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Photocleavable Initiator Nucleotide Substrates for an Aldolase Ribozyme

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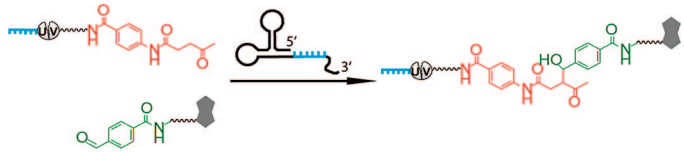

We have previously reported the in vitro selection of a ribozyme that catalyzes an aldol reaction between a levulinic amide aldol donor and a benzaldehyde substrate. The selection scheme involved the priming of the RNA library with a levulinic amide aldol donor group that was introduced via transcription priming in the presence of a modified guanosine mononucleotide derivative. Here we provide a detailed description of the synthesis of the ribozyme substrates and the substrate oligonucleotides used for its isolation and characterization. The aldol donor group was attached to the phosphate moiety of guanosine monophosphate via a photocleavable linker molecule. This initiator nucleotide was efficiently incorporated into RNA molecules of differing sizes and composition by transcription priming with T7 RNA polymerase. With this method modified RNA oligonucleotides as small as a 6-mer sequence can be generated. A temperature profile of the intermolecular reaction indicates that the modified RNA hexamer binds the ribozyme largely by Watson−Crick pairing and only to a minor extent via the non-RNA moiety, whereas the ribozyme appears to have evolved a specific binding site for the aldehyde substrate.

Introduction

An impressive number of new oligonucleotide-based catalysts for a variety of chemical reactions have been isolated from complex oligonucleotide libraries by in vitro selection. Ribozymes, deoxyribozymes, and chemically modified oligonucleotide catalysts have been described[1–19] and have demonstrated the versatility of nucleic acids in promoting diverse chemical transformations of biological relevance. The ability to isolate and evolve complex ribozymes in the laboratory increases the plausibility of the RNA world hypothesis—a theory which proposes that during early evolution life forms based on ribonucleic acids predated the current DNA/RNA/protein-based organisms. The plethora of nucleic acid-based catalysts show that, in principle, multiple metabolic reactions were possible, allowing the generation of the components necessary for replication from simpler building blocks.[20–22]

Unlike the isolation of catalytic antibodies, which are usually obtained by immunization with a chemically accessible transition state analogue,[23] nucleic acid-based catalysts are isolated entirely in vitro by direct screening for catalytic activity.[24–28] For example, direct selections allow for the screening of oligo-

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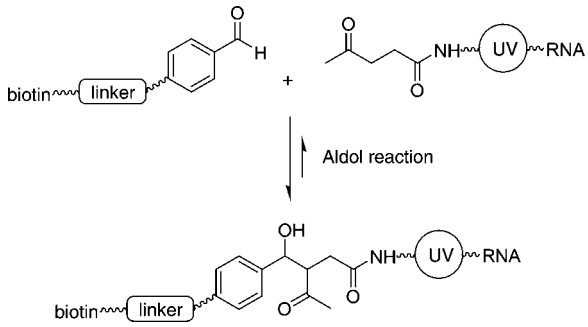
SCHEME 1. Aldol Reaction of a Biotinylated Aldehyde with a Levulinic Amide As Aldol Donor, Coupled to the Ribozyme via a Linker and a Photocleavable Group (UV)



nucleotide catalysts that catalyze reactions between two substrates. This requires covalent attachment of one of the reactants to every individual member of the starting pool, while the other one needs to carry an affinity tag that modifies only those members of the library which are catalytically active. This tag can then be used for immobilization of these species, allowing the application of stringent conditions to remove inactive library members. The attachment of functional groups to a nucleic acid via a cleavable linker such as disulfide bridges[8,29] or photosensitive groups[12] allows for the specific recovery of the desired immobilized active sequences. Alternatively, Pfander et al. have recently shown that reversible site-specific tagging of enzymatically synthesized RNAs using aldehyde−hydrazine chemistry and protease-cleavable linkers is, in principle, a strategy for reversibly introducing chemical groups into RNA molecules.[30]

We recently described the isolation of a ribozyme that catalyzes an aldol reaction of a biotinylated aldehyde with a levulinic amide aldol donor, coupled to the ribozyme via a photocleavable linker[31](Scheme 1; see also ref 33 for a commentary). In an RNA world, C−C bond-forming reactions that occurred in an aldol reaction are thought to have been the main source for the synthesis of ribose via a cross-aldol reaction between simple precursors such as glycolaldehyde and glyceraldehydes.[20,32–34]

In the selection procedure shown in Scheme 2, the levulinic amide moiety was covalently attached to the 5′-end of each RNA molecule contained in the RNA library. The 5′-end modification of the RNA was achieved co-transcriptionally by transcription priming[35] using T7 RNA polymerase and the initiator nucleotide **1**. In this way, ribozymes that catalyze the aldol reaction between the biotinylated aldehyde **2** are modified with the biotin tag that can be used to separate the active species from inactive ones via streptavidin chromatography. The presence of the photocleavable group immediately at the 5′-phosphate group of the RNA not only allows for the convenient elution of streptavidin-bound active RNAs after UV irradiation at 366 nm but also facilitates the release of the reaction product from the RNA catalyst for analytical purposes and characterization.[36]

We report here a versatile and concise synthetic strategy for the preparation of the initiator nucleotide **1** with the levulinic amide substrate and the photocleavable linker separated by a triethylene glycol spacer, and a series of the biotinylated aldehyde **2** and substrate derivatives used for determining the substrate specificity of the ribozyme (Scheme 3). We also report studies on the efficiency of incorporation of **1** into the 5′-position of the RNA library used previously for the selection of the aldolase ribozyme, as a function of nucleotide triphosphate concentrations, and the efficiency of the removal of the 5′-end modification by photocleavage at 366 nm. Finally, a temperature profile of the catalyzed intermolecular reaction between a **1**-modified RNA hexamer substrate and **2** is reported.

Results and Discussion

The synthesis of the multifunctional compound **1** starts from the photosensitive precursor **3**, which was obtained from 4-ethyl benzoic acid after nitration, esterification with MeOH, and subsequent bromination at the benzylic position in an adapted method of Barany and Albericio.[37] Hydrolysis of **3** with aqueous KOH in acetone yielded 4-(1-hydroxyethyl)-3-nitrobenzoic acid **4**. The carboxylic acid derivative **4** was activated with *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and coupled to the tetraethylene glycol spacer **5**. The latter was obtained in analogy to known procedures,[38] starting from tetraethylene glycol by two-fold tosylation, substitution with azide, and subsequent partial reduction under Staudinger conditions. The coupled azide intermediate was converted to the corresponding primary amine in a Staudinger reduction, and the photosensitive compound **6** was obtained in 36% overall yield starting from **3**.

Since 4-amino benzoic acid **7** could not be coupled to EDC·HCl activated levulinic acid, 4-(4-oxopentanoylamino)benzoic acid **8** was prepared by a ring-opening reaction from 5-methyl-3*H*-furan-2-one (Angelica lactone) **9** and **7** under solvent-free conditions in 61% yield and high purity after two recrystallization steps. Subsequently, benzoic acid derivative **8** was coupled with amine **6** in an EDC·HCl-mediated reaction at ambient temperature, and benzamide **10** was isolated in 40% yield.

