we were able to obtain a sample of GNHP with a much reduced content of 5′-amino-5′-deoxyguanosine, where amine and phosphoramidate were present in a 1 : 11.1 ratio by ^{1}H NMR spectroscopy, Fig. 4. The small amount of 5′-amino-5′-deoxyguanosine likely resulted from hydrolysis of GNHP during desalting.

With the purified material in hand, we began to assess the effect of purification and, more significantly, the effect of amine *vs.* phosphoramidate on transcription performance.

Purified GNHP was used under the same transcription conditions as we employed for the crude material. A series of experiments, where each transcription experiment contained a different concentration of the purified GNHP, was performed. At the end of the 2.5 h incubation period the RNA from each experiment was purified by urea-PAGE. The transcription procedure incorporated a ^{32}P radiolabel thus the level of radioactivity from each RNA band on the purification gel gives a direct measure of the amount of RNA produced in each experiment, Fig. 5.

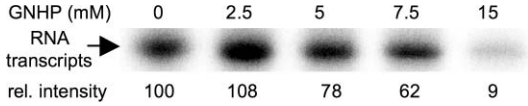

Fig. 5 Phosphorimage of a purification gel of transcription reactions containing increasing concentrations of purified GNHP.

As the concentration of purified GNHP was increased the amount of RNA produced increased initially, but then quickly tailed off as higher concentrations were attained. The gel pieces containing the RNA transcripts were then excised from the purification gel and the RNA was eluted passively from the gel pieces. During this time the remaining phosphoramidate groups were expected to hydrolyse completely, thus revealing 5′-amino groups on a proportion of the RNA transcripts. A biotinylation assay was then used in order to assess the level of incorporation of 5′-amino groups in the RNA transcripts from each transcription experiment. The transcripts from each experiment were derivatised using 3-sulfo-*N*-hydroxysuccinimidyl biotin, and the level of biotinylation (which corresponds to the level of 5′-amine label in the RNA transcripts) was assessed using a streptavidin (SAv)-dependent gel shift assay. The biotinylated RNA molecules bind to the streptavidin and their mobilities are reduced relative to those RNA molecules that are not biotinylated (*i.e.* those RNAs that were initiated with GTP rather than our modified initiator molecule). The biotinylation assays revealed that the level of incorporation of 5′-amino group increased steadily as the concentration of GNHP was increased in the corresponding transcription experiments, Fig. 6.

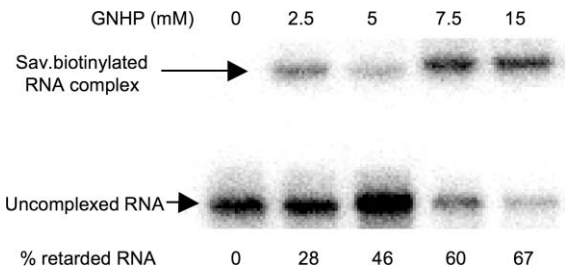

Fig. 6 Phosphorimage of a gel of biotinylated transcription products from transcription reactions containing increasing concentrations of purified GNHP.

In order to confirm that the observed gel shifts arose as a result of the presence of 5′-amino groups in the RNAs, which in turn arose from the use of GNHP in transcriptions, a series of control experiments were also performed. Transcriptions and subsequent biotinylations were performed where GNHP, biotinylating agent and/or streptavidin were omitted and the resulting matrix of experiments was analysed by urea-PAGE, Fig. 7. The experiments show clearly that the shifted RNA band is seen only when all components are present, and thus we conclude that the band arose as a result of the presence of 5′-amino groups in the RNAs.

The results of the series of transcription and biotinylation experiments illustrated in Figs. 5 and 6 have been combined to produce Fig. 8B. This figure shows that total RNA yield tails off very quickly as the concentration of purified GNHP is increased in

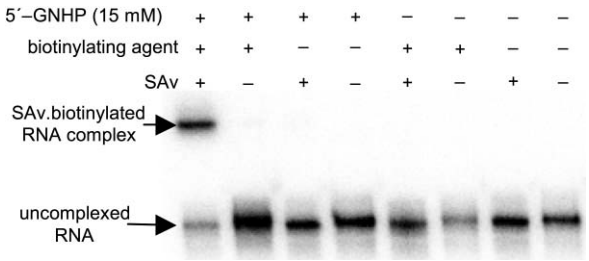

Fig. 7 Phosphorimage of gel-shift assays on transcription products from reactions containing (lanes 1–4) or lacking (lanes 5–8) purified GNHP. A '+' symbol signifies the presence of a reagent, whereas a '–' symbol signifies its absence.

