Synthesis of guanosine 5′-conjugates and their use as initiator molecules for transcription priming†

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We have synthesised two guanosine derivatives that are linked to biotinylated adenosine moieties by using two different strategies, one that includes synthetic steps on the solid phase and another one that is performed entirely in solution. The synthesised derivatives were shown to function as initiator molecules in transcription priming experiments. The incorporation efficiency was determined to be approximately 2%. Even though this value is rather low, the use of either molecule in selection experiments seems reasonable. Basically, RNA libraries with sequence complexities of 10^{15} to 10^{16} can be generated. Labelling of such a library with our initiator molecule would still produce 10^{13} to 10^{14} labelled/functionalised sequences, and thus sufficient sequence space for selection.

Introduction

The discovery that RNA is capable of catalysing a wide variety of chemical reactions has significantly supported the hypothesis of an 'RNA world', where RNA molecules were carriers of information as well as functional players in the amplification, adaptation and realisation of this program.[1] To further extend the repertoire of RNA-catalysed reactions, a number of studies have been carried out using the powerful method of *in vitro* selection, whereby a catalyst for a specific reaction is selected from a random RNA library. Thus, RNAs that catalyse classical organic reactions such as, for example, a Diels–Alder reaction,[2] a Michael addition[3] or an aldol reaction,[4] have been developed.

The typical assay for selection of a catalyst to enhance reaction between two reactants A and B requires linking of a potential reactant to the members of the RNA library, preferably *via* a flexible tether, while the other reactant is decorated with a suitable affinity tag, *e.g.* biotin. RNAs that accelerate the reaction of the attached reactant with the second reactant carrying the tag become tagged themselves, and subsequently can be separated by affinity chromatography. While biotinylation is rather easily accomplished, conjugation of a potential reactant to the members of the RNA library is a more difficult task. *In vitro* selection involves iterative rounds of selection and amplification requiring enzymatic procedures for reverse transcription of RNA into DNA and transcription of amplified DNA back into RNA. Therefore, modification of the RNA molecules in the library has to be repeated in each round. The most common strategies involve post-transcriptional protocols, attaching the specific reactant either to the 3′-end or to the 5′-end of RNAs in the library. 3′-End modification uses the unique properties of the 3′-terminal *cis*-diol. It can be specifically oxidised with periodate, and the obtained dialdehyde can be reacted with an amine or hydrazine-functionalised derivative of the compound of interest to yield the reactant–RNA conjugate.[5] Alternatively, the reactant can be conjugated to the 5′-end of the RNA molecules of the library. A possible route to this goal is 5′-thiophosphorylation by chemical or enzymatic means, and subsequent reaction of the nucleophilic thiol with a suitably activated derivative of the compound to be attached.[6,7]

In addition to post-transcriptional strategies, 5′-end modification of RNA can be also achieved co-transcriptionally by transcription priming.[8] T7 RNA polymerase is the most widely used enzyme for preparation of RNA by run-off transcription *in vitro*. It has been shown that 5′-modified guanosine derivatives are accepted instead of GTP as starting nucleoside in the initiation step, but owing to the missing 5′-triphosphate, cannot be used during elongation.[9] Thus, conjugates of guanosine or guanosine monophosphates have been shown, if provided in excess, to be incorporated at the 5′-end of the produced RNA molecules by T7 RNA polymerase.[4,6,9–13]

Within a project that focuses on the selection of ribozymes that support deamination of nucleosides as an important step in RNA editing, we have synthesised biotinylated adenosine and cytidine derivatives and linked those to guanosine. The obtained conjugates were subsequently used for transcription priming in order to generate a library of RNA molecules carrying for example compounds **1** or **2** (Fig. 1) at their 5′-end. In the strategy we have devised, selection will not rely on reaction of an attached reactant with a tagged reactant as described above. On the contrary, we intend to immobilise the entire library on a solid phase and to select for species that are released into solution upon reaction. For this purpose, the exocyclic amino group at C-6 of the adenine residue is linked to biotin. This decoration allows for a selection procedure involving immobilisation of the biotinylated RNA library on a streptavidin-coated surface. Upon incubation of the immobilised library under suitable reaction conditions, beneficial variants will be released into solution and can be filtered from non-functional members of the library. Here we describe the synthetic route to

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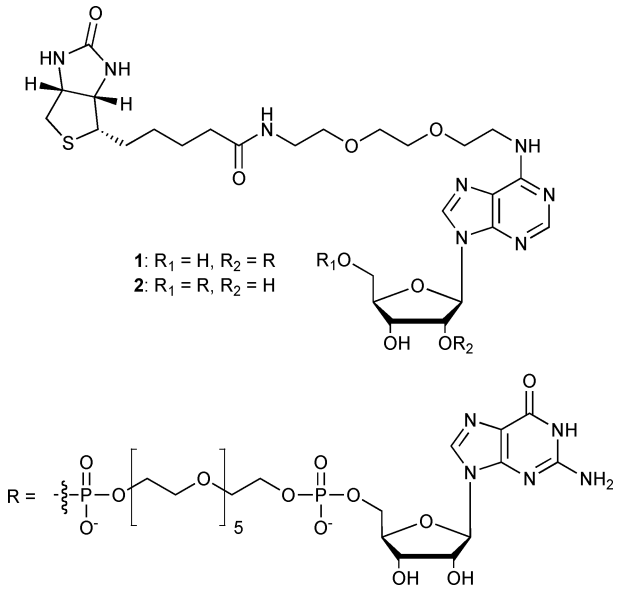

Fig. 1 Adenosine–guanosine conjugates used in transcription priming reactions. **1** was obtained by solid phase phosphoramidite chemistry; **2** was synthesised in solution.

compounds **1** and **2** and their evaluation as initiator molecules in transcription priming.

Results and discussion

In our effort to synthesise initiator molecules that allow for the attachment of a functionalised adenosine residue to the 5′-end of RNA by transcription priming, we have prepared two different adenosine–guanosine conjugates, **1** and **2**. Synthesis of the initiator molecules was accomplished by modifying the guanosine and adenosine moieties separately and eventually coupling them *via* a hexaethylene glycol tether.[14]

Both compounds **1** and **2** carry a biotin residue at the N^6-position of the adenine base. The guanosine moiety is linked to hexaethylene glycol *via* its 5′-phosphate. The glycol linker in turn is connected to a 2′-phosphate (in the case of **1**) or 5′-phosphate (in the case of **2**) of the sugar moiety of adenosine. Two related strategies were applied to the synthesis of the guanosine 5′-conjugates **1** and **2**, one involving solid phase coupling of nucleoside derivatives, the other involving full solution chemistry.

Strategy 1: Solid phase coupling of functionalised adenosine to guanosine

In our first approach, coupling of a suitably modified guanosine derivative **4** to the biotinylated adenosine **3** was carried out on the solid phase using phosphoramidite chemistry and an automated DNA/RNA-synthesiser, yielding **1** as a 2′,5′-tethered dinucleotide derivative in nanomolar quantity (Fig. 2).