The alcohol **7** was converted to the corresponding phosphoramidite **11** using 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite **12** in the presence of Hünig's base. Coupling of phosphoramidite **11** to the 5′-OH group of the "transiently protected"[39,40] guanosine nucleoside **13** was performed under standard DNA coupling conditions, using tetrazole as activator and I_2 to oxidze the intermediate phospite to the phosphate ester. After removal of the protecting groups under first strongly basic conditions followed by aqueous trifluoroacetic acid, the crude was purified by RP-MPLC and the desired aldol donor derivatized guanosine initiator nucleotide **1** was isolated in 16% yield starting from **11**.

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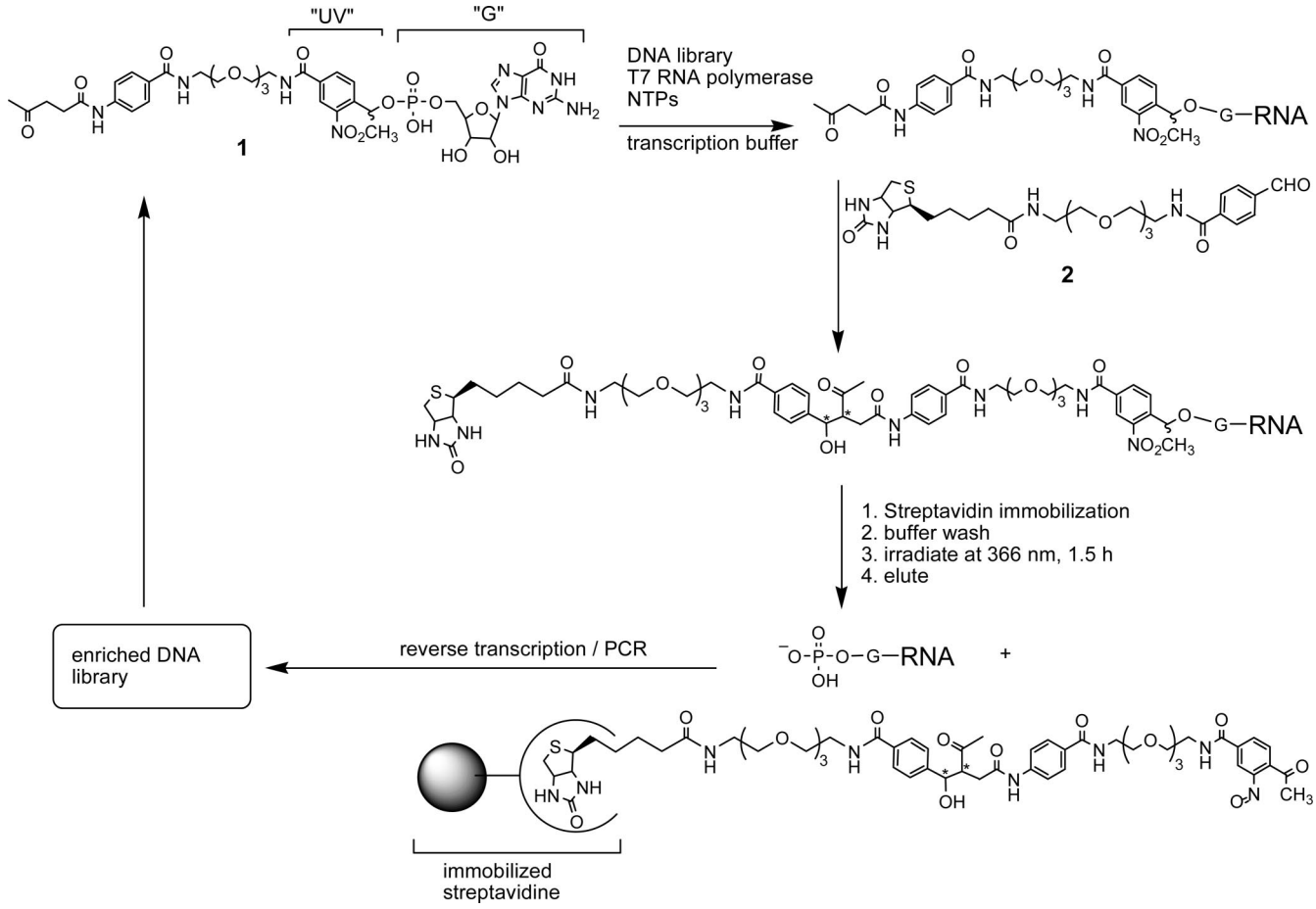
SCHEME 2. Schematic for the In Vitro Selection of Ribozymes That Catalyze an Aldol Reaction[a]



[a] NTPs: Nucleotide triphosphates.

Transcription Priming with the Initiator Nucleotide 1. The incorporation of the initiator nucleotide **1** into the 5′-end of RNA was done by transcription priming in vitro, catalyzed by recombinant RNA polymerase from bacteriophage T7 (T7 RNA polymerase). This DNA-dependent polymerase binds specifically to the double-stranded T7 promoter sequence and starts transcription immediately at the promoter region, by using ribonucleotide triphosphates (NTPs) and the DNA template.[41] The transcription initiation step occurs with less precision than the other steps, rendering this polymerase fairly tolerant toward modifications at the priming guanosine residue.[42] This property has previously been used for the selective incorporation of guanosine monophosphate analogues into RNA molecules at the 5′-end to introduce chemical modifications.[8,19,29,36,43–45]

To quantify the transcription priming efficiency of **1** by polyacrylamide gel electrophoresis (PAGE), we used the previously described 99-mer DNA sequence K61 (Figure 1).[46] This DNA template was chosen because we found that the electrophoretic mobility of the chemically modified 82-mer RNA transcripts of this length differed sufficiently from that of the unmodified RNA to allow quantification by phosphorimaging (Figure 1a, left panels). The sequence used for the in vitro selection of the aldolase ribozyme was a 194-mer RNA containing two randomized regions of 70 and 72 nucleotides, respectively, separated by two 6-mer restriction endonuclease cleavage sites (Figure 1b).[31] In parallel, we also determined the efficiency of UV cleavage of the photocleavable linker group (Figure 1a, right panels).

The left panel of Figure 1a shows the results of the transcription reaction with the K61 DNA template in the presence of varying amounts of GTP and the modified guanosine monophosphate **1**, separated by denaturing PAGE. The T7 RNA polymerase generates RNA 82-mer transcripts in a standard transcription reaction (no modified guanosine **1** added) that exhibits a high electrophoretic mobility ("GTP-primed"). As the concentration of **1** was increased, a new band corresponding to aldol donor modified 82-mer RNA appeared that showed decreased mobility in the electric field ("**1**-primed"). We also found that the overall yield of transcribed RNAs was higher at a concentration of 3.5 mM of the remaining NTPs (ATP, CTP, UTP) as compared to 5.0 mM NTP concentrations. Maximum amounts of modified RNA transcripts, as compared to total amounts of RNA generated, were obtained when the ratio of GTP:**1** was 1:4 at 5.0 mM NTPs and 1:6 at 3.5 mM NTPs, respectively. We did not observe a significant inhibitory effect

