



Synthesis, Incorporation Efficiency, and Stability of Disulfide Bridged Functional Groups at RNA 5'-Ends

Gerhard Sengle,^a Andreas Jenne,^b Paramjit S. Arora,^c Burckhard Seelig,^d
James S. Nowick,^c Andres Jäschke^d and Michael Famulok^{a,*}

^a*Kekulé Institut für Organische Chemie und Biochemie, Universität Bonn, Gerhard-Domagk-Straße 1, 53121 Bonn, Germany*

^b*Institut für Biochemie-Genzentrum, Feodor-Lynen-Straße 25, 81377 München, Germany*

^c*Department of Chemistry, 535B Rowland Hall, University of California at Irvine, Irvine, CA 92697-2025, USA*

^d*Institut für Chemie/Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany*

Received 29 September 1999; accepted 27 January 2000

Abstract—Modified guanosine monophosphates have been employed to introduce various functional groups onto RNA 5'-ends. Applications of modified RNA 5'-ends include the generation of functionalized RNA libraries for in vitro selection of catalytic RNAs, the attachment of photoaffinity-tags for mapping RNA–protein interactions or active sites in catalytic RNAs, or the non-radioactive labeling of RNA molecules with fluorescent groups. While in these and in similar applications a stable linkage is desired, in selection experiments for generating novel catalytic RNAs it is often advantageous that a functional group is introduced reversibly. Here we give a quantitative comparison of the different strategies that can be applied to reversibly attach functional groups via disulfide bonds to RNA 5'-ends. We report the preparation of functional groups with disulfide linkages, their incorporation efficiency into an RNA library, and their stability under various conditions. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

The targeted modification of RNA molecules with arbitrary chemical functionalities at defined sites has the potential for broad applications in molecular biology.^{1,2} One of the most straightforward methods to introduce a particular functionality into an RNA molecule is the derivatization with guanosine monophosphorothioate (GMPS) at the 5'-end and the subsequent chemical modification of the nucleophilic sulfur at the phosphothioate group. For example, Burgin and Pace have attached a photoaffinity tag to a 5'-thiophosphorylated tRNA^{Phe} for mapping the active site of RNaseP.³ Another potential application of this technology is the mapping of RNA–protein interactions in large RNA/protein complexes.

5'-Modified RNA molecules have probably experienced their widest application in the field of in vitro selection of novel catalytic RNAs. Various ribo- and deoxyribozymes have been isolated by screening large combinatorial nucleic acid libraries.^{4–6} In a typical in vitro

selection experiment, a pool of randomized RNA or DNA molecules is subjected to iterative selection/amplification steps which allow the enrichment of active nucleic acid catalysts until they dominate the library even though they initially may have been represented in a very low proportion.

By far the most successful strategy for the isolation of RNA- and DNA-based catalysts involves the direct screening of nucleic acids libraries for catalytic activity. In these direct selections, nucleic acids capable of catalyzing a particular chemical transformation modify themselves with an affinity-tag or other characteristic that allows its preferential enrichment over those molecules which are catalytically inactive. This strategy also allows for the screening of nucleic acids that catalyze reactions between two small substrates if one of the reactants is covalently attached to every individual member of the starting RNA pool while the other reactant in solution is carrying an affinity tag. Consequently, only those RNA species within the library that catalyze bond formation between the two reactants become tagged and therefore can be separated from non-active molecules, e.g., by affinity chromatography.⁷ If cleavable linkers have been used to attach the functional group to the RNA, active catalysts can be recovered specifically

*Corresponding author. Tel.: +49-228-731-787; fax: +49-228-735-388; e-mail: m.famulok@uni-bonn.de

after their immobilization. This has been achieved for example, by using a photosensitive group⁸ or a disulfide bridge that can be easily cleaved to liberate the RNA from the solid support.^{9,10}

In this paper, we report the preparation of functional groups with disulfide linkages, their incorporation efficiency into an RNA library, and their stability under various conditions. We have applied two different strategies in this study: (a) The direct incorporation of GMP-derived initiator nucleotides that already contain a disulfide linkage into RNA by in vitro transcription, and (b) the incorporation of GMPS (guanosine-5'-monophosphorothioate)³ into RNA by GMPS-primed transcription and subsequent coupling of the RNA with a 2-thiopyridine-activated substrate to form a disulfide linkage. A third strategy (c), involving the coupling of GMPS-primed RNA by a sulfide bond to a substrate containing a disulfide group, has been published previously^{9,10} and was not investigated in this study. Figure 1 illustrates these strategies.