Synthesis of the adenosine moiety started with acetylation of the 2′-, 3′- and 5′-hydroxy groups of adenosine **5** (Fig. 3). Although it does not obstruct the following reactions, the aminoacetylated side product was removed by silica gel chromatography to isolate the pure triacetylated product **6**. Synthesis of the 6-iodo derivative

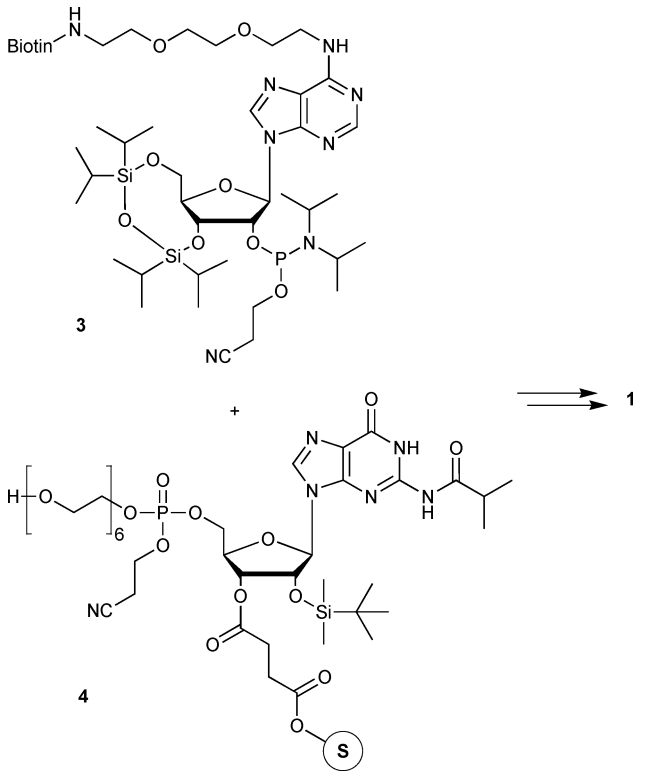

Fig. 2 Solid phase synthesis of **1**. Coupling of components **3** and **4** by phosporamidite chemistry and successive deprotection yields compound **1**.

7 was then accomplished by photochemically induced substitution of the exocyclic amino group by iodine.

To this end, **6** was reacted with isopentyl nitrite in an iodine-donating solvent (CH_2I_2) at 60 °C followed by irradiation with visible light according to a procedure introduced by Nair and Richardson.[15] This procedure allows for the synthesis of purinyl halogenides, which are well suited for the introduction of alkyl residues at the N^6-position of adenosine. There are other ways of fulfilling this task,[16] many of which, however, require the use of HMPT, known to be a rather strong carcinogen.[17] Synthesis of the iodine derivative, as carried out here, avoids the use of this highly carcinogenic solvent and thus seemed to be the more convenient procedure. Furthermore, it has been successfully applied by other groups and it was reported that successful substitution can be achieved even in the absence of irradiation.[18] Thus, we were able to obtain the acetylated 9-iodoadenosine **7** in 43% yield.

Next we introduced a linker to the N^6-position to be used for attachment of biotin. The 3,6-dioxa-8-*N*′-BOC-aminooctyl linker was synthesised from unprotected 2,2′-(ethylenedioxy)bis-(ethylamine) by reaction with di-*tert*-butyldicarbonate based on a method reported by Sigal *et al.*[19] The BOC-protected linker was coupled to **7** by reaction in ethanol under reflux for 6 hours. The concomitant deacetylation of the ribose moiety was completed by treatment with 25% aqueous ammonium hydroxide, and the product **8** was obtained after chromatographic purification in 64% yield.

Further synthetic steps involved renewed protection of the 3′- and 5′-OH groups, removal of the BOC group from the aliphatic linker amino function, coupling it with biotin, and

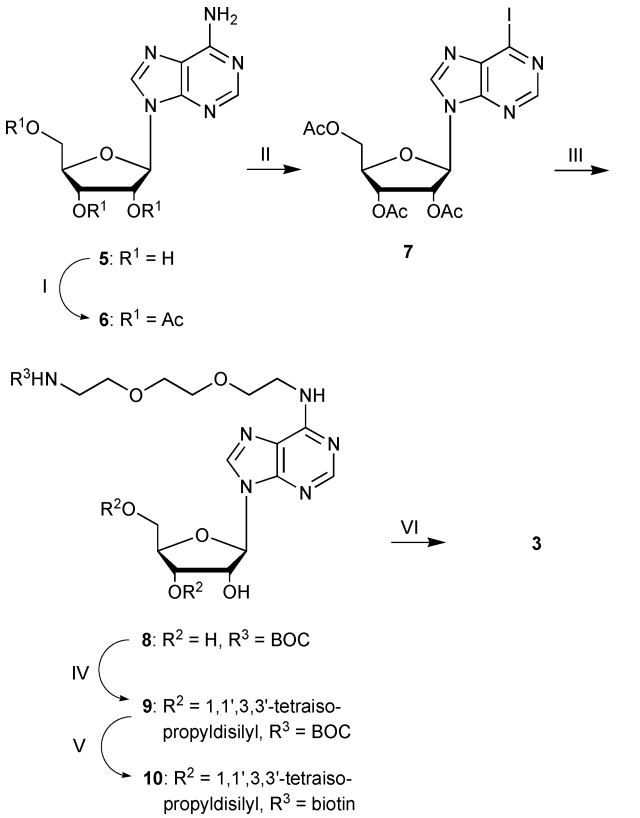

Fig. 3 Introduction of the 3,6-dioxa-8-*N*′-BOC-aminooctyl linker at the N^6-position of the adenine base. *Reagents and conditions*: I) 4 eq. acetic anhydride, 4-DMAP, pyridine, 24 h, RT, 50%. II) 30 eq. methylene iodide, 19 eq. isoamyl nitrite, 5 h, 60 °C, 43%. III) 4.8 eq. *N*-BOC-2,2′-(ethylenedioxy)bis(ethylamine), ethanol, 25% NH_4OH, 64%. IV) 1 eq. 1,3-dichloro-1,1′,3,3′-tetraisopropyldisiloxane, pyridine, 2 h, RT, 84%. V) 50% CF_3COOH in $CHCl_3$, 20 min, RT; 0.8 eq. 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU), 0.8 eq. biotin, overnight, RT, 67%. VI) 1.8 eq. (2-cyanoethyl-*N*,*N*-diisopropyl)-chlorophosphoramidite, 7.5 eq. diisopropylethylamine, CH_2Cl_2, ethyl acetate, 76%.

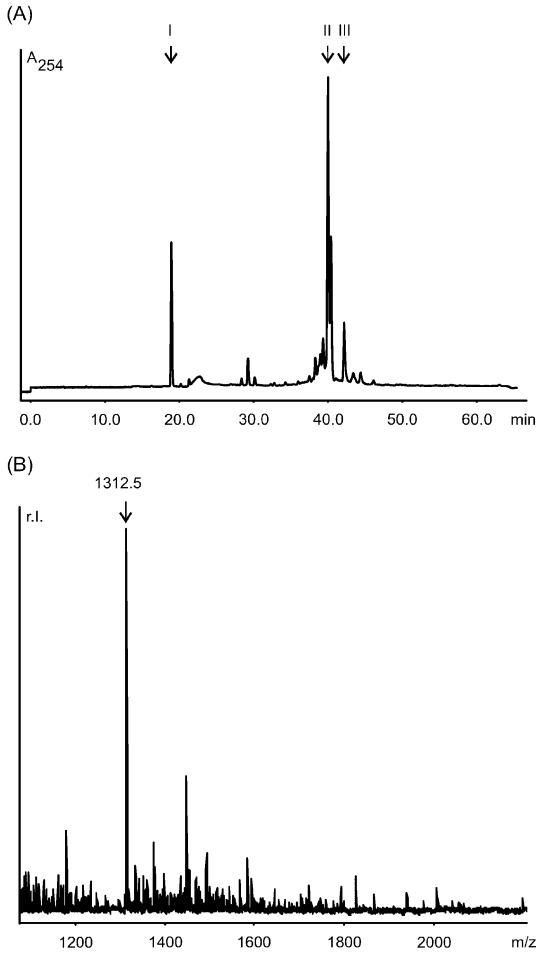

Fig. 4 **A)** HPLC reverse phase purification of **1**; A_{254} represents the absorption at 254 nm. **B)** ESI-spectrum of the pooled HPLC-fractions from peak II; r.I. represents the relative intensity. The peak at 1312.5 corresponds to molecule **1**. For further details refer to main text.

finally preparation of the 2′-phosphoramidite. As shown in Fig. 3, selective 3′,5′-OH protection of **8** was accomplished with the Markiewicz silyl group to yield **9**. The BOC group was removed from the amino linker using 50% CF_3COOH in $CHCl_3$, and the resulting primary amino group was coupled to biotin that was functionalised as an active succinimidyl ester. The resulting nucleoside derivative **10** was then reacted with (2-cyanoethyl-*N*,*N*-diisopropyl)chlorophosphoramidite to yield building block **3**. This was then coupled to immobilised derivatised guanosine **4** on the solid phase to generate initiator molecule **1** (Fig. 2). First, CPG loaded with 1 μmol guanosine was reacted with hexaethylene glycol phosphoramidite in an automated synthesiser using the standard protocol,[20] followed by coupling of **3** without changes.