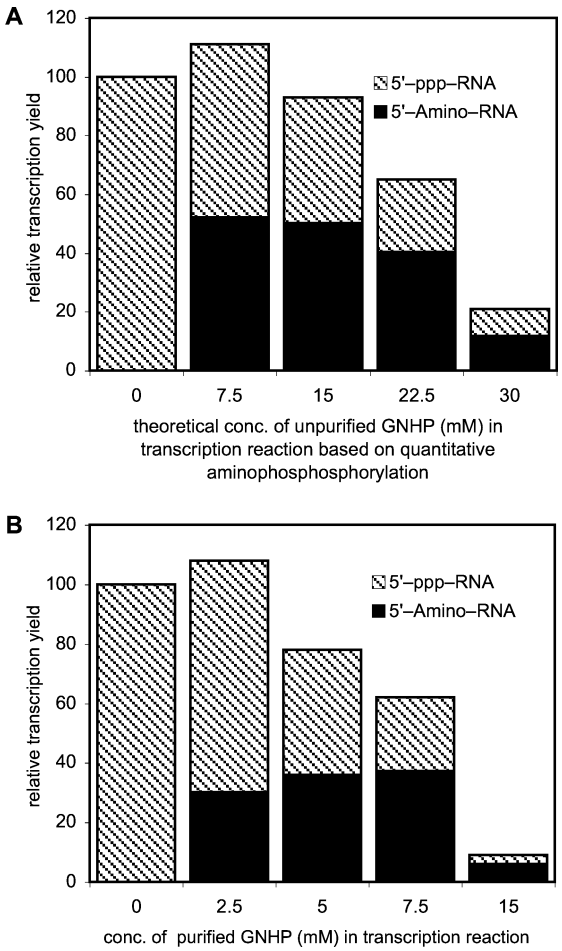

Fig. 8 Transcription results. The overall yield of RNA relative to control transcriptions that do not contain GNHP are represented by the heights of the light grey bars. The level of incorporation of the 5′-amino group into transcripts is represented by the height of the darker bars within the shaded bars. Data in charts **A** and **B** correspond to results from experiments on unpurified GNHP and purified GNHP respectively. The concentrations of unpurified GNHP were based on the assumption that complete conversion of 5′-amino-5′-deoxyguanosine to GNHP occurred during synthesis.

the transcription experiments. The analogous results for the crude GNHP, Fig. 8A, show a more gradual tailing off of RNA yield at higher concentrations of GNHP, even when correcting for the fact that the crude GNHP contained only *ca.* 64% GNHP. In terms of incorporation of 5′-amino groups, the results between the two sets of experiments are similar, with both series reaching a maximum of *ca.* 60% of the RNA-containing 5′-amino groups at the higher GNHP concentrations. The result from the crude GNHP system at 7.5 mM is particularly interesting as the overall RNA yield is high *and* the level of incorporation of the 5′-amino group is also reasonable. We have to remember that the concentration of 7.5 mM was based on the assumption that the material used in the synthetic procedure was quantitatively converted to the phosphoramidate, which we have shown not to be the case. The results of the chromatography study (Section 3) revealed that the crude material contained the 5′-amino-5′-deoxyguanosine and the GNHP in approximate proportions of 18% and 64%, respectively. Thus the actual concentrations of 5′-amino-5′-deoxyguanosine and GNHP in this experiment were closer to 1.35 mM and 4.8 mM, respectively. Although the amine is sparingly soluble it is an effective initiator, and, when combined with GNHP, both systems contribute towards initiation of transcription with both systems leading to the production of 5′-amino-RNA. The transcription results from our studies with purified GNHP, where the concentration of any residual 5′-amino-5′-deoxyguanosine is very much lower (<10%), show inhibition of RNA production at much lower concentrations of initiator.

We believe that the anionic phosphoramidate, whilst an effective inhibitor, may also inhibit the elongation phase of T7 RNA polymerase. Martin and Coleman demonstrated that the anionic phosphate groups of GTP and GMP are not required during transcription initiation.[24] Indeed, guanosine shows similar initiation properties to its phosphorylated homologues. The triphosphate portions of NTPs are, however, bound in the elongation phase of polymerisation, and so the phosphoryl group of GNHP may also bind in competition with GTP in the elongation phase. As GNHP does not possess the same pyrophosphate leaving group as the NTPs, its binding to the elongation active site is unproductive and will lead to inhibition and reduced yield. Famulok and co-workers have observed similar inhibition effects when using higher concentrations of phosphorylated guanosine analogues as initiators.[7] Although limited by its poor solubility, the amine is not charged, and therefore we believe that it does not inhibit the elongation phase in the same way as the phosphoramidate; thus RNA production with crude GNHP, which contains a greater proportion of the amine, is significantly greater. This is a challenging hypothesis to prove as uncharged nucleosides are likely to be intrinsically limited in their solubility properties, and thus we are unable to explore the effect of high concentrations on transcription. We are, however, investigating the transcription properties of an uncharged guanosine analogue that shows reasonable solubility properties,[25] and we hope to investigate the properties of cationic initiator nucleosides in the future.