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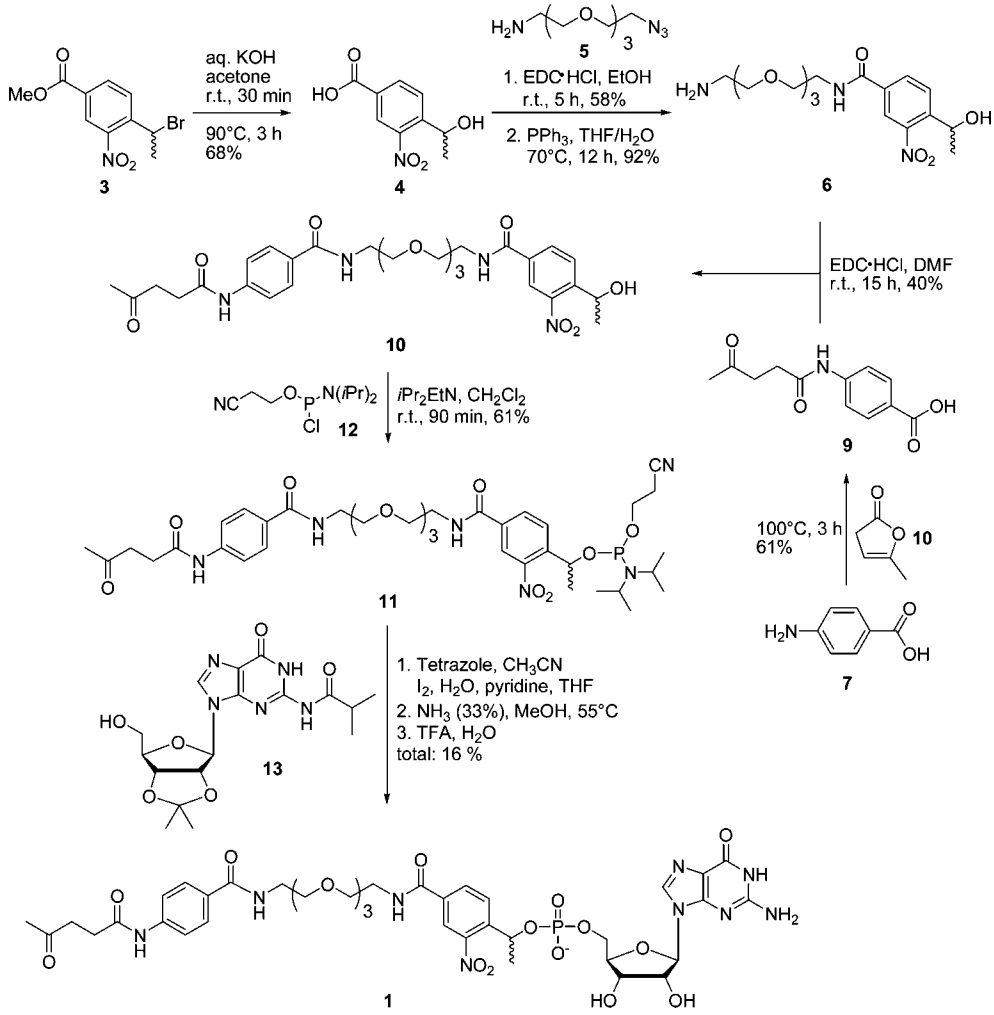
SCHEME 3. Synthesis of the Initiator Nucleotide 1



by **1** on the T7 polymerase activity, as seen occasionally for other initiator nucleotides.[36]

We then analyzed the efficiency of transcription priming using the DNA library that was also used for the actual ribozyme selection experiment. This DNA is 220 nucleotides long and leads to a 194-mer RNA transcript.[4,12] The transcription reactions were performed at 3.5 mM NTP concentrations with varying ratios of GTP:**1**. As shown in Figure 1b, the bands corresponding to modified and unmodified RNA do not separate well under denaturing PAGE conditions due to the increased length of the resulting transcripts. When the ratio of GTP:**1** was 4:1, approximately equal amounts of modified and unmodified RNA are obtained. At a ratio of GTP:**1** of 3:2, however, the main product was the modified RNA, but the total amount of RNA transcripts were clearly reduced. This indicates that the efficiency of transcription priming depends on the nature and length of the resulting RNA to some degree, at least for the initiator nucleotide studied here.

The right panel of Figure 1a shows that UV irradiation (366 nm, 1.5 h) allows successful cleavage of the aldol donor from the modified RNAs. The cleavage efficiency of the photocleavable linker of the initiator nucleotide was greater than 90% under these conditions, which can be considered to be quantitative. This experiment also shows that the RNA molecules did not degrade under these cleavage conditions. The same in vitro transcription conditions as applied for the generation of the **1**-primed RNA library were also used to synthesize a hexameric RNA substrate **1**−GGAGA-3′, modified with **1** at its 5′-end (Figure 2a), which was used as a substrate for the selected ribozyme. This short RNA was generated by transcription priming using the two synthetic DNA oligonucleotides D2T.1-6:5′ and D2T.1-6:3′, which spontaneously assembled to give the T7 promoter-containing DNA double strand required for T7 polymerase transcription initiation. As shown in Figure 2b, the in vitro transcription performed under these conditions led to a similar ratio of primed with **1** versus GTP-primed RNA as was also observed in case of the longer DNA templates K61 and the DNA library. The ribozyme used to catalyze the reaction between this modified RNA hexamer (Figure 2) was truncated by the same six nucleotides at its 5′-end to allow the formation of a catalytically active complex between the truncated ribozyme and the hexameric RNA substrate.[31] We refer to this ribozyme as $11DA_{7\text{-}194}$.

We have previously found that further truncation of $11DA_{7\text{-}194}$ by 20 nucleotides at the 3′-end led to a considerable improvement of the rate of catalysis by this variant which was designated as $11DA_{7\text{-}174}$.[31] A possible explanation for this effect may be that the additional 20 nucleotides result in conformational instability of the ribozyme in the context of the 5′-deletion by six nucleotides, due to mis-hybridization. This might result in a smaller fraction of the RNA folding into the correct conformation required for catalysis.

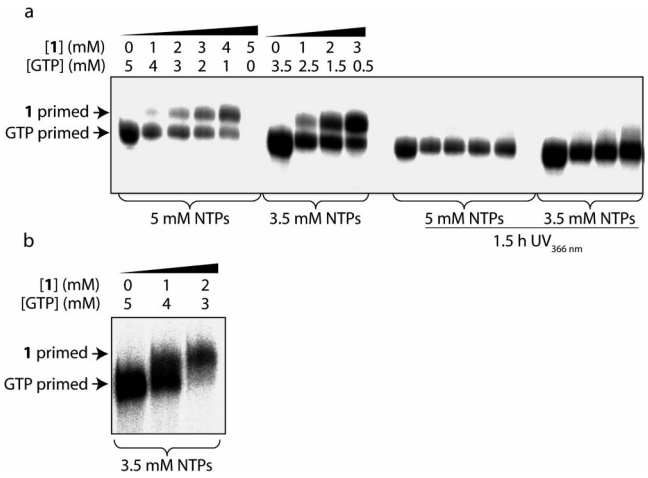


FIGURE 1. Enzymatic incorporation of the initiator nucleotide **1** by transcription priming using two different DNA templates. (a) Dependence of transcription priming of K61 RNA with **1** on the ratio of **1**:GTP in the presence of 5 mM of the NTPs ATP, CTP, and UTP and 3.5 mM of these NTPs (left panels). Right panels: Efficiency of cleavage of the primed RNAs after UV irradiation at 366 nm for 1.5 h. (b) Dependence of transcription priming of 196-mer randomized RNA with **1** on the ratio of **1**:GTP in the presence of 3.5 mM of the NTPs ATP, CTP, and UTP. In (a) and (b), the upper bands correspond to the RNA with incorporated **1**, whereas the lower bands represent GTP-primed RNAs.

In the final transcription reactions, we used the various optimized combinations of **1**, GTP, at 3.5 mM of the remaining nucleotide triphosphates, for the different DNA templates described herein. These combinations are summarized in Table 1.