After deprotection and cleavage from the solid phase, **1** was purified by reverse phase chromatography (Fig. 4A). The product was assigned to the large peak with a retention time of 40 min (peak II) in the HPLC diagram. Further mass spectrometric analysis (MALDI) clearly identified the product as the desired adenosine–guanosine conjugate **1**. A smaller peak (peak I, retention time 20 min) presumably represents the guanosine–hexyl adduct resulting from uncoupled conjugate **4**. Smaller peaks surrounding the main peak may result from incompletely deprotected initiator molecule **1** (peak III). The still present protecting groups lead to a higher hydrophobicity, resulting in a longer retention time relative to the fully deprotected product.

Since it can be carried out in part by an automated process, the method described represents a relatively quick route for synthesis of the initiator molecule **1**. However, it has to be noted that a large excess of the phosphoramidite **3** (15-fold over solid phase reactant) is required for coupling in solid phase synthesis. Furthermore, solid phase coupling with our facilities is limited to the μmol scale, thus requiring iterative coupling reactions in order to produce **3** in larger amounts. In order to overcome this restriction, a second method was developed, which is performed in solution and requires equimolar amounts of coupling partners. Additionally, the solution-based method allows for synthesis of initiator molecules on higher scales.

Strategy 2: Coupling of functionalised adenosine to guanosine in solution

As an alternative to solid phase coupling as described above, the synthetic route in solution involves coupling of a guanosine

5′-phosphoramidite to the 5′-OH group of the biotinylated adenosine derivative *via* a hexaethylene glycol bridge (product **2** in Fig. 1).

First, we aimed to couple an equimolar amount of mono-DMT-protected hexaethylene glycol to the protected adenosine phosphoramidite **3**. However, we did not observe any coupling product. Possibly, steric hindrance due to the bulky silyl groups at the 5′- and 3′-OH hampers reaction at the neighbouring 2′-*O*-phosphoramidite in solution. Also, problems concerned with loss of the rather labile cyanoethyl group at the phosphorus in solution chemistry have been reported.[21] Therefore, we changed the synthetic strategy for the adenosine part, as depicted in Fig. 5. Instead of the labile cyanoethyl group, we chose the more stable methyl group for protection of the phosphite, and decided to couple the adenosine and guanosine moieties *via* the primary 5′-OH groups of both sugar residues.

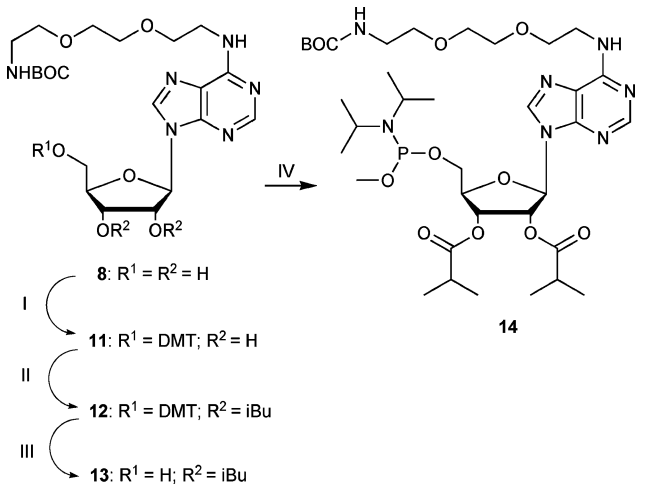

Fig. 5 Synthesis of the adenosine derivative phosphoramidite **14** for coupling in solution. *Reagents and conditions*: I) 1.4 eq. DMTCl, 3.6 eq. NEt_3, 4-DMAP, pyridine, 24 h, RT, 76%. II) 13 eq. isobutyric anhydride, pyridine, 24 h, RT, 93%. III) 5% CCl_3COOH in $CHCl_3$, 20 min, RT, 73%. IV) 1.8 eq. (methyl-*N*,*N*-diisopropyl)chlorophosphoramidite, 7.5 eq. diisopropylethylamine, 1 h, used as crude product, quantitative yield assumed from TLC analysis.

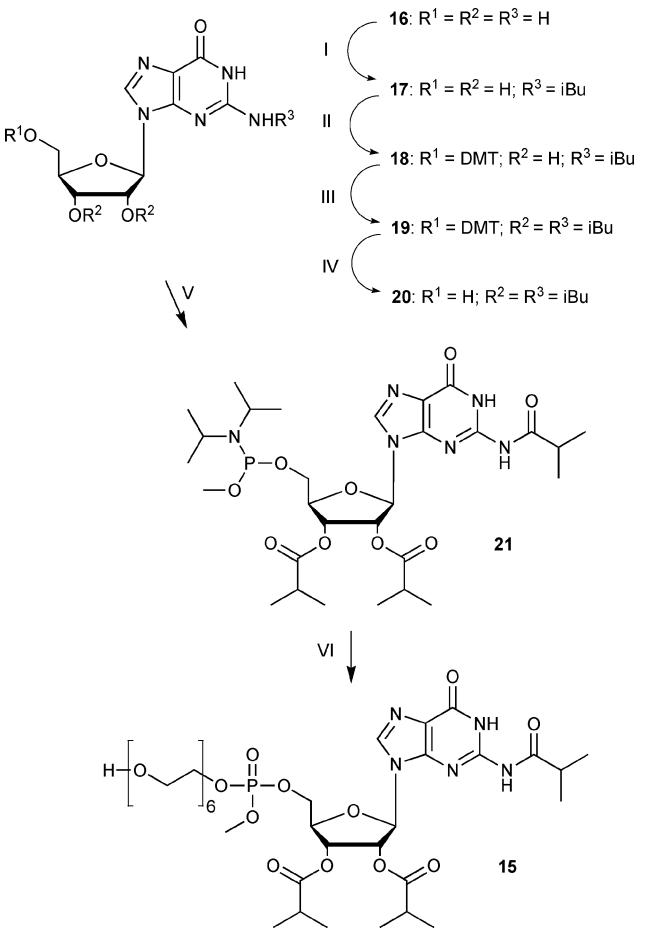

Fig. 6 Synthesis of the guanosine–hexaethylene glycol conjugate **15**. *Reagents and conditions*: I) 8 eq. TMSCl, 10 eq. isobutyric anhydride, 25% NH_4OH, RT, 26%. II) 1.3 eq. DMTCl, NEt_3, pyridine, overnight, RT, 41%. III) 10 eq. isobutyric anhydride, pyridine, 78%. IV) 5% CCl_3COOH in CH_2Cl_2, 20 min, pyridine, RT, 49%. V) 1.8 eq. (methyl-*N*,*N*-diisopropyl)chlorophosphoramidite, 7.5 eq. diisopropylethylamine, 1 h, CH_2Cl_2, used as crude product, quantitative yield assumed from TLC analysis. VI) 1.0 eq. DMT-hexaethylene glycol, acetonitrile, 0.45 M tetrazole solution; 5% CCl_3COOH in $CHCl_3$, 20 min, RT, 35%.

Thus, **8** was selectively protected with a DMT-group at the 5′-OH to provide **11**, followed by isobutyrylation of the remaining free 2′- and 3′-OH functionalities to result in **12**. After removal of the DMT-group (**13**), the 5′-methyl-*N*,*N*-diisopropylphosphoramidite **14** was prepared and used without further purification for the coupling reaction with the guanosine moiety **15**. The guanosine moiety **15** was synthesised as shown in Fig. 6.