Strategy (a), designated as the *incorporation-method* throughout the text, has recently been established to be a useful method for the direct and site-specific incorporation of functional groups into RNA transcripts.^{11,22,24} Strategy (b), designated as the *GMPS-coupling-method*

throughout the text, produces RNA transcripts which allow the modification of RNA at the 5'-end through phosphorothioate disulfide linkages. In studying these strategies, we were concerned that the stability of the phosphorothioate sulfide linkage might not be sufficient for in vitro selection experiments. In such experiments the library is often incubated for several hours and therefore the linkage has to be inert to decomposition, otherwise the desired activity cannot be enriched. This paper describes the implementation and evaluation of these methods.

Results and Discussion

We have established and analyzed two different strategies to attach certain functional units via cleavable disulfide bridges onto RNA molecules (see Fig. 1). For both methods, the efficiency of disulfide-incorporation and the stability of different disulfide linkages at different buffer conditions were investigated. For the *GMPS-coupling-method*, a number of 2-thiopyridine activated compounds (**8**, **10**, **14**) were synthesized. These compounds were linked to the 5'-phosphorothioate group of an RNA library generated by T7 transcription in the presence of GMPS.¹² To investigate the stability of the resulting 5'-RNA phosphorothioate sulfide linkage

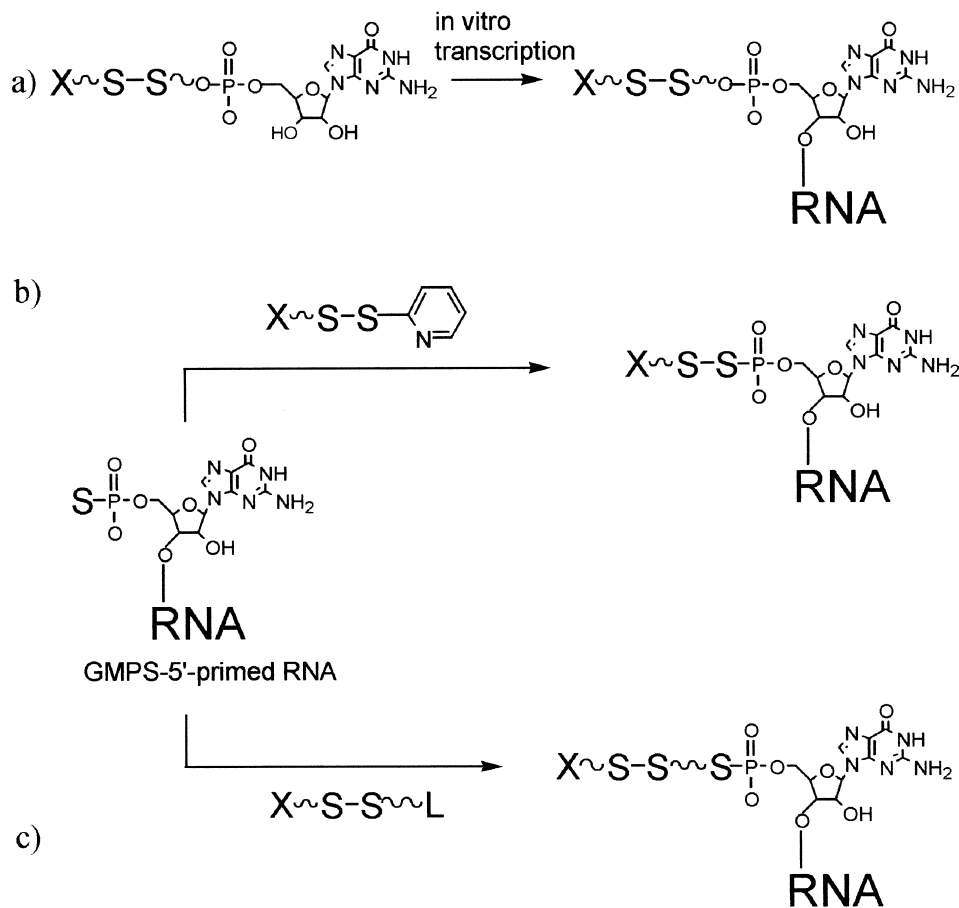


Figure 1. There are different strategies to introduce functional groups via cleavable disulfide bridges onto RNA 5'-ends. In this study, methods (a) and (b) were investigated in detail. Various functionalized RNAs were prepared, and incorporation efficiency and stability of the disulfide linkages were analyzed. 'X' stands for a functional group, 'L' stands for a leaving group (e.g., a leaving group in an active ester).