TABLE 1. Ratios of 1 and GTP Used for Transcription Priming of Different DNA Templates

DNA template	concentration[a] (mM) **1**	GTP
K61	3.0	0.5
Pool DNA	2.0	1.5
D2T.1−6:5′/:3′	2.0	1.5

[a] Concentration of the remaining NTP is 3.5 mM each.

Synthesis of Biotinylated Aldehyde Substrates. The straightforward synthetic strategy of the biotinylated aminobenzaldehyde derivative **2**, in which the biotin group was separated from the aminobenzaldehyde moiety by a tetraethylene glycol group, is shown in Scheme 4. Compound **2** was used as substrate for the in vitro selection of RNA $11DA_{1\text{-}194}$, the aldolase ribozyme.[31] The tetraethylene glycol spacer was chosen not only to position the biotin moiety distant from the reactive aldehyde group but also to increase the water solubility of the aldol acceptor benzaldehyde derivative.

We first started by coupling biotin **14** to the monoaminotetra(ethylene glycol)azide **5**, the same spacer group that was also used in the synthesis of the initiator nucleotide **1**, in the presence of EDC·HCl. The azide intermediate was then converted into the amino group by a Staudinger reduction using triphenyl phosphine in THF to give **15** in 88% yield.

The question at this point was whether to perform the coupling of 4-formylbenzoic acid to the primary amine **15** without protecting the formyl group to avoid the formation of a Schiff base with the primary amine in **15** or whether to perform

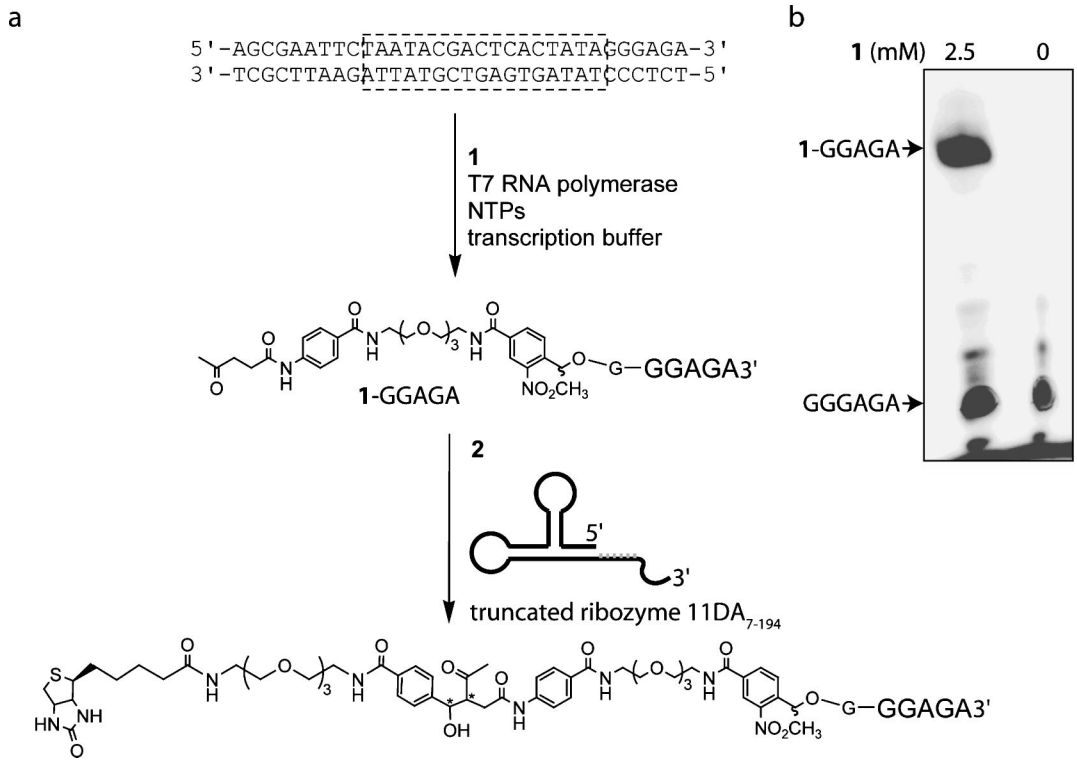
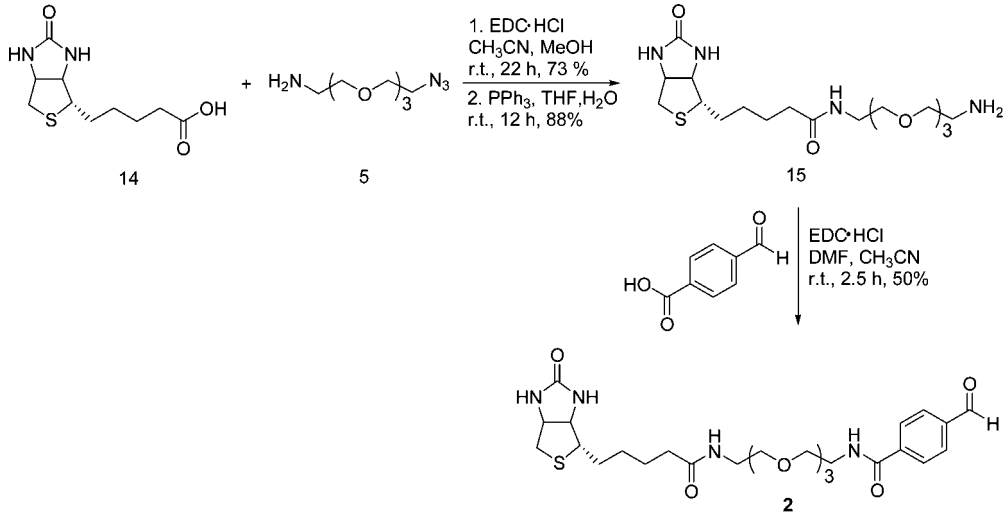


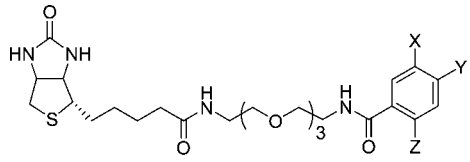
FIGURE 2. Ribozyme catalysis of the aldol reaction using the free substrates **2** and the **1**-primed RNA hexamer substrate. (a) The hexameric RNA−substrate oligonucleotide modified with the initiator nucleotide **1** is obtained by transcription priming of the double-stranded DNA (top) in presence of **1**, NTPs, and T7 RNA polymerase in transcription buffer. The dashed frame in the DNA sequence represents the T7 promotor sequence. The resulting **1**-primed RNA hexamer substrate **1**−GGAGA is converted to the product of the aldol reaction in the presence of **2**, catalyzed by the truncated ribozyme $11DA_{7\text{-}194}$. (b) Autoradiography of a 20% polyacrylamide gel of the transcription reaction shown in (a). The upper band corresponds to the **1**-primed RNA hexamer substrate **1**−GGAGA, the main lower band to the unmodified hexamer GGAGA. The identity of both RNA hexamers was verified by ESI-MS (see Experimental Section).

SCHEME 4. Synthesis of Biotinlyated Aldehyde 2



14 5 15 2

SCHEME 5. Derivatives Used to Investigate the Substrate-Specificity of the Aldolase Ribozyme



16: X = CHO, Y, Z = H
17: X, Z = H, Y = CH_2-OH
18: X = CHO, Y = H, Z = OH

the reaction with the free aldehyde. We decided to try this step by using the unprotected aldehyde to circumvent the protection and deprotection steps. The EDC·HCl-mediated coupling was performed in DMF. After removal of the solvent in vacuo, the crude was purified first on silica gel and then by preparative RP-MPLC, which afforded compound **2** in 50% yield. This procedure, however, is only advantageous if the synthesis scale is below 1 g. For preparations of larger amounts of **2**, the use of a protected aldehyde is recommended. The same procedure was also used to synthesize derivatives **16**−**18** that differ from **2** in their substitution pattern (Scheme 5). These compounds were used to investigate the substrate specificity of the aldolase ribozyme.[31] However, we found the yields of **16**−**18** under these conditions to be considerably lower compared to those of the synthesis of **2**.