Guanosine **16** was isobutyrylated at the N^2-position and crystallised from water to give sufficiently pure N^2-isobutyrylguanosine **17**. The 5′-position was protected with a DMT-group (**18**) followed by isobutyrylation of the 2′- and 3′-OH groups (**19**). The following removal of the DMT-group by treatment with 5% trichloroacetic acid delivered **20**, which was converted into the 5′-*O*-methylphosphoramidite **21** and subsequently coupled with a mono-DMT-protected hexaethylene glycol linker. Final detritylation delivered building block **15** in reasonably good yield. This was then coupled to the adenosine derivative **14** as shown in Fig. 7.

The coupling product **22** was BOC-deprotected to give **23** and biotinylated to provide **24** in a manner similar to that described for the synthesis of compound **3** (Fig. 3). Removal of the methyl group from the phosphate was performed using disodium 2-carbamoyl-2-cyanoethylen-1,1-dithiolate ("Na_2S_2") followed by treatment with a 1 : 1 mixture of 32% aqueous ammonium hydroxide and methylamine to deprotect the 2′- and 3′-OH functionalities.[22] The obtained initiator molecule **2** was purified by HPLC on a reverse phase column. Fig. 8 shows the reverse phase HPLC diagram and the MALDI spectra. Peak III was identified as the desired compound **2** by mass spectrometry and NMR. Peak II presumably results from uncoupled, deprotected compound **15**, which is in analogy to observed peak II in Fig. 5. The intense peak I results from the UV-visible reagent Na_2S_2, which was used in excess during the deprotection procedure.

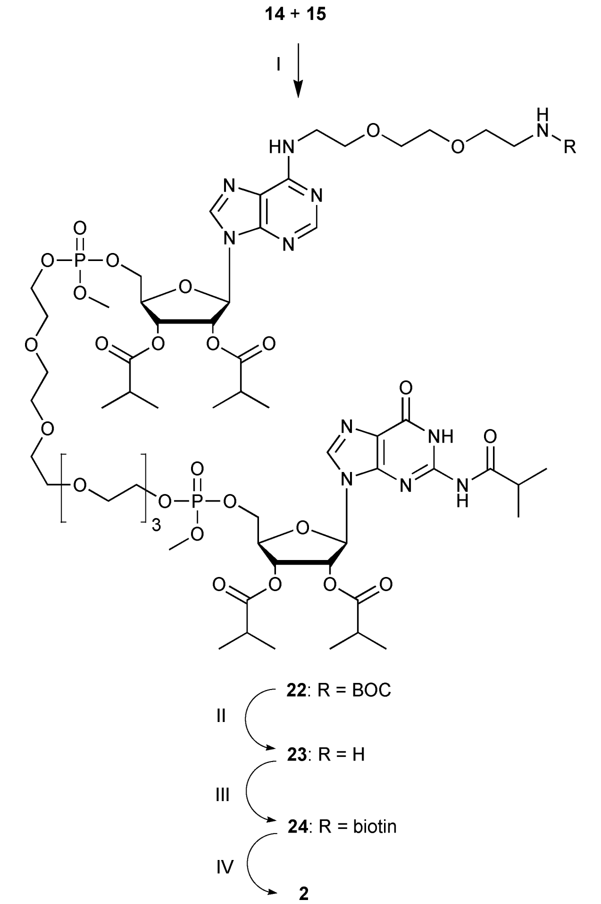

Fig. 7 *Reagents and conditions*: I) 0.45 M tetrazole, acetonitrile, 83%. II) 30% CF_3COOH in CH_2Cl_2, quantitative yield. III) TSTU, biotin, 52%. IV) Na_2S_2, 32% NH_4OH, $MeNH_2$, quantitative yield assumed from TLC analysis.

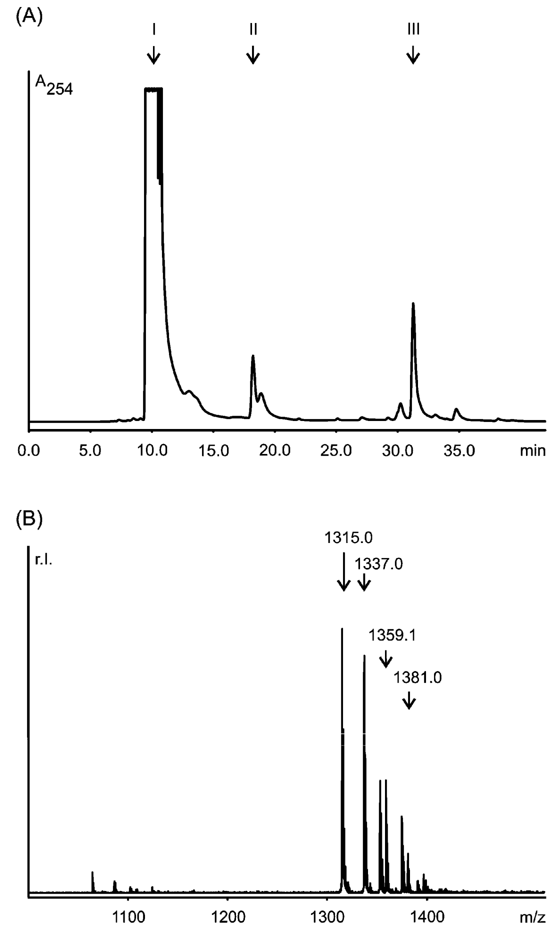

Fig. 8 **A)** HPLC reverse phase diagram of biotinylated adenosine–guanosine conjugate **2**. A_{254} represents the absorption at 254 nm. **B)** MALDI-spectrum of the pooled HPLC-fractions from peak III; r.I. represents the relative intensity. Peaks at 1315.0, 1337.0, 1359.1 and 1381.0 correspond to the molecular ion and the molecule with 1, 2, or 3 bound Na^+ ions, respectively.

Transcription priming with initiator molecules 1 and 2

After extensive qualitative analysis of both compounds **1** and **2** to ensure their identity, we carried out initial experiments on transcription priming. To this end, derivatives **1** or **2**, respectively, were added in different molar ratios to a transcription mixture containing the four natural nucleoside triphosphates, a model DNA template and T7 RNA polymerase. After reaction, product RNAs were separated by electrophoresis through 10% denaturing polyacrylamide gels and subsequently blotted onto a nylon membrane. The membrane was incubated with streptavidin-conjugated alkaline phosphatase. Thus, after several washing steps, alkaline phosphatase remains bound to the membrane only at the sites containing biotinylated RNA. Upon incubation with a solution of a pro-luminescent compound (CDP-Star), biotinylated RNA can be identified due to alkaline phosphatase-induced conversion of CDP-Star into a chemiluminescence marker.

Fig. 9 shows examples of transcription priming reactions of a 55-mer RNA (HP-WTL) with initiator molecule **2**. In individual experiments, different concentrations of **2** were applied, whereas the concentrations of NTPs were kept constant. Fig. 9A shows the effect of augmentation of the initiator concentration on total RNA yield: the higher the initiator concentration, the smaller the total yield of transcription product. This is in agreement with

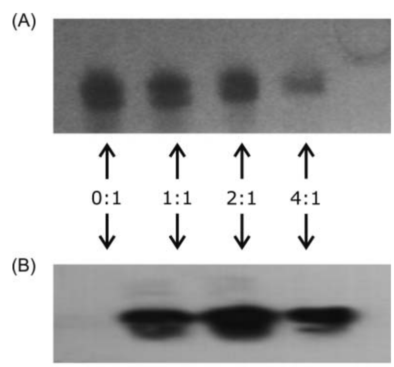

Fig. 9 Transcription reactions in presence of different concentrations of **2** (RNA: HPWTL 55-mer). Figures represent the ratio of initiator concentration to GTP concentration (2 mM) in standard transcription reactions (concentrations of ATP, CTP, and UTP were also 2 mM). **A)** Standard reaction analysed by denaturing polyacrylamide gel electrophoresis. Bands are visualised by UV shadowing. RNA yield diminishes with growing initiator concentration. **B)** Chemiluminescence proof of incorporation of the biotin-carrying initiator molecule. Note that there is no spot for the transcription reaction without initiator molecule.

similar results obtained for other derivatives.[14] Fig. 9B shows the incorporation of the initiator molecule by detecting the present biotin with a chemiluminescence assay. Obviously, an excess of initiator molecule **2** over natural GTP does not significantly enhance the yield of primed RNA. Thus, it can be concluded that a 1 : 1 molar ratio of initiator **2** to natural GTP is most suitable for both overall yield of the transcript and incorporation of the initiator. Similar results have been obtained with initiator **1**.