(alkyl-S-S-PO₃-RNA), we synthesized the corresponding model compounds **1a**, **3a** or directly analyzed **2b** in which the functional group is attached to the 5'-end of RNA molecules (see Fig. 2). As a representative molecule for related investigations into the second strategy, the *incorporation-method*, we synthesized the initiator nucleotide **4a**, a molecule containing disulfide bridge flanked by alkyl groups (alkyl-S-S-alkyl-GMP) and analyzed its incorporation by T7 RNA-polymerase^{13,14} and the stability of the resulting 5'-modified RNA.

Syntheses

The GMPS-linked benzoylcysteine **1a** was prepared in two steps from *N*-hydroxy succinimide (NHS)-activated ester of benzoic acid (Scheme 1). Coupling of the NHS-activated

ester **7** with thiopyridine-derivatized cysteine **6** in DMSO and triethylamine (TEA) afforded thiopyridine-activated **8** in 43% yield. The thiopyridine group of **8** was exchanged with GMPS to obtain the GMPS-cysteine analogue **1a** in 62% yield after purification by preparative HPLC. This reaction is conveniently monitored by analytical HPLC, which indicates the GMPS to be consumed and **1a** to form within 10 min of mixing of 7 equivalents of **8** with GMPS at 10 mM thiopyridine-activated substrate concentrations.

Scheme 1 also illustrates the synthesis of the thiopyridine-activated biotinylated cysteine **10**, which was used to afford biotin linked RNA **2b** in subsequent experiments. The biotin-derivative **10** was prepared from commercially available NHS-activated biotin and thiopyridine-activated