While the alcohol **17** was used as a negative control which, as expected, did not result in the formation of any product, we found that, among the aldehyde substrates that we tested in a reaction catalyzed by $11DA_{7\text{-}174}$ and the hexameric **1**-primed RNA substrate, only **2** and, to a slightly lesser extent, **16** reacted within 5 h to form the corresponding aldol products.[31] Under otherwise identical reaction conditions, the catalyzed aldol reaction using **16** as a substrate gave 77% of the corresponding product obtained with **2**. When using compound **18**, which contains an additional OH group ortho to carboxamide (Z) and the aldehyde group in Y, no product was formed under these conditions. This points to a certain specificity of $11DA_{7\text{-}174}$ for the substrate (**2**) that was used during the in vitro selection (Scheme 2). It is possible that the ribozyme forms a binding site that optimally evolved for the recognition of **2** and exhibits less tolerance for slight modifications such as moving the aldehyde functionality from the *para*-position in **2** to the *meta*-position in **16**. Introduction of additional substituents into **16**, such as the *ortho*-OH group, is excluded either for steric or electronic reasons.

Temperature Dependence of the Ribozyme $11DA_{7\text{-}174}$. The ribozyme $11DA_{2\text{-}174}$, which was truncated only by the 5′-G nucleotide, did not catalyze the aldol reaction between **2** and **1**, which had prompted us to perform the reaction in which the ribozyme acts as a true catalyst on two external substrates with **2** and the RNA hexamer 5′-GGGAGA-3′, primed with the initiator nucleotide **1**.[31]

Due to the short size of the **1**-primed RNA hexamer substrate which exhibits a theoretical melting temperature of only 18 °C, the reaction catalyzed by $11DA_{7\text{-}174}$ should strongly depend on the temperature. We found that the full-length ribozyme $11DA_{1\text{-}174}$ to which **1** was covalently attached performed best within a temperature range between 35 and 40 °C, lost 60% of its activity at 45 °C and was completely inactive at 50 °C (data not shown). We determined the initial reaction rate v_0 at 5 mM of **2**, using $11DA_{7\text{-}174}$ and the **1**-primed RNA hexamer substrate at various temperatures and found a temperature optimum between 20 and 25 °C (Figure 3). This temperature range is only slightly higher than the theoretical melting temperature for Watson−Crick hybridization of the hexamer RNA, which might indicate that the RNA substrate recognizes the ribozyme largely by Watson−Crick pairing and only to a minor extent by forming a binding pocket that recognizes the non-RNA moiety of this substrate. This temperature profile would also explain the lack of catalysis of $11DA_{2\text{-}174}$ observed when **1** and **2** were used as substrates.

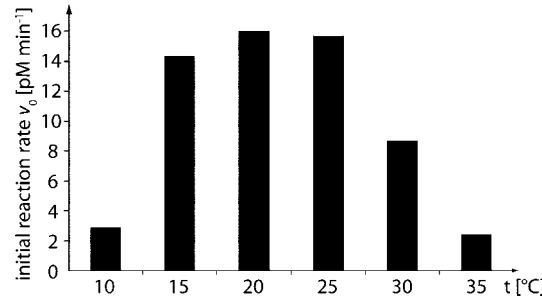


FIGURE 3. Temperature profile of the reaction between **2** and the **1**-primed RNA hexamer substrate oligonucleotide, catalyzed by $11DA_{7\text{-}174}$.

Conclusion

The incorporation of chemically modified guanosine monophosphate initiator nucleotides at the 5′-ends of RNA transcripts by T7 RNA polymerase priming works efficiently for RNAs of different lengths, complex RNA libraries, and even for very short transcripts as small as the hexameric RNA sequence GGGAGA used in this study. We have previously reported the selection of a ribozyme that catalyzes an aldol reaction as an example for an RNA catalyst for a complex organic reaction with relevance for both cellular metabolism and hypothetical RNA-based metabolism in the RNA world.[31] The designed selection scheme utilized a photoinduced ribozyme isolation procedure; this method proved to be useful in the isolation of other ribozymes as it allows for the separation of active RNA molecules from the mixture in a mild and specific manner. We describe here the design and synthesis of the molecules required for the in vitro selection experiment and for the characterization of the isolated ribozyme and its derivatives. Our study shows that small molecule functionalities attached to the phosphate of guanosine monophosphate via a photocleavable linker moiety can be conveniently attached to RNA molecules of a broad size range via transcription priming using T7 RNA polymerase. The preparation of the modified RNA hexamer by runoff transcription is a particularly useful achievement and provides a highly valuable means for the characterization of the aldolase ribozyme and its conversion into a true enzyme. The molecules and methods described here promise to be a generally applicable procedure for the generation and characterization of other RNA molecules that can catalyze complex organic reactions.

Experimental Section

Materials. Commercial grade reagents and solvents were used without further purification, except as indicated below. Dichloromethane was distilled from calcium hydride. Tetrahydrofuran was distilled from sodium and benzophenone under nitrogen. Reverse-phase HPLC experiments were conducted with 4.6 × 250 mm (analytical scale) or 21.4 × 250 mm (preparative scale) Rainin C_{18} reverse-phase columns. All reactions were stirred magnetically in flame- or oven-dried glassware under a positive pressure of Ar or N_2.

Syntheses. 4-(1-Hydroxyethyl)-3-nitrobenzoic acid 4. To a solution of 4-(1-bromoethyl)-3-nitrobenzoic acid methyl ester **3** (5.01 g, 17.4 mmol, 1.0 equiv) in acetone (100 mL) and H_2O (25 mL) was added KOH (2.92 g, 52.1 mmol, 3.0 equiv) dissolved in H_2O (80 mL) dropwise. The reaction mixture was allowed to stir at rt for 30 min, and during this time, the pale yellow solution turned brown. The reaction mixture was stirred at 90 °C for 3 h, and the volatile solvent was evaporated. The reaction mixture was washed with ether (2 × 100 mL) and was acidified by adding 2 N HCl to pH 2. The reaction mixture was extracted with ethyl acetate (3 × 150 mL), dried over sodium sulfate, and evaporated. The crude product was purified by flash chromatography (CH_2Cl_2:MeOH: acetic acid, 95:5:0.5 v/v/v) to afford the product as yellow solid (2.50 g, 68%): TLC (CH_2Cl_2:MeOH:acetic acid, 95:5:0.5 v/v/v) R_f = 0.38; ^{1}H NMR (400 MHz, CD_3OD) δ 8.41 (d, J = 1.6 Hz, 1H), 8.26 (dd, J = 8.2 Hz, J = 1.6 Hz, 1H), 7.97 (d, J = 8.2 Hz, 1H), 5.35 (q, J = 6.4 Hz, 1H), 1.49 (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 167.5, 149.2, 147.6, 134.9, 132.3, 129.3, 126.3, 66.2, 25.1; FAB (*m*/*z*) calcd 212, found 212.0 $[M + H]^+$; ESI-HRMS $C_9H_9NO_5Na^+$ $[M + Na]^+$ calcd 234.0372, found 234.0360.