In order to better quantify the efficiency of incorporation of the synthesised initiator molecule, we chemically synthesised a 39-mer RNA HHR6-St. The phosphoramidite **3** of biotinylated adenosine was coupled *via* a hexaethylene glycol unit to the 5′-end of this RNA by solid phase chemistry. Thus, the chemically synthesised RNA HHR6-St is identical to the RNA HHR6 that was functionalised statistically by transcription priming experiments with initiator **1**, and nearly identical to RNA HHR6 that was prepared and primed with initiator **2** (Fig. 10A).

The chemically synthesised 39-mer was used as a standard to estimate the extent of incorporation of initiator in a transcription priming reaction. To this end, defined amounts of statistically labelled 39-mer HHR6 obtained by transcription priming with **2** were blotted together with defined amounts of the fully labelled synthetic standard RNA HHR6-St. The chemiluminescence that appeared upon incubation with streptavidin-linked alkaline phosphatase and the pro-luminescent compound CDP-Star as described above was analysed in a photo-system, and quantified by analysis of the chemiluminescence intensities. By comparison between the standard and the priming product chemiluminescence, the efficiency could be determined to be ~2%. Changes in the ratio of concentrations of initiator **2** and GTP during the priming reactions did not significantly improve this efficiency. The rather low incorporation efficiency could be due to a comparably low hydrophobicity of the initiator molecule, which has been shown to reduce incorporation.[11,23] Additionally, the steric hindrance of both **1** and **2** might also diminish efficiency.

Even though transcription priming with the initiator molecules **1** and **2** has so far delivered only about 2% of the RNA population as primed products, the strategy has the potential to be used for functionalization of RNA libraries. The sequence complexity of RNA libraries is supposed to be about 10^{15} to 10^{16} molecules; 2% of this library still corresponds to 10^{13} to 10^{14} molecules. Therefore, initial transcription priming with 2% yield decreases the size of the library by two orders of magnitude, and even though the major part of the initial population is lost, the remaining part is still well in the range of what can be termed a library.[24] Protein libraries for example may involve only 10^{7} to 10^{10} members and yet have been extensively used in molecular evolution with impressive results.

To the best of our knowledge, the molecules presented here belong to the largest initiator molecules that have been used so far for transcription priming. We have developed an efficient synthetic route to biotinylated adenosine–guanosine conjugates that are accepted by T7 RNA polymerase and thus incorporated at the 5′-end of RNAs prepared by run-off transcription. Further studies to improve the incorporation efficiency are under way.

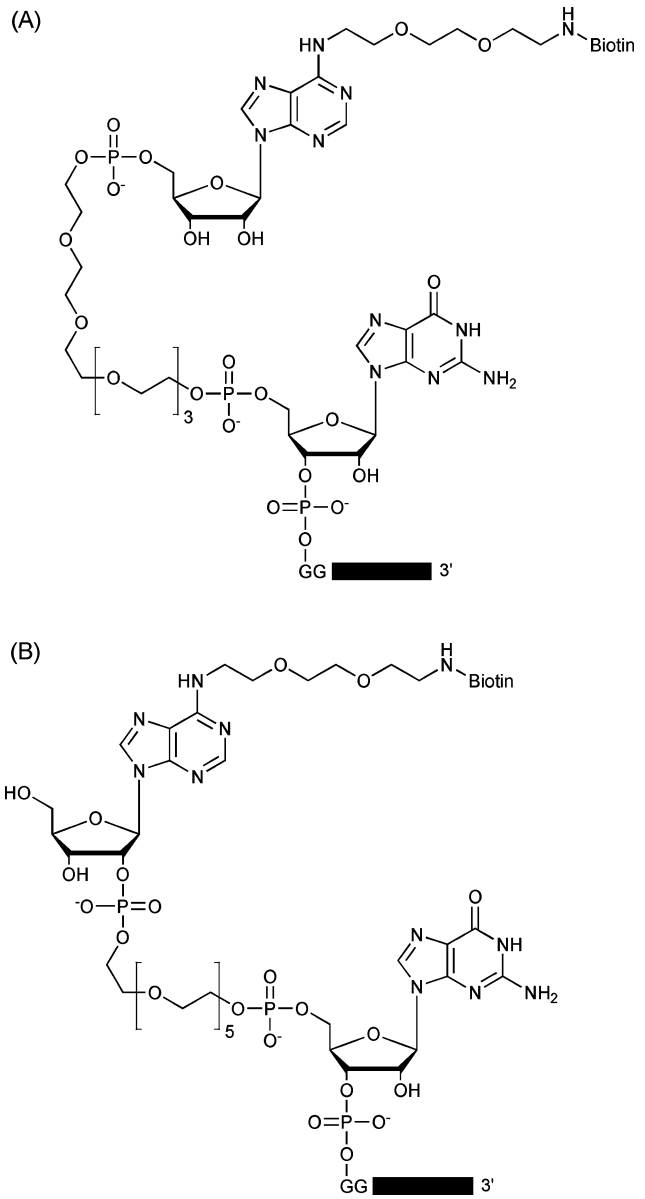

Fig. 10 **A)** Chemical structure of the 5′-end of RNA obtained by transcription priming experiments with initiator **2**. **B)** Chemical structure of the 5′-end of a standard RNA (HHR6-St) synthesised on the solid phase using phosphoramidite **3** in the last coupling step. Note that in A) this label is incorporated depending on the incorporation efficiency, whereas in B) it is introduced chemically during synthesis and therefore conjugated to all RNA molecules.

Experimental

General

Dry methylene chloride and dry methanol were obtained from Fluka. Pyridine was dried overnight over KOH, heated to reflux for 2 h, distilled off and stored over molecular sieves. Diisopropylethylamine was stored over calcium hydride and distilled before use. All other reagents, chemicals, buffers and solvents were obtained as the highest commercially available grade and used without further purification. Silica gel for column chromatography (0.063–0.2 mm) was obtained from Sigma-Aldrich. Reactions were

carried out at room temperature unless stated otherwise, and in the case of moisture-sensitive compounds under an atmosphere of argon. HPLC spectra were performed on an Äkta Purifier (Amersham Biosciences) with the columns described. NMR spectra were recorded with TMS as the internal standard on the following machines: 300 MHz: ARX-300; 400 MHz: Bruker DRX-400; 600 MHz: Bruker DRX-600. *J* values are given in Hz. Mass spectra were recorded on a Bruker Autoflex MALDI-TOF, VG Autospec (FAB), and ESI Bruker Esquire LC (Ion Trap). UV measurements were obtained using an Ultrospec 2100 pro (Amersham Biosciences) Cary 1E or a Varian Nano Drop® ND-1000 spectrophotometer.