Conclusions

We have devised and optimised a convenient method for the preparation of a previously unknown nucleoside phosphoramidate. Our procedure uses a simple aqueous approach to solubilise 5′-amino-5′-deoxyguanosine followed by a chemoselective *N*-phosphorylation. The use of alkali may represent a general strategy for the solubilisation of recalcitrant guanosine derivatives and we are currently exploring its use with 5′-deoxy-5′-iodoguanosine

and other guanosine systems. The nucleoside phosphoramidate was then purified by a combination of ion exchange and gel filtration chromatographies. The identity of the phosphoramidate was confirmed by kinetic and spectroscopic techniques before being used in T7 RNA polymerase-catalysed transcriptions. We found that crude GNHP gave a superior performance in transcription studies, and we believe that the uncharged nucleoside 5′-amino-5′-deoxyguanosine acts as a supplementary initiator. We are currently exploring the use of another uncharged guanosine derivative that displays better solubility characteristics than 5′-amino-5′-deoxyguanosine.

Experimental

5′-Amino-5′-deoxyguanosine

Prepared in three steps from guanosine using Dean's procedure.[15] 5′-Amino-5′-deoxyguanosine was recrystallised from water in order to produce analytically pure material as off-white crystals. mp = 218–220 °C (dec.); lit., 219–220 °C[15] or 221 °C[14]; (Found C, 39.8; H, 5.4; N, 27.7. $C_{10}H_{14}N_6O_4{\cdot}H_2O$ requires C, 40.0; H, 5.3; N, 28.0%); ν_{max}(KBr disc)/cm^{-1} 2622br (OH), 1702 (C=O); ^{1}H NMR, δ_H(500 MHz, DMSO-d_6; Me_4Si): 2.72 (1 H, ABX system, J_{AB} 13.5, J_{BX} 5.5, 5′-CH_AH$_B$), 2.77 (1H, ABX system, J_{AB} 13.5, J_{AX} 4.5, 5′-CH$_A$$H_B$), 3.77–3.81 (1H, m, 4′-H$_X$), 4.08 (1H, t, J 4.5, 3′-H), 4.44 (1H, t, J 6.8, 2′-H), 5.66 (2H, d, J 6.5, 1′-H), 6.52 (1H, br s, NH$_2$), 7.93 (1H, s, 8-H): δ_C(100.6 MHz, DMSO-d_6; Me_4Si): 43.5 (5′-C), 70.6 (3′-C), 73.11 (2′-C), 85.5 (4′-C), 86.2 (1′-C), 116.7 (5-C), 135.71 (8-C), 151.3 (4-C), 153.6 (2-C), 156.7 (6-C)): m/z (ES$^+$) 283.1 ([M + H]$^+$).

Preliminary experiment using mixed aqueous–DMSO solvent system

5′-Amino-5′-deoxyguanosine (20 mg, 68.8 μmol) was dissolved in DMSO (500 μL, 5.82 mmol), and the solution was then diluted with water (500 μL) and cooled to 0 °C. Phosphorus oxychloride (7.04 μL, 75 μmol) dissolved in dry THF (34.4 μL) was added dropwise with sufficient sodium hydroxide solution (stock of 109 mg, 2.7 mmol in 1.1 mL water) in order to ensure that the pH was always above 10 (by indicator paper). When the addition of the phosphorus oxychloride was complete, the reaction mixture was diluted with water to a final volume of 750 μL and the mixture was transferred to an NMR tube. ^{31}P [^{1}H coupled] NMR, δ_P(121 MHz): 8.52 (t, OP(O)$_2$–NH–CH$_2$), 2.31 (s, inorganic phosphate): m/z (LC–ES$^-$) 361 ([GNHP^{2-} + H$^+$]).

Optimisation of DMSO–water ratio

5′-Amino-5′-deoxyguanosine (10 mg, 35 μmol) was added to DMSO (where applicable) and stirred for 5 min, over which time full dissolution of the solid occurred. Water was added to the solution, in most cases resulting in the precipitation of a white solid. The reaction vessel was then cooled to 0 °C (in an ice-bath), before addition of NaOH$_{(aq)}$ (3.75 mmol) resulted in the dissolution of all the solid to give a clear solution. Additions (10 μL) of phosphorus oxychloride solution (22.8 μL $POCl_3$ in 77.2 μL dry THF]) were made at 60 s intervals over the course of 10 min. The solvents were removed under reduced pressure to give white solids, which were each dissolved in water (1 mL) and transferred to NMR tubes. Product distribution was then assessed by ^{31}P NMR spectroscopy.