***N*-(2-{2-[2-(2-Aminoethoxy)ethoxy]ethoxy}ethyl)-4-(1-hydroxyethyl)-3-nitrobenzamide 6.** A solution of 4-(1-hydroxyethyl)-3-nitrobenzoic acid **4** (1.47 g, 6.96 mmol, 1 equiv), 2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethylamine **5** (1.52 g, 6.96 mmol, 1 equiv), and EDC·HCl (1.61 g, 8.40 mmol, 1.2 equiv) in ethanol (50 mL) was stirred at rt for 5 h. The reaction mixture was concentrated to dryness, and the residue was dissolved in CH_2Cl_2 (200 mL) and was washed with acetic acid (5%, 100 mL), saturated $NaHCO_3$, (150 mL) and brine. The organic extract was dried over magnesium sulfate and evaporated. The residue was purified by silica gel chromatography to afford the azide product as viscous yellow oil (1.65 g, 58%): TLC (CH_2Cl_2:MeOH, 90:10 v/v) R_f = 0.58; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, J = 1.6 Hz, 1H), 7.89 (dd, J = 8.2 Hz, J = 1.6 Hz, 1H), 7.80 (d, J = 8.2 Hz, 1H), 7.33 (t, J = 5.0 Hz, 1H), 5.34 (q, J = 6.4 Hz, 1H), 3.63−3.57 (m, 14H), 3.30 (t, J = 5.0 Hz, 2H), 1.47 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.4, 147.3, 144.8, 134.2, 131.8, 128.2, 122.9, 70.7, 70.6, 70.5, 70.2, 70.0, 69.6, 65.2, 50.6, 40.2, 24.7; FAB (*m*/*z*) $C_{17}H_{26}N_5O_7$ $[M + H]^+$ calcd 412.17, found 412.2.

The azide compound *N*-(2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}-ethyl)-4-(1-hydroxyethyl)-3-nitrobenzamide (0.89 g, 2.15 mmol, 1 equiv), triphenyl phosphine (0.68 g, 2.60 mmol, 1.2 equiv), and water (0.35 mL, 19.4 mmol, 9 equiv) were dissolved in THF (15 mL). The reaction mixture was left to stir at 70 °C for 12 h. Volatile solvents were evaporated, and the residue was purified by silica gel chromatography to afford the product **6** as viscous yellow oil (0.76 g, 92%): TLC (CH_2Cl_2:MeOH:TEA, 80:20:1 v/v/v) R_f = 0.28; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, J = 1.6 Hz, 1H), 8.04 - 8.01 (dd, J = 1.7 Hz, J = 8.0 Hz, 1H) and (s, 1H), 7.83 (d, J = 8.0 Hz, 1H), 5.30 (q, J = 6.5 Hz, 1H), 3.61−3.48 (m, 12H), 3.38 (t, J = 5.1 Hz, 2H), 2.68 (br. t, J = 5 Hz, 2H), 1.47 (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.5, 147.5, 145.1, 134.5, 131.9, 128.3, 123.2, 72.7, 70.6, 70.5, 70.3, 70.1, 69.8, 64.9, 41.3, 40.2, 24.8; FAB (*m*/*z*) calcd 386.19, found 386.2 $[M + H]^+$; ESI-HRMS $C_{17}H_{28}N_3O_7^+$ $[M + H]^+$ calcd 386.1922, found 386.1909.

4-(1-Hydroxyethyl)-3-nitro-*N*-{2-[2-(2-{2-[4-(4-oxopentanoylamino)benzoylamino]ethoxy}ethoxy)ethoxy]ethyl}benzamide 10. A solution of **6** (0.76 g, 1.97 mmol, 1 equiv), benzoic acid derivatized acid **8** (0.51 g, 2.17 mmol, 1.1 equiv), and EDC·HCl (0.45 g, 2.35 mmol, 1.2 equiv) in DMF (15 mL) was stirred at rt for 15 h. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and was washed with dilute HCl (10%, 50 mL), saturated $NaHCO_3$ (100 mL), and brine. The organic extract was dried over magnesium sulfate, the solvent was evaporated, and the residue was purified by silica gel chromatography to afford the product **10** as viscous yellow oil (0.482 g, 40%): TLC (CH_2Cl_2:MeOH, 80:20 v/v) R_f = 0.28; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, J = 1.6 Hz, 1H), 8.18 (br, 1H), 7.94 (dd, J = 8.2 Hz, J = 1.6 Hz, 1H), 7.85 (d, J = 8.2 Hz, 1H), 7.60 (d, J = 8.6 Hz, 2H), 7.45 (d, J = 8.7 Hz, 2H), 6.83 (t, J = 5.2 Hz, 1H), 5.39 (q, J = 6.4 Hz, 1H), 3.66−3.52 (m, 16H), 2.89 (t, J = 6.4 Hz, 2H), 2.63 (t, J = 6.3 Hz, 2H), 2.23 (s, 3H), 1.53 (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.3, 170.9, 167.1, 165.4, 147.5, 145.1, 141.0, 134.6, 131.9, 128.4, 128.2, 123.1, 120.9, 119.4, 70.72, 70.71, 70.4, 70.3, 69.9, 69.8, 65.6, 40.2, 39.9, 38.7, 31.4, 30.1, 24.6; FAB (*m*/*z*) calcd 603.3, found 603.2 $[M + H]^+$; ESI-HRMS $C_{29}H_{38}N_4O_{10}Na^+$ $[M + Na]^+$ calcd 625.2480, found 625.2457.

4-(4-Oxopentanoylamino)benzoic acid (8). 4-Aminobenzoic acid **7** (5.0 g, 36.5 mmol, 1 equiv) was suspended in 5-methyl-3*H*-furan-2-one **9** (13.1 mL, 145.8 mmol, 4 equiv) and stirred for 3 h at 100 °C. The excess lactone was then removed in vacuo, and the residue was recrystallized twice from hot MeOH to afford 5.20 g (22.1 mmol, 61%) of compound **8** as pale yellow crystals: TLC (CH_2Cl_2:MeOH, 90:10 v/v) R_f = 0.39; ^{1}H NMR (300 MHz, DMSO) δ 12.65 (s, 1H), 10.24 (s, 1H), 7.86 (d, J = 8.7 Hz, 2H), 7.67 (d, J = 8.7 Hz, 2H), 2.75 (t, J = 6.6 Hz, 2H), 2.55 (t, J = 6.6 Hz, 2H), 2.10 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 207.3, 170.9, 166.9, 143.3, 130.4, 124.8, 118.1, 37.5, 30.2, 29.7; FAB (*m*/*z*) calcd 236.1, found 236 $[M + H]^+$; ESI-HRMS $C_{12}H_{13}NO_4Na^+$ $[M + Na]^+$ calcd 258.0737, found 258.0743.