2′-*O*-,3′-*O*-,5′-*O*-Triacetyl-6-iodopurine-9-β-D-riboside 7

17 g of 2′-*O*-,3′-*O*-,5′-*O*-triacetyladenosine **6** (43 mmol) were suspended in 110 mL methylene iodide (1.3 mol) and 110 mL isopentyl nitrite (816 mmol). The suspension was stirred at 60 °C for 5 h, then at room temperature overnight. The solvent was removed *in vacuo* and the residue dissolved in $CHCl_3$ and worked up as described for **6** in the ESI†. The residue was purified chromatographically (silica gel, hexane to remove iodine, then $CHCl_3 \rightarrow CHCl_3$–MeOH = 95 : 5). **7** was obtained as a colourless foam (9.3 g, 18.4 mmol, 43%); δ_H(400 MHz; DMSO-d_6; Me_4Si) 2.01, 2.04, 2.12 [9 H, 3 × s, 3 × (C=O)–CH_3], 4.29 (1 H, dd, *J* 12.9 and 6.4, *H*-5′), 4.40–4.44 (2 H, m, *H*-4′, *H*-5′), 5.63–5.66 (1 H, m, *H*-3′), 6.02–6.05 (1 H, m, *H*-2′), 6.33 (1 H, d, *J* 5.1, *H*-1′), 8.69 (1 H, s, *H*-2), and 8.85 (1 H, s, *H*-8); δ_C(400 MHz; DMSO-d_6; Me_4Si) 20.0, 20.2, 20.3 [3 × (C=O)–*C*H_3], 62.5 (*C*-5′), 69.8 (*C*-3′), 71.9 (*C*-2′), 79.6 (*C*-4′), 86.1 (*C*-1′), 123.2 (*C*-5), 138.6 (*C*-8), 145.2 (*C*-6), 147.3 (*C*-4), 151.9 (*C*-2), 169.1, 169.2, and 169.8 [3 × (C=O)–CH_3]; FAB$^+$-MS: calculated for $C_{16}H_{17}IN_4O_7$ [M + H$^+$]: 505.2; found: 505.1.

N^6-(3,6-Dioxa-8-*N*′-BOC-aminooctyl)adenosine 8

22 g *N*-BOC-2,2′-(ethylenedioxy)bis(ethylamine) (88 mmol) were added to a solution of 9.3 g 2′-*O*-3′-*O*-,5′-*O*-triacetyl-6-iodopurine-9-β-D-riboside **7** (18.4 mmol) in 50 mL ethanol. The mixture was heated to 78 °C and stirred for 6 h under reflux. The solvent was removed *in vacuo*, the residue dissolved in $CHCl_3$, and the organic layer was worked up as described for **7**. 30 mL of aqueous ammonium hydroxide (25%) were added and the solution was stirred for 1 h. Afterwards, the solvent was removed *in vacuo*, and the residue dissolved in $CHCl_3$ and worked up as described for **7**. The crude product **8** was purified by chromatography (silica gel, $CHCl_3$–MeOH = 9 : 1) and obtained as a colourless foam (5.7 g, 11 mmol, 64%); δ_H(400 MHz; DMSO-d_6; Me_4Si) 1.36 (9 H, s, BOC), 2.66–2.69, 3.05–3.09, 3.37–3.40 (12 H, 3 × m, ether-*H*), 3.60–3.69 (2 H, m, *H*-5′), 3.96-3.99 (1 H, m, *H*-4′), 4.15–4.17 (1 H, m, *H*-3′), 4.59-4.62 (1 H, m, *H*-2′), 5.90 (1 H, d, *J* 6.1, *H*-1′), 8.31 (1 H, s, *H*-2), and 8.35 (1 H, s, *H*-8); δ_C(400 MHz; DMSO-d_6; Me_4Si) 28.1 [BOC–(C=O)–O–(*C*H_3)$_3$], 61.5 (*C*-5′), 69.0, 69.4 (2 × ether), 70.5 (*C*-3′), 72.3 (*C*-2′), 79.0 (C_q-BOC), 85.8 (*C*-4′), 87.9 (*C*-1′), 122.5 (*C*-5), 139.7 (*C*-8), 148.5 (*C*-4), 152.2 (*C*-2), 155.5 (*C*-6), and 157.9 [BOC–(*C*=O)–O–(CH$_3$)$_3$]; FAB$^+$-MS: calculated for $C_{21}H_{34}N_6O_8$ [M + H$^+$]: 499.5; found: 499.3.

3′-*O*-,5′-*O*-(1,1′,3,3′-Tetraisopropyldisiloxan-1,3-diyl)-N^6-(3,6-dioxa-8-*N*′-biotinylaminooctyl)adenosine 10

1.3 g **8** (2.6 mmol) was coevaporated three times with 10 mL of dry pyridine and finally dissolved in 15 mL of dry pyridine. The mixture was cooled and 0.8 mL of 1,3-dichloro-1,1′,3,3′-tetraisopropyldisiloxane (0.8 g, 2.5 mmol) in 2 mL of dry DMF were added. The mixture was stirred overnight and quenched by adding 5 mL of water. The solvent was removed *in vacuo* and the residue worked up as described for **7**. Chromatographic purification ($CHCl_3$–MeOH = 92 : 8) yielded 1.6 g of **9** as a yellow oil (2.1 mmol, 84%). This oil was dissolved in 6 mL of 50% CF_3COOH in $CHCl_3$, stirred for 20 min, diluted with 80 mL of diethyl ether, and neutralised with a saturated solution of sodium hydrogen carbonate. The aqueous phase was extracted with diethyl ether (3 × 60 mL) and the organic phase removed. 0.4 g biotin (1.6 mmol), 0.5 g TSTU (1.7 mmol) and 0.7 mL diisopropylethylamine (0.5 g, 4 mmol) were dissolved in 5 mL of DMF and 5 mL of dioxane. This mixture was added to the yellow oil and stirred overnight at room temperature. After removal of the solvent, the residue was directly subjected to silica gel chromatography with $CHCl_3$–MeOH = 9 : 1 as solvent. After removal of the solvent, 0.9 g of a colourless oil **10** was obtained (1.0 mmol, 67%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 1.05 [24 H, m, Si–CH–(CH_3)$_2$], 1.26–1.61 (6 H, m, biotin-*H*-4′, biotin-*H*-5′, biotin-*H*-3′), 2.06 (2 H, t, *J* 7.2, biotin-*H*-2′), 2.81 (1 H, m, biotin-*H*-6x), 3.08 (1 H, m, biotin-*H*-6y), 3.17 (1 H, m, biotin-*H*-4), 3.36-3.60 (12 H, m, ether-*H*), 3.92-4.14 (3 H, m, 4′-*H*, 2 × *H*-5′), 4.28 (1 H, m, biotin-*H*-3a), 4.50 (1 H, m, biotin-*H*-6a), 4.79 (1 H, m, *H*-2′), 5.10 (1 H, m, *H*-3′), 5.88 (1 H, m, *H*-1′), 6.36 (1 H, s, biotin-N-*H*1), 6.42 (1 H, s, biotin-N-*H*3), 8.16 (1 H, s, *H*-2), and 8.23 (1 H, s, *H*-8); δ_C(400 MHz; DMSO-d_6; Me_4Si) 12.0, 12.2, 12.4, 12.7 [Si–*C*H–(CH$_3$)], 16.8, 16.9, 16.9, 17.0, 17.2, 17.2, 17.3, 17.4 [Si–CH–(*C*H_3)], 25.2 (biotin-*C*-3), 28.0, 28.2 (biotin-*C*-5, biotin-*C*-4), 35.1 (biotin-*C*-2), 38.4 (biotin-*C*-9), 55.4 (biotin-*C*-6), 59.2 (biotin-*C*-8), 60.8 (*C*-5′), 61.0 (biotin-*C*-7), 68.7, 69.2, 69.5, 69.5, 69.8 (ether-*C*, *C*-3′), 73.7 (*C*-2′), 81.9 (*C*-4′), 89.4 (*C*-1′), 119.7 (*C*-5), 139.1 (*C*-8), 147.9 (*C*-4), 152.3 (*C*-2), 154.5 (*C*-6), 162.7 (biotin-*C*-10), 172.1, and 172.8 (biotin-*C*-1); MALDI$^+$-MS: calculated for $C_{38}H_{66}N_8O_9Si_2$ [MH$^+$]: 867.2; found: 867.9.