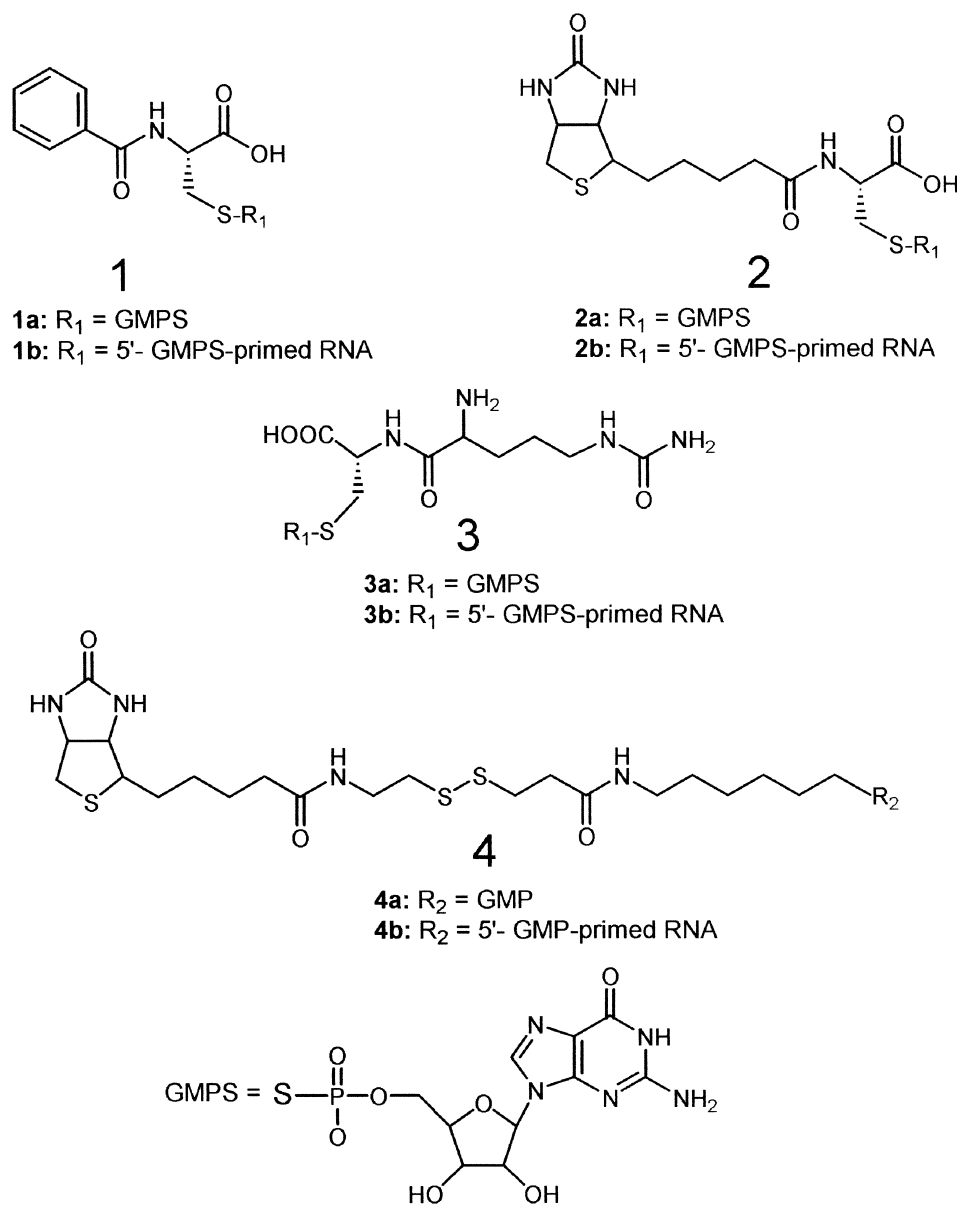
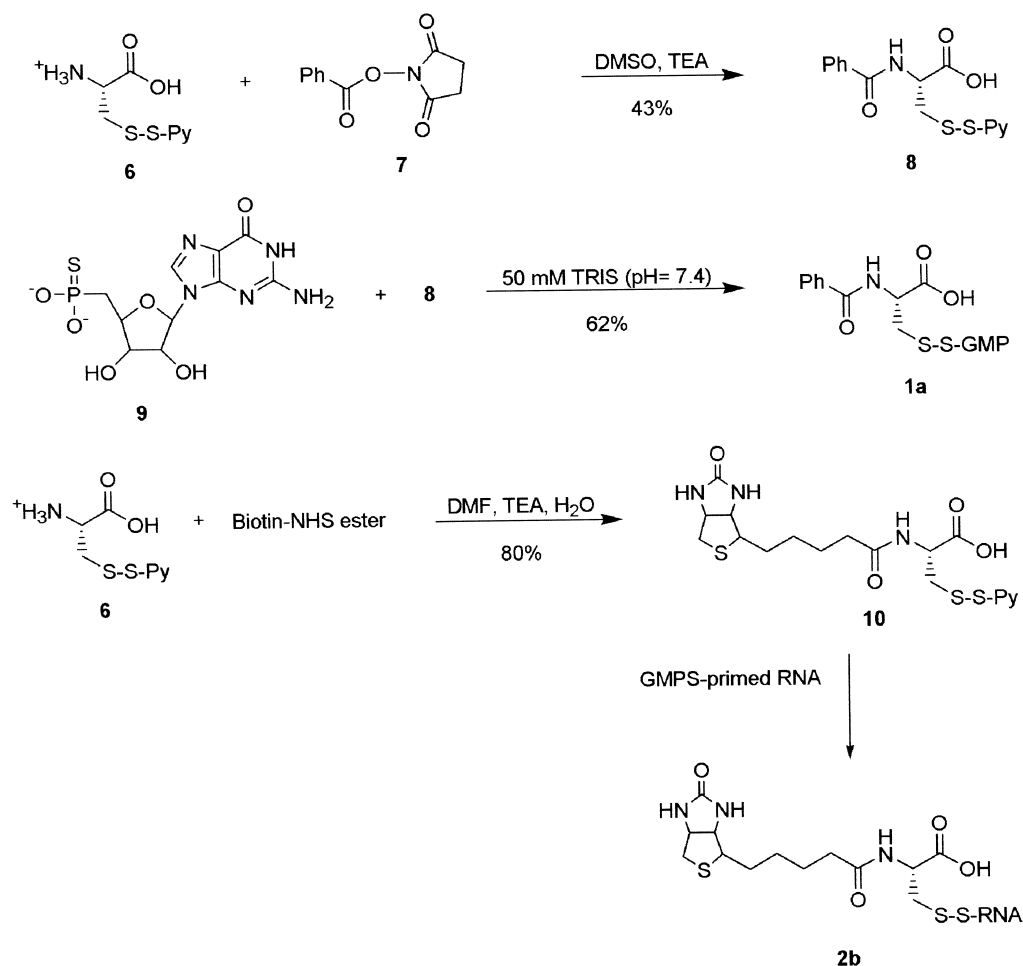