Diisopropylphosphoramidous acid 2-cyanoethyl ester 1-(2-nitro-4-{2-[2-(2-{2-[4-(4-oxopentanoylamino)benzoylamino]ethoxy}-ethoxy)ethoxy]ethylcarbamoyl}phenyl)ethyl ester (11). To a sol-

ution of **10** (0.147 g, 0.24 mmol, 1 equiv) in anhydrous CH_2Cl_2 (10 mL) was added *N*,*N*′-diisopropylethylamine (125 μL, 0.73 mmol, 3 equiv) in argon atmosphere. The mixture was cooled to 0 °C, and 2-cyanoethyl diisopropylchlorophosphoramidite **12** (0.116 g, 0.49 mmol, 2 equiv) was added dropwise. The reaction mixture was stirred at rt for an additional 90 min and diluted with ethyl acetate (50 mL). The solution was washed with saturated $NaHCO_3$ (50 mL) and brine and was dried over sodium sulfate. The organic extract was evaporated, and the residue was purified by flash chromatography (CH_2Cl_2:MeOH:TEA, 95:4:1 v/v/v) to afford the product **11** as a white foam (0.120 g, 61%): TLC (CH_2Cl_2:MeOH:TEA, 95:5:1 v/v/v) $R_f = 0.34$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (t, $J = 1.7$ Hz, 1H), 8.04−7.99 (m, 1H), 7.86−7.81 (m, 1H), 7.63 (d, $J = 8.7$ Hz, 2H), 7.47 (dd, $J = 8.7$ Hz, $J = 1.8$ Hz, 2H), 5.51−5.40 (m, 1H), 3.84−3.73 (m, 1H), 3.66−3.54 (m, 17H), 3.48−3.42 (m, 2H), 2.79 (t, $J = 6.4$ Hz, 2H), 2.61 (dd, $J = 6.5$ Hz, $J = 0.6$ Hz, 1H), 2.56 (t, $J = 6.3$ Hz, 2H), 2.45−2.41 (m, 1H), 2.13 (s, 3H), 1.50 (dd, $J = 6.3$ Hz, $J = 1.0$ Hz, 3H), 1.14−1.06 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.94, 169.76, 166.15, 166.02, 164.26, 164.03, 146.10, 145.98, 142.54, 142.52, 142.24, 142.21, 140.18, 133.64, 133.52, 130.95, 103.94, 128.01, 127.94, 126.98, 121.98, 121.86, 118.06, 69.50, 69.24, 69.22, 69.19, 68.70, 68.67, 68.51, 66.53, 66.37, 66.34, 66.17, 57.63, 57.44, 57.35, 57.15, 42.30, 42.17, 39.04, 38.75, 37.32, 29.97, 28.90, 24.08, 24.05, 23.96, 23.92, 23.67, 23.63, 23.60, 23.55, 23.49, 23.42, 23.32, 23.25, 20.02, 19.44, 19.37, 19.31, 19.23; ^{31}P NMR (162 MHz, $CDCl_3$) δ 151.2, 150.3. Molecular formula: $C_{38}H_{55}N_6O_{11}P$.

N^2-Isobutyryl-2′,3′-isopropylidene guanosine (13). A solution of N^2-isobutyrylguanosine[36] (10 g, 28.3 mmol), 2,2′-dimethoxypropane (200 mL, 1.63 mol), and *p*-toluenesulfonic acid monohydrate (0.6 g, 3.15 mmol) in DMF (200 mL) was stirred under a nitrogen atmosphere. After 10 h, the reaction mixture was concentrated to dryness and the residue partitioned between equal volumes (100 mL) of CH_2Cl_2 and saturated aqueous $NaHCO_3$. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (MeOH/CH_2Cl_2, 5:95) to afford protected guanosine **13** (9.95 g, 87%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 12.12$ (s, 1H), 9.94 (s, 1H), 7.90 (s, 1H), 5.86 (d, $J = 3.9$ Hz, 1H), 5.12 (dd, $J = 6.2$, 3.9 Hz, 1H), 5.10−5.08 (m, 1H), 5.00 (dd, $J = 6.2$, 2.3 Hz, 1H), 4.41 (app q, $J = 2.3$ Hz, 1H), 3.92 (d, $J = 12.2$ Hz, 1H), 3.80−3.76 (m, 1H), 2.69 (app sept, $J = 6.9$ Hz, 1H), 1.59 (s, 3H), 1.35 (s, 3H), 1.27 (d, $J = 7.0$ Hz, 3H), 1.25 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 180.1, 155.6, 148.1, 138.7, 129.7, 113.8, 90.5, 86.8, 84.0, 81.1, 62.1, 35.7, 26.9, 24.8, 18.7; IR ($CHCl_3$) 3250−3075, 1695, 1685, 1610 cm^{-1}; ESI-HRMS (*m*/*z*) for $C_{17}H_{24}N_5O_6^+$ $[M + H]^+$, calcd 394.1726, found 394.1724.

Phosphoric acid 5-(2-amino-6-oxo-1,6-dihydropurin-9-yl)-3,4-dihydroxytetrahydrofuran-2-ylmethylester1-(2-nitro-4-{2-[2-(2-{2-[4-(4-oxopentanoylamino)benzoylamino]ethoxy}ethoxy)ethoxy]ethylcarbamoyl}phenyl)ethyl ester (1). To an ice-cooled solution of protected guanosine **13** (0.570 g, 1.45 mmol, 1 equiv) and tetrazole (0.45 M, 16.7 mL, 7.52 mmol, 4 equiv) in CH_3CN (4 mL) was added phosphoramidite **11** (1.512 g, 1.88 mmol, 1.3 equiv) in CH_3CN (4 mL) dropwise. The reaction mixture was stirred at 0 °C for 10 min and then at rt for 45 min. The reaction mixture was again cooled to 0 °C, and iodine (0.367 g, 1.45 mmol, 1 equiv) in a mixture of H_2O/pyridine/THF (2:20:80) was added dropwise and allowed to stir at 0 °C for 10 min and then at rt for 25 min. The reaction mixture was concentrated in vacuum and diluted with ethyl acetate (30 mL) and sodium thiosulfate (0.5 M, 30 mL). Isopropanol was added until the solution was clear, and the organic layer was washed with water. The organic extract was dried over sodium sulfate and evaporated. The residue was flash chromatographed (CH_2Cl_2:MeOH; 90:10 v/v) to yield a pale white foam (0.926 g, 57%): TLC (CH_2Cl_2:MeOH 90:10 v/v) $R_f = 0.27$.

The coupled product (0.90 g) was dissolved in a mixture of concentrated NH_3 (33%, 12 mL) and methanol (1.5 mL) and incubated at 55 °C for 14 h. The reaction mixture was evaporated and washed with ethyl acetate (2 × 25 mL). The acetal deprotection was performed in 50% aqueous TFA (4 mL) for 6 h at rt. The reaction mixture was evaporated and was washed with ethyl acetate (2 × 25 mL). The crude product was then applied to a reverse-phase column and eluted with 100 mM triethylammonium acetate buffer and CH_3CN (10−35%). The collected fractions were lyophilized to yield the product (0.122 g, overall 16%): ^{1}H NMR (300 MHz, D_2O) δ 8.14 (d, $J = 7.7$ Hz, 1H), 8.03 (d, $J = 7.5$ Hz, 1H), 7.82−7.69 (m, 3H), 7.50 (d, $J = 8.3$ Hz, 1H), 7.31−7.25 (m, 2H), 5.81−5.61 (m, 2H), 4.44−4.33 (m, 1H), 4.22−4.13 (m, 1H), 4.09−3.84 (m, 3H), 3.64−3.62 (m, 12H), 3.52−3.48 (m, 4H), 2.86 (t, $J = 6.4$ Hz, 2H), 2.59 (t, $J = 5.7$ Hz, 2H), 2.21 (s, 3H), 1.47 (d, $J = 6.2$ Hz, 3H); ^{13}C NMR (75 MHz, D_2O) δ 216.20, 180.49, 175.90, 172.12, 171.78, 169.68, 169.64, 169.42, 169.37, 161.01, 156.25, 156.16, 148.46, 148.41, 144.93, 144.83, 143.28, 140.41, 136.08, 136.0, 135.81, 134.80, 131.34, 131.24, 131.07, 130.68, 130.51, 125.78, 125.69, 122.07, 94.92, 89.97, 89.67, 85.81, 85.71, 76.39, 76.24, 72.69, 72.63, 72.53, 72.48, 72.30, 72.25, 72.17, 72.12, 71.48, 71.38, 67.85, 67.79, 67.17, 67.13, 42.29, 42.10, 40.44, 36.99, 32.81, 31.79, 28.11, 26.30, 26.23; ^{31}P NMR (121 MHz, D_2O) δ 0.0, −0.18; ESI-TOF (*m*/*z*) calcd 948.3, found 948.29 $[M + H]^+$, 970.29 $[M + Na]^+$, 986.25 $[M + K]^+$; ESI-HRMS $C_{39}H_{50}N_9O_{17}PNa^+$ $[M + Na]^+$ calcd 970.2954, found 970.2947.