Solid phase synthesis of initiator 1 and RNA HHR6

For the synthesis of **1**, phosphoramidite **3** was synthesised from **10** as described in the general procedure (ESI†). Initiator **1** and RNA HHR6 were prepared by solid phase phosphoramidite chemistry as described previously.[26] In the case of initiator **1**, two syntheses (2 × 1 μmol scale) were performed, in which solid bound guanosine [ChemGenes G (*N*-PAC) 3′-tBDSilyl 2′-lcaa CPG 1000 Å] was first coupled to a hexaethylene glycol phosphoramidite (ChemGenes DMT-hexaethyloxy-glycol phosphoramidite) and phosphoramidite **3** in the second step. Afterwards, the solid phase was treated overnight with 2 mL of ammoniacal methanol and separated from the solid support. The solvent was removed and the residue treated with 400 μL triethylamine tris(hydrofluoride)–DMF = 3 : 1 (v/v) for 90 min at 55 °C. The solvent was removed *in vacuo* and the residue was dissolved in 1 mL saturated aqueous $NaHCO_3$. **1** was purified by reverse phase chromatography [column: Macherey Nagel VP 250/10 Nucleodur 100-5 C18 ec; buffers

used were (A) 0.1 M triethylammonium acetate (pH 7.0) and (B) 0.1 M triethylammonium acetate (pH 7.0), 50% acetonitrile; flow rate 4 mL min^{-1}; gradient: 0% B for 5 min 30 s, linear augmentation to 100% B over 50 min, 100% for 5 min, 0% for 5 min]. The retention time of **1** was 40 min. The fractions containing **1** were pooled, the solvent was removed, the residue dissolved in 1 mL of water, filtered (0.2 μm), and the yield was determined by UV ($\varepsilon = 27\,100\ cm^{-1}\ M^{-1}$) to be 240 nmol; ESI^--MS: calculated for $C_{48}H_{77}N_{13}O_{24}P_2S$ [M^-] 1313.2; found: 1312.5.

HHR6St (5′-ΦGGG CAG CUG AUG AGC UCC AAA UAG AGC GAA AGU UAC ACC-3′, where Φ denotes a modification as shown in Fig. 10) was synthesised by solid phase synthesis on a Gene Assembler Special (Pharmacia) with PAC-phosphoramidites, using hexaethylene glycol phosphoramidite (ChemGenes DMT-hexaethyloxy-glycol phosphoramidite) and phosphoramidite **3** in the second-to-last and final coupling steps, respectively. The obtained RNA was deprotected as described[26] and purified by electrophoresis using a 10% denaturing gel for RNA purification. Elution was carried out using 0.5 M LiOAc followed by acetone precipitation.

Hexaethylene glycol-2′,3′,N^2-triisobutyrylguanosine conjugate 15

Mono-BOC-protected DMT-hexaethylene glycol (4 mmol) was coevaporated twice with 10 mL of dry pyridine and stored *in vacuo* overnight. Then it was dissolved in 5 mL of dry acetonitrile and mixed with 4 mmol of dried 2′-*O*-,3′-*O*-,N^2-triisobutyrylguanosine (methyl-*N*,*N*-diisopropyl)phosphoramidite **21**, which was also dissolved in 1 mL of dry acetonitrile (the synthesis of **21** is described in the ESI†). The mixture was cooled in an ice bath and 8.5 mL of a 0.45 M solution of tetrazole (4 mmol) in acetonitrile was added. The mixture was stirred for 30 min and 5.5 mL of a 0.1 M solution of iodine in collidine–THF–H_2O = 2 : 2 : 1 was added dropwise. After a further 5 min, excess iodine was reduced with 1 M $Na_2S_2O_3$. The solvent was removed *in vacuo*, the residue was dissolved in $CHCl_3$, worked up and purified chromatographically as described for **6** ($CHCl_3$ → $CHCl_3$–MeOH = 95 : 5). The purified coupling product was DMT-deprotected with 5% CCl_3COOH in CH_2Cl_2 and purified chromatographically as described for **12**. Compound **15** was obtained as a colourless oil (1.3 g, 1.4 mmol, 35%); δ_H(400 MHz; DMSO-d_6; Me_4Si) 1.01, 1.04 [2 × 3 H, 2 × d, *J* 7.0, 3′-O–CH–(CH_3)$_2$], 1.13-1.17 [12 H, m, 2′-O–CH–(CH_3)$_2$, NH–CH–(CH_3)$_2$], 2.50 [1 H, sept., *J* 7.0, 3′-O–C*H*–(CH_3)$_2$], 2.64 [1 H, sept., *J* 7.0, 2′-O–C*H*–(CH_3)$_2$], 2.79 [1 H, sept., *J* 6.8, NH–(C=O)–C*H*–(CH_3)$_2$], 3.32-3.72 (m, 20 H, ether-*H*), 3.57-3.62 (2 H, m, *H*-5′), 3.67 (3 H, s, O–CH_3), 4.05–4.10, 4.31–4.35 (2 × 2 H, 2 × m, ether-*H*), 4.51-4.53 (1 H, m, *H*-4′), 5.51-5.54 (1 H, m, *H*-3′), 5.83–5.87 (1 H, m, *H*-2′), 6.10 (1 H, d, *J* 6.8, *H*-1′), and 8.22 (1 H, s, *H*-8); δ_C(400 MHz; DMSO-d_6; Me_4Si) 18.3–18.7 (6 × isobutyryl-CH_3), 32.7 [3′-O–*C*H–(CH_3)$_2$], 33.0 [2′-O–*C*H–(CH_3)$_2$], 34.7 [NH–*C*H–(CH_3)$_2$], 54.1 (P–O–CH_3), 60.0, 66.6, 69.5 (ether-*C*), 71.9 (*C*-2′), 70.2 (*C*-3′), 80.9 (*C*-4′), 84.6 (*C*-1′), 120.4 (*C*-5), 137.6 (*C*-8), 148.2, 148.5 (*C*-2, *C*-4), 154.6 (*C*-6) 174.6, 174.8 [O–(*C*=O)], and 180.0 [N–(*C*=O)]; MALDI$^+$-MS: calculated for $C_{35}H_{58}N_5O_{17}P$ [MH^+]: 852.8; found: 852.9.