Figure 2. Compounds synthesized for and analyzed in this study. **1–3** were generated by reaction of thiopyridine activated compounds **8**, **10**, **14** with GMPS or GMPS-primed RNA. **4b** is the RNA conjugate prepared by transcription reaction in the presence of initiator nucleotide **4a**. While compounds **1**, **2**, and **3** contain a phosphorothioate sulfide linkage, compound **4** contains a 'natural' alkyl disulfide bond.



Scheme 1. Synthesis of compounds **1** and **10**.

cysteine **6** in 80% yield. Incubation of compound **10** with GMPS-primed RNA afforded biotin-linked RNA **2b**.

Compound **14** was prepared from activated-citrulline **11** in three steps (Scheme 2). Coupling of 4-nitrophenyl ester of *N*-Boc-protected citrulline¹⁵ **11** with *S*-tritylcysteine **12** followed by deprotection of the trityl group afforded the citrullinyl-cysteyl dipeptide **13** in 61% yield. The activated 2-thiopyridyl compound **14** was synthesized from **13** by reaction with 2,2'-dipyridyl disulfide in 79% yield. Compound **14** was subsequently condensed with GMPS or GMPS-primed RNA, to afford **3a** or **3b**, respectively. Completeness of the reaction was determined by quantification the UV absorbance of the liberated thiopyridone.

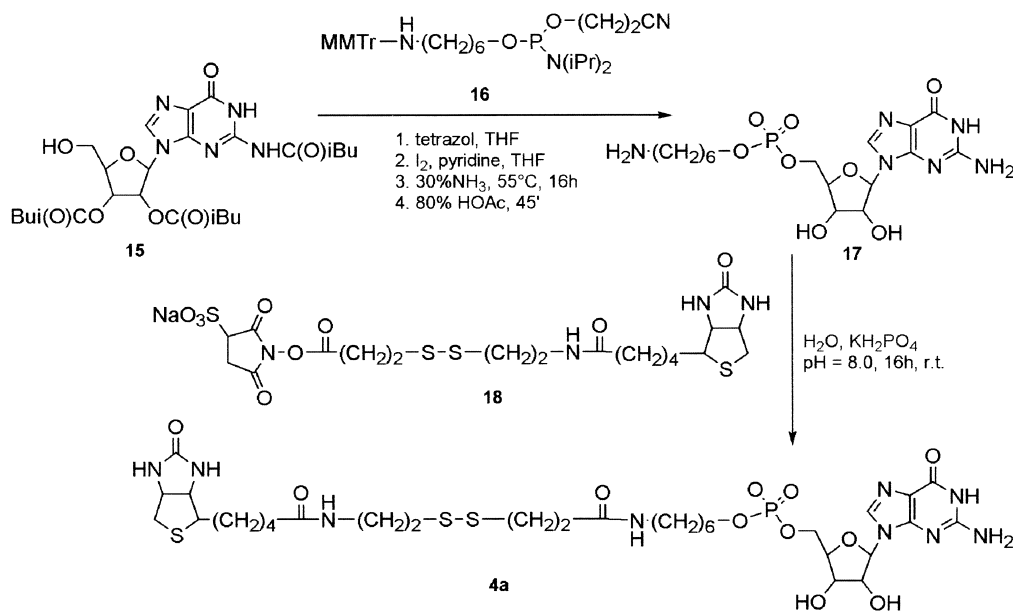
The synthesis of **4a** is shown in Scheme 3. Coupling of protected guanosine **15** and the phosphoramidite **16** followed by iodine oxidation, and basic and acidic deprotection steps, afforded hexylamino guanosine-5'-monophosphate **17**. Subsequent coupling of sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate **18** (a water soluble *N*-hydroxysuccinimide ester which is routinely used for the biotinylation of proteins such as IgG¹⁶ or cell surface proteins of lymphocytes) with **17** in KH₂PO₄-buffer at pH 8.0 for 16 h yielded **4a**.

The purity of **4a** was quantified by the HABA (4-hydroxyazobenzene-2-carboxylic acid)-test¹⁷ which allows quantification of biotin contained in a solution. Using the HABA-test, we calculated the yield of HPLC-purified **4a** to be 53%.