5-(2-Oxohexahydrothieno[3,4-*d*]imidazol-4-yl)pentanoic acid (2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethyl)amide (15). A solution of biotin **14** (6.41 g, 26.3 mmol, 1.05 equiv), monoaminotetra(ethylene glycol) azide **5** (5.46 g, 25 mmol, 1 equiv), and EDC·HCl (6.23 g, 32.5 mmol, 1.3 equiv) in CH_3CN/MeOH (3:1, 240 mL) was stirred at rt for 22 h. The reaction mixture was concentrated to dryness, and the residue was purified by silica gel chromatography to afford the biotinylated tetraethylene glycol azide (8.14 g, 73%): TLC (CH_2Cl_2:MeOH, 90:10 v/v) $R_f = 0.41$.

A solution of biotinylated tetraethylene glycol azide (8.14 g, 18.3 mmol, 1 equiv) and triphenyl phosphine (5.42 g, 20.13 mmol, 1.1 equiv) dissolved in THF (70 mL) and H_2O (20 mL) was stirred at rt for 12 h. The solvent was evaporated, and the residue was washed with ether (3 × 150 mL) and CH_2Cl_2 (2 × 100 mL) and dried in vacuum to yield the biotinylated tetraethylene glycol amine **15** as a pale white solid (6.72 g, 88%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22 (t, $J = 5.6$ Hz, 1H), 6.78 (br, 1H), 6.09 (br, 1H), 4.43 (dd, $J = 7.6$ Hz, $J = 4.6$ Hz, 1H), 4.24 (dd, $J = 7.6$ Hz, $J = 4.6$ Hz, 1H), 3.92 (br, 2H), 3.57−3.48 (m, 12H), 3.38−3.32 (m, 2H), 3.10−3.04 (m, 1H), 2.85 (t, $J = 5.1$ Hz, 2H), 2.81 (d, $J = 4.7$ Hz, 1H), 2.68 (d, $J = 12.6$ Hz, 1H), 2.16 (t, $J = 7.4$ Hz, 2H), 1.72−1.53 (m, 4H), 1.42−1.32 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 164.5, 71.9, 70.5, 70.2, 70.1, 62.0, 60.4, 55.9, 41.1, 40.7, 39.3, 35.9, 28.4, 28.2, 25.7; FAB (*m*/*z*) calcd 419.2, found 419.2 $[M + H]^+$; ESI-HRMS $C_{18}H_{35}N_4O_5S^+$ $[M + H]^+$ calcd 419.2323, found 419.2322.

4-Formyl-*N*-{2-[2-(2-{2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-4-yl)pentanoylamino]ethoxy}ethoxy)ethoxy]ethyl}benzamide (2). Biotinylated tetraethylene glycol amine **15**(0.966 g, 2.3 mmol, 1.0 equiv), 4-carboxybenzaldehyde (0.725 g, 4.8 mmol, 2.1 equiv), and EDC·HCl (0.973 g, 5.1 mmol, 2.2 equiv) were dissolved in DMF/CH_3CN (1:1, 50 mL) and left to stir for 2.5 h at rt. Solvents were evaporated in vacuum, and the residue was purified on a reverse-phase column (15−50%, CH_3CN in H_2O) to afford the product, biotinylated aldehyde **2**, as a white foam (0.64 g, 50%): TLC (CH_2Cl_2:MeOH, 85:15 v/v) $R_f = 0.60$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.08 (s, 1H), 8.70 (t, $J = 5.4$ Hz, 1H), 8.04−7.97 (m, 4H), 7.77 (t, $J = 5.4$ Hz, 1H), 6.37 (br, 1H), 6.32 (br, 1H), 4.30 (dd, $J = 7.6$ Hz, $J = 5.2$ Hz, 1H), 4.14−4.10 (m, 1H), 3.58−3.36 (m, 14H), 3.20−3.14 (m, 2H), 3.11−3.05 (m, 1H), 2.81 (dd, $J = 12.4$ Hz, $J = 5.1$ Hz, 1H), 2.57 (d, $J = 12.4$ Hz, 1H), 2.06 (t, $J = 7.6$ Hz, 2H), 1.67−1.39 (m, 4H), 1.34−1.23 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 192.8, 172.1, 165.4, 162.6, 139.4,

137.7, 129.3, 127.9, 69.7, 69.6, 69.5, 69.1, 68.7, 61.0, 59.2, 55.3, 35.0, 28.1, 28.0, 25.2; FAB (*m*/*z*) calcd 551.2, found 551.2 $[M + H]^+$; ESI-HRMS $C_{26}H_{38}N_4O_7SNa^+$ $[M + Na]^+$ calcd 573.2353, found 573.2352.

In Vitro Transcription. Independent of the double-stranded DNA templates, we used 100−300 pmol of DNA in a 100 μL in vitro transcription reaction.[47] For radioactive labeling, 10−20 μCi α-[^{32}P]-labeled guanosine triphosphate (3000 Ci/mmol) was added. In case of the K61 and pool DNA templates, DNA generated in a PCR reaction was used. For the transcription of the hexameric RNA modified RNA substrate, two synthetic cDNA oligonucleotides D2T.1-6:5′ (5′-AGC GAA TTC TAA TAC GAC TCA CTA TAG GGA GA-3′) and D2T.1-6:3′ (5′-TCT CCC TAT AGT GAG TCG TAT TAG AAT TCG CT-3′) were added, which spontaneously formed a duplex, as determined by agarose gel electrophoresis (data not shown). Because of the short size of the resulting RNA, we added up to 150 μCi α-[^{32}P]-labeled guanosine triphosphate (3000 Ci/mmol) to achieve sufficient radioactive labeling of the **1**−GGAGA RNA. The 100 μL transcription reaction was incubated for at least 4 h, up to 16 h at 37 °C in the presence of various amounts of **1** and GTP as specified in Table 1, 3.5 mM ATP, CTP, and UTP (Roche), 0.8 U/μL RNasin (Promega), 0.2 μL inorganic pyrophosphatase (0.2 U/μL, New England Biolabs), 100−200 pmol DNA template, and 1.2 U/μL T7 RNA polymerase (Stratagene) in transcription buffer (40 mM Tris-HCl, pH 8.0, 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, and 30 mM DTT).

The identity of **1**−GGAGA was verified by ESI-MS after purification of the transcript by electrophoresis on a 20% denaturing polyacrylamide gel. Two major peaks of the modified hexamer **1**−GGAGA were obtained that corresponded to $[M - 3H]^{3-}$ (calcd 880.3, found 879.5) and $[M - 2H]^{2-}$ (calcd 1320.4, found 1319.7). For the unmodified hexamer 5′-GGGAGA-3′, we obtained two major peaks that corresponded to $[M - 3H]^{3-}$ (calcd 738.7, found 737.8) and $[M - 2H]^{2-}$ (calcd 1108.1, found 1107.2).

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Supporting Information Available: Proton and carbon NMR spectra of reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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