Preparation and purification of GNHP

5′-Amino-5′-deoxyguanosine (50 mg, 177 μmol) was dissolved in a mixture of NaOH$_{(aq)}$ (885 μL of a 1 M volumetric standard, 885 μmol (5 eq)) and water (1 mL), and then cooled to 0 °C on an ice bath. Phosphorus oxychloride (33 μL, 177 μmol) in anhydrous THF (1 mL) was then added dropwise to the solution over the course of 10 min. The reaction mixture was diluted with water (3 mL) and loaded on to a Capto Q strong anion exchange chromatography column (25 mL bed volume) attached to an Äkta Prime Plus automated liquid chromatography system. Materials were eluted from the column using a gradient starting with eluent A (0.1 M NaOH) and running to 25% eluent B (1 M NaOH), and the absorbance at 254 nm of the eluted material was recorded, Fig. 3. Fractions containing material from each peak were combined and lyophilised. The residues from each peak were then dissolved in water (0.75 mL) and subjected to ^{31}P NMR spectroscopy. The material from the peak **B**, Fig. 3, was then subjected to gel filtration chromatography (Äkta Prime Plus automated liquid chromatography system, Sephadex G10, 150 mL bed volume, 5 mL min^{-1} of water) in order to remove salt. Fractions containing material absorbing at 254 nm were combined and lyophilised. The material was then repeatedly lyophilized from D_2O in order to reduce the large residual water signal in the ^{1}H NMR spectrum. Integration of the ^{1}H NMR spectrum showed that the sample contained 8% 5′-amino-5′-deoxyguanosine. 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$), 4.20 (1H, t, J 4, 3′-H), 4.61 (1H, t, J 5.5, 2′-H), 5.66 (2H, d, J 6.5, 1′-H), 7.74 (1H, s, 8-H): ^{13}C NMR δ_C(100.6 MHz, D_2O; Me_4Si): 44.4 (5′-C), 71.5 (3′-C), 73.1 (2′-C), 85.4 (d, J_{CP} 10.1, 4′-C), 86.9 (1′-C), 117.9 (5-C), 136.2 (8-C), 151.8 (4-C), 161.1 (2-C), 167.8 (6-C): ^{31}P NMR δ_P(121 MHz, D_2O; H_3PO_4): 10.2 (t, 88%, phosphoramidate), 4.1 (s, 12%, inorganic phosphate), see ESI for spectra: FT–MS m/z (ES$^-$): found 361.06661 at a FWHH resolving power of 10^6; $C_{10}H_{14}N_6O_7P^-$ requires 361.06671.

Kinetic study on the hydrolysis of GNHP

Owing to the great range of reactivity covered from pH 3 to 10.5, kinetic experiments were performed using three different methods. For experiments between pH 7.2 and 9, GNHP (final concentration 30 mM) was dissolved in buffer (0.5 M MES pH 7.2, 0.5 M sodium bicarbonate pH 8.0 or 0.5 M sodium borate pH 9.0 where the pHs were measured after dissolution of GNHP) and the samples were transferred to the NMR machine (equilibrated to 37 °C). Spectra were acquired regularly at fixed time points using the automated software. For experiments at lower pHs (3 and 3.5) GNHP was dissolved to a final concentration of 30 mM in 0.5 M sodium formate buffer and the pH was measured (one experiment at pH 3.0, one at pH 3.5). Aliquots of were withdrawn from the stock solutions at 5 min intervals and were quenched directly into sodium hydroxide solution. The quenched mixtures were then subjected to ^{31}P NMR spectroscopy. At higher pHs (9.8 and 10.5)

GNHP was dissolved to a final concentration of 30 mM in either 0.5 M sodium carbonate buffer or sodium hydroxide solution and the pH was measured as pH 9.8 or pH 10.5 respectively. The samples were transferred to a water bath set at 37 °C. The samples were periodically removed for ^{31}P NMR analysis and then returned to the water bath. All spectra were integrated and the ratio of the ^{31}P NMR signal corresponding to GNHP relative to that inorganic phosphate *and* GNHP was calculated for each time point. The fraction of GNHP within the mixture was then plotted as a function of time, and the data were found to fit simple first order decay curves. The observed pseudo first order rate constants are plotted in logarithmic form in Fig. 2. The data were found to fit eqn (1), which represents a simplified version of the expression used by Benkovic and Sampson.[12] Fitted values of the constants were $k_H = 26\ M^{-1}\ s^{-1}$, $k_0 = 6 \times 10^{-3}\ s^{-1}$ and $pK_a = 8.6$.

$$k_{obs} = k_H \times 10^{-pH} + \frac{k_0}{1 + 10^{pH-pK_a}} \quad (1)$$

Transcription studies and biotinylation assays

These experiments were performed as described previously.[13]

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