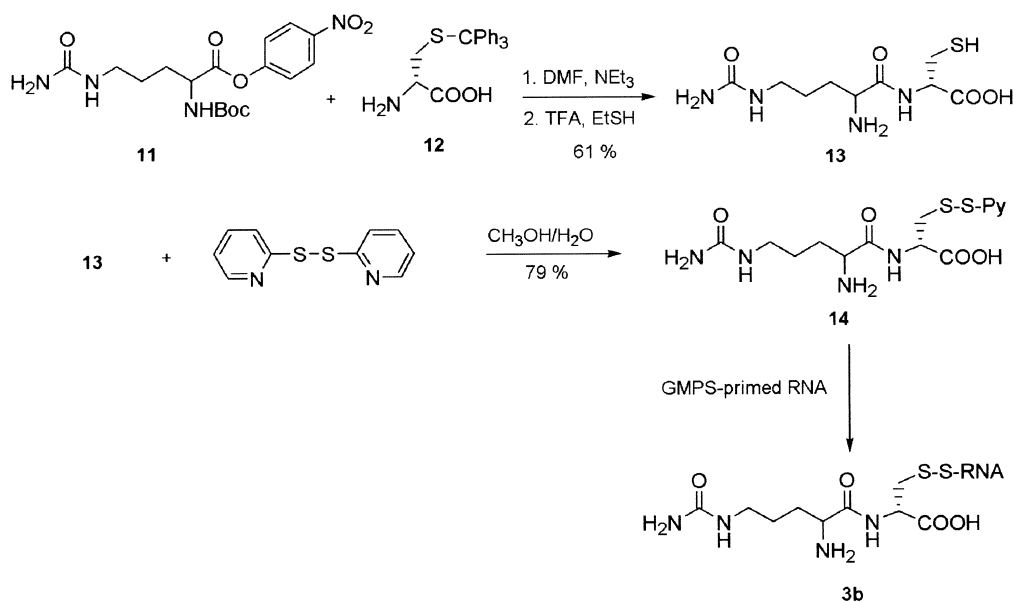
Incorporation studies

We performed a set of different analyses to determine the efficiency of incorporation of the various functional groups into RNA 5'-ends by using both the *incorporation-method* and the *GMPS-coupling-method* (see Fig. 1). These analyses included affinity chromatography and gel-retardation assays of functionalized RNA.

For the *GMPS-coupling-method*, we first determined the efficiency of GMPS-incorporation by T7-transcription of a random-dsDNA library in the presence of various amounts of GMPS (Fig. 3). The percentage of GMPS-primed RNA was determined by separation of the thiomodified ³²P-labeled RNA from non-modified RNA by [(β-acryloylamino)phenyl]mercuric chloride (APM polyacrylamide gel affinity electrophoresis)^{18,23} and subsequent quantification of the retarded band on a phosphor imager. Surprisingly, we observed that GMPS had a stimulating effect on the transcription reaction: the yields of RNA were generally higher when GMPS



Scheme 2. Synthesis of 3b.



Scheme 3. Synthesis of 4a and precursors.

was present up to a 4-fold excess over the NTPs used in the standard transcription (Fig. 3). At a GMPS-concentration of 5–15 mM and a concentration of 5 mM of the other four NTPs the total yield of RNA comprised >80% GMPS-primed transcripts. Similar results in priming efficiency have been obtained by other groups using lower amounts of GMPS.^{19,20}

For comparison we also performed similar experiments with the mononucleotides GMP and AMP. Whereas AMP had no effect on the transcription yield at concentrations below 10 mM, the presence of GMP in equal concentrations or moderate molar excess over the four NTPs clearly led to an increase of RNA yields. At a concentration of 5 mM GMP, corresponding to an

equal molarity of each NTP, the transcription reaction was enhanced by a factor of 3.5. When the mononucleotide concentration exceeded 50–60 mM we observed inhibition of transcription.

To compare the efficiency of the second step of the reaction, i.e., coupling of the thiopyridine-activated compounds to the 5'-phosphorothioate group in GMPS or GMPS-primed RNA, respectively, we used HPLC, UV-spectroscopy or affinity chromatography. The rate of disulfide formation of the GMPS model-substrate was determined by HPLC-quantification of the 2-thiopyridone by-product formed during the coupling step. At the reaction conditions described in the synthesis section less than 5% of the initial GMPS concentration

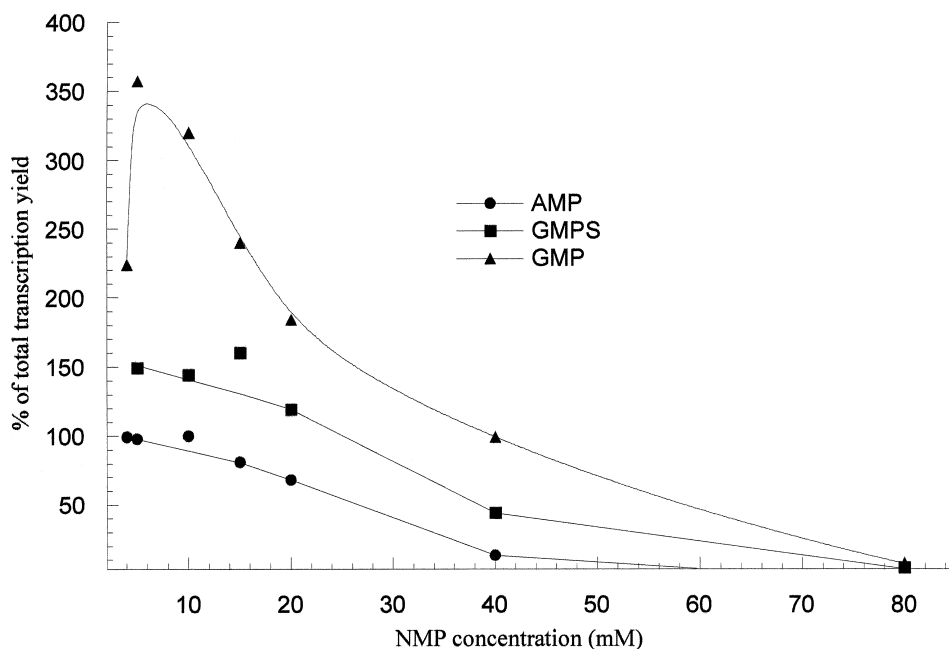


Figure 3. Dependence of transcription yields on the concentration of mononucleotides GMP, GMPS and AMP. Percentage values of the various transcription reactions (NMP concentration: 4–80 mM) were normalized to a standard *in vitro* transcription containing each NTP at a concentration of 5.0 mM without any mononucleotide added.

remained in the reaction mixture after 5 min. We also performed similar experiments with a 25- to 50-fold excess of the activated dipeptide **14** using both GMPS-primed RNA and GMPS alone. The analysis of the coupling efficiency of these reactions yielding compounds **3a** and **3b** was performed by UV spectroscopy. After a 5 min incubation time the UV-vis-spectra in the range between 210 and 420 nm were recorded and subtracted by a spectrum obtained with a reference sample containing the dipeptide. From the absorption at 343 nm the amount of 2-thiopyridone was calculated providing an indirect but nevertheless very accurate measure of the coupling efficiency. This analysis established that the reaction was quantitative since the molar amount of liberated thiopyridone was approximately equivalent to the RNA concentration used (see methods). When using GMPS instead of GMPS-primed RNA the formation of the phosphorothioate sulfide bridge also appeared to be quantitative (data not shown).

We then wanted to investigate the coupling efficiency of activated thiols to GMPS-primed RNA (see above for **1a**, **3a**) by analyzing the formation of compound **2b**. This compound contains a biotin moiety which enabled the determination of streptavidin-bound ^{32}P -radio-labeled RNA by scintillation counting. At 300-fold excess of thiopyridine-activated biotinylated cysteine **10** the amount of phosphorothioate sulfide formation appeared to be at least 70% which was around 25% lower than for **2a**. This diminished yield may result from inaccessibility of the 5'-phosphorothioate group in the folded RNA for the reaction with **10** or non-quantitative coupling of biotinylated RNA to the column resin. However, with a GMPS priming rate for the RNA of more than 85% and a reaction efficiency for the

successive coupling step of at least 70%, the overall yield of phosphorothioate sulfide derivatized RNA was determined to be at least 60%. Taken together the results obtained with the activated thiols **1–3** confirm that the coupling of GMPS/ GMPS-primed RNA with thiopyridine-activated molecules is fast, selective, and quantitative.

To compare the data obtained with the *GMPS-coupling-method* we now analyzed the *incorporation-method* (see Fig. 1a). It is known that T7 RNA polymerase tolerates GMP,¹¹ GMPS,³ a series of modified nucleotides,^{13,22} and various dinucleotides [NpG²¹] for initiation of transcription. However, it has been also observed that trimers or short oligonucleotides are only incorporated with moderate success.^{14,28} To investigate whether the initiator nucleotide **4a** was accepted by T7 RNA polymerase, we added **4a** to standard transcription reactions and reduced the concentration of GTP accordingly. In addition to the ratio of initiator nucleotide and GTP the concentration of the DNA template and that of Mg^{2+} -ions are also important parameters for the general efficiency of transcription with T7 RNA polymerase. Therefore, it was necessary to find reaction conditions that would lead to both a high ratio of initiator-nucleotide-primed to unprimed RNA and a high overall transcription yield.

For a randomized 213mer DNA-library we varied both the template and Mg^{2+} -concentrations and determined the ratio for the concentrations of GTP and **4a** where highest absolute yields of primed RNA were obtained. Highest yields of RNA were obtained at template concentrations between 4.0 and 5.0 μM and 12 mM MgCl_2 (data not shown). Under these conditions we determined the ratio of $[\text{GTP}]/[\mathbf{4a}]$ to be optimal at 8 mM/3 mM.

The results of this analysis are shown in Figure 4. While relatively high yields of total RNA are obtained at concentrations of **4a** at or below 3 mM, the yield dramatically dropped when the concentration of **4a** was raised. At a GTP/**4a**-ratio of 1:4, using 1.0 mM GTP and 4.0 mM **4a**, the total RNA yield dropped to 10% of the RNA yield obtained under standard conditions for T7 transcription, while with the same ratio at higher concentrations (2.5 mM GTP, 10 mM **4a**), no transcribed RNA was detected. This indicates that **4a** strongly inhibits transcription.

Stability studies

For many applications of disulfide-derivatized RNAs it is critical that the modification remains stably coupled to the RNA. To analyze the stability of the phosphorothioate sulfide linkage resulting from the *GMPS-coupling-method* we performed a time course experiment, in which the decomposition of **1a** was monitored by HPLC. We also analyzed the more complex substrate **3a** that includes a nucleophilic primary NH_2 -group in close proximity to the phosphorothioate sulfide, which we assumed to affect the stability of this linkage. Furthermore, we investigated the phosphorothioate sulfide linkage in the context of an RNA (**2b**). To compare the stability of the phosphorothioate sulfide linkage with that of a disulfide bridge flanked by short alkyl chains we also analyzed compounds **4a** and **4b** by HPLC and RNA affinity chromatography, respectively.

Compounds **2b**, **3a**, **4a,b** and **4b*** (obtained from **4b** after disulfide reduction with 0.2 M DTT and then

coupled to thiopyridine-activated sepharose) were incubated at conditions typical for an *in vitro* selection experiment. Aliquots were taken at 6–8 different time points and analyzed. The results are shown in Figure 5. Compound **1a** shows no detectable decomposition after incubation for 72 h in buffer (50 mM TRIS 7.4, 5 mM MgCl_2 , 500 mM NaCl; data not shown). In buffered solution at a physiological pH there seems to be only a slight difference in stability of a phosphorothioate sulfide linkage compared to the alkyl-flanked disulfide-bond in **4**. The detected differences in stability for model compounds **1a**, **3a**, **4a**, compared to **2b**, **4b**, **4b***, most likely are due to a certain extent of RNA degradation. As similar curves were obtained for **4b** and **4b*** it is obvious that the biotin-anchor-group in **4b** had no influence on the stability of the disulfide-bond.

The result obtained with **2b** suggests that the stability of the phosphorothioate sulfide-linkage is slightly decreased compared to the alkyl-disulfide bridge. Despite this slightly decreased stability it appears, however, that the stability of an RNA library modified with the disulfide linkage is sufficient for an incubation period of several hours in buffered solutions around a neutral pH. With model compound **3a** it could also be shown that nucleophilic side chains which might attack the linkage had no effect on the stability (see insert Fig. 5).

To investigate, whether buffer, temperature or salt conditions affect the stability of the phosphorothioate sulfide-linkage, we performed a time course experiment in which GMPS-primed ^{32}P -labeled RNA was coupled to thiopyridine-activated sepharose and incubated the