Initiator 2

13 (0.5 mmol) was reacted with (methyl-*N*,*N*-diisopropyl)-chlorophosphoramidite to yield the respective methyl phosphoramidite **14** as described in the ESI†. 0.23 mmol of **15** were coupled to **14** in the same way as described for the coupling of **21** and mono-DMT-hexaethylene glycol. The resulting coupling product **22** was purified chromatographically ($CHCl_3$–MeOH = 95 : 5; $R_f = 0.4$) and gave 0.19 mmol of **22** (83%); MALDI$^+$-MS: calculated for $C_{65}H_{105}N_{11}O_{29}P_2$ [MH^+]: 1567.5; found: 1566.6. **22** (1.0 g, 63 μmol) was treated with 30% CF_3COOH in CH_2Cl_2 for 20 min. The mixture was diluted with 50 mL CH_2Cl_2, neutralised with saturated $NaHCO_3$ solution (50 mL), and washed with brine (50 mL). The aqueous solution was extracted with CH_2Cl_2 (3 × 50 mL). The organic solvent was removed and the crude product **23** was used in the following steps without further purification. A solution of 14 mg biotin (55 μmol), 17 mg TSTU (55 μmol), and 28 μL (20 mg, 160 μmol) diisopropylethylamine in a mixture of 0.5 mL dioxane–DMF = 1 : 1 was stirred for 10 min and added to **23**. The mixture was stirred overnight at room temperature, and worked up as described for **10**. Chromatographic purification was performed with $CHCl_3$–MeOH = 9 : 1. Compound **24** was obtained as a colourless oil (48 mg, 28 μmol, 52%); δ_H(600 MHz; DMSO-d_6; Me_4Si) 1.01, 1.05, 1.15 [30 H, 3 × m, 5 × –CH–(CH_3)$_2$], 1.31 (2 H, m, biotin-*H*-4′), 1.50, 1.62 (4 H, m, biotin-*H*-3′, biotin-*H*-5′), 2.07 (2 H, m, biotin-*H*-2′), 2.52–2.67 [5 H, 3 × m, –C*H*–(CH_3)$_2$], 2.74 (1 H, m, biotin-*H*-6x), 2.90 (1 H, m, biotin-*H*-6y), 3.10 (1 H, m, biotin-*H*-4), 3.19, 3.40 (4 H, 2 × m, ether-*H*), 3.48–3.67 (6 H, m, P–O–CH_3; 28 H, m, ether-*H*), 4.06, 4.13, 4.31 (10 H, 3 × m, 1 biotin-*H*-6a, 1 biotin-*H*-3a, 4 ether-*H*, 2 guanosine-*H*-5′, 2 adenosine-*H*-5′), 4.41 (2 H, m, guanosine-*H*-4′, adenosine-*H*-4′), 5.53 (1 H, m, guanosine-*H*-3′), 5.67 (1 H, m, adenosine-*H*-3′), 5.85 (1 H, m, guanosine-*H*-2′), 6.00 (1 H, m, adenosine-*H*-2′), 6.10 (1 H, d, *J* 6.8, guanosine-*H*-1′), 6.23 (1 H, d, *J* 5.5, adenosine-*H*-1′), 6.33 (1 H, s, biotin-N*H*-3), 6.39 (1 H, s, biotin-N*H*-1), 8.23 (1 H, s, adenosine-*H*-2), 8.26 (1 H, s, guanosine-*H*-8), and 8.36 (1 H, s, adenosine-*H*-8); δ_C(600 MHz; DMSO-d_6; Me_4Si) 18.9, 19.0, 19.1, 19.3 (6 × isobutyryl-CH_3), 25.4, 28.6, 28.7, 31.1, 31.2, 33.4, 33.6 [6 × (C=O)–*C*H–(CH_3)$_2$], 35.3, 35.6, 36.2, 38.7, 38.9, 54.4, 54.6, 55.4, 55.9, 59.7, 61.5, 66.4, 66.7, 66.9, 67.0, 67.1, 67.2, 67.3, 69.6, 69.7, 70.0, 70.2, 70.5, 70.7 (ether, *C*-3′, *C*-5′, biotin), 72.5, 72.6 (2 × *C*-2′), 80.8, 81.5 (2 × *C*-4′), 85.1, 86.1 (2 × *C*-1′), 120, 121 (2 × *C*-5), 138.2, 140.0 (*C*-8), 148.8, 149.1 (*C*-2, *C*-4), 155.1, 155.2 (*C*-6), 162.8, 163.3 (biotin-*C*-10), 172.6 (biotin-*C*-1), 175.2 [isobutyryl-O–(*C*=O)], 175.4 [isobutyryl-O–(*C*=O)], and 180.6 [isobutyryl-N-(*C*=O)]. For the deprotection of **24**, 200 mg of disodium-2-carbamoyl-2-cyanoethylen-1,1-dithiolate was dissolved in 2 mL of dry DMF and added to 48 mg (28 μmol) of **24**. The solution was stirred for 10 min and the solvent removed *in vacuo*. The residue was dissolved in 5 mL 32% NH_4OH–CH_3NH_2 = 1 : 1 (v/v) and stirred at 50 °C for 90 min. The solvent was removed *in vacuo*, and the residue dissolved in water and purified by HPLC. A reverse phase column was used (Knauer, Nucleosil-120, C_{18}, 10 μm; buffers used were (A) 0.1 M triethylammonium acetate, pH 7.0 and (B) 0.1 M triethylammonium acetate, pH 7.0, 50% acetonitrile; flow rate 3 mL min^{-1}; gradient: 0% B for 8 min 30 s, linear augmentation to 100% B over 24 min, 100% for 9 min, 0% for 6 min). The retention time was 32 min. The fractions containing **2** were pooled, the solvent was removed, the residue was dissolved in 1 mL of water, filtrated (0.2 μm) and the yield was determined by UV ($\varepsilon = 27\,100\ cm^{-1}M^{-1}$) to be 9.2 μmol; δ_H(600 MHz; DMSO-d_6; Me_4Si) 1.35 (2 H, m, biotin-*H*-4′), 1.56 (4 H, m, biotin-*H*-3′, biotin-*H*-5′), 2.13 (2 H, m, biotin-H-2′), 2.63,

2.66 (2 H, 2 × m, biotin-*H*-6x, -*H*-6y), 3.15 (1 H, m, biotin-*H*-4), 3.24 (2 H, m, ether-*H*), 3.45, 3.54, 3.67 (30 H, m, ether-*H*), 3.82 (4 H, m, ether-*H*), 3.87-3.99 (4 H, m, guanosine-*H*-5′, adenosine-*H*-5′), 4.05, 4.11 (2 × 1 H, 2 × m, adenosine-*H*-4′, guanosine-*H*-4′), 4.21 (2 H, m, adenosine-*H*-3′, biotin-*H*-3a), 4.26 (1 H, m, guanosine-*H*-3′), 4.36 (1 H, m, biotin-*H*-6a), 4.67, 4.56 (2 × 1 H, 2 × m, guanosine/adenosine-*H*-2′), 5.77 (1 H, d, *J* 9.1, guanosine-*H*-1′), 6.00 (1 H, d, *J* 8.8, adenosine-*H*-1′), 6.42 (1 H, s, biotin-N*H*-3), 6.47 (1 H, s, biotin-N*H*-1), 7.98, 8.29, and 8.50 (3 H, 3 × s, adenosine-*H*-8 and -*H*-2, guanosine-*H*-8); MALDI$^+$-MS: calculated for $C_{48}H_{77}N_{13}O_{24}P_2S$ [MH$^+$]: 1315.2; found: 1315.0.

Transcription priming reactions

HHR6 (5′-GGG CAG CUG AUG AGC UCC AAA UAG AGC GAA AGU UAC ACC-3′) and HPWTL (5′-G GGA GAA AGA GAG AAG UGA ACC AGA GAA ACA CAC GUU GUG GUA UAU UAC CUG GUA-3′) were labelled with initiator molecules **1** or **2** by *in vitro* transcription from a double-stranded DNA template. The DNA templates were obtained from two overlapping synthetic DNA primers (Purimex) by enzymatic strand extension using Klenow Fragment exo$^-$ (MBI Fermentas).[25] In a standard transcription reaction (50 µl), the concentrations of the components were as follows: 2 mM NTPs, 50 pmol ds DNA, 1 × HEPES, 5 U µl^{-1} T7 RNA polymerase. Initiator concentrations (**1** or **2**) depended on the specific reaction and varied between 0 and 8 mM. Reaction components were mixed and incubated for 2 h at 37 °C. The mixture was subjected to a phenol–chloroform extraction, the transcription product obtained was precipitated with ethanol, purified by denaturing polyacrylamide gel electrophoresis (10%), visualised by UV-shadow, excised, eluted with 0.5 M LiOAc, and precipitated with acetone. Determination of yield was accomplished by UV measurement.

Blotting of biotin-containing samples

The biotinylated RNA probe was purified on a denaturing polyacrylamide gel (10%, 8 × 10 cm) and transferred onto a same-sized nylon membrane by electrophoresis. The membrane was heated to 75 °C for 5 min and washed using the following solutions: washing buffer (WP): 130 mM NaCl, 3 mM KCl, 12 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.5% SDS, pH 7.4; blocking buffer (BP): same as washing buffer, plus 2 g l^{-1} coffee whitener; assay buffer (AP): 0.1 M Tris, 0.1 M NaCl, pH 9.5. The membrane was covered with the respective buffer solution in a container and moved gently. The protocol used was as follows: 2 × WP 5 min; 2 × BP 5 min, 1 × BP 30 min, 1 × BP 30 min + 1 µL streptavidin-alkaline phosphatase (Promega); 1 × 15 min BP, 3 × 10 min WP, 2 × 2 min AP. After further washing steps, the membrane was incubated with a 0.25 M solution of 2-chloro-5-{5′-chloro-4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo(3.3.1.1^{3,7})decan]-4-yl} dihydrogenphosphate ("CDP-Star", Roche). For qualitative detection, chemiluminescence was detected with an X-ray film. For quantitative detection, chemiluminescence was detected in a photo-system (Vilber Lourmat Darkroom-CN-3000) and integrated using the program "Image J".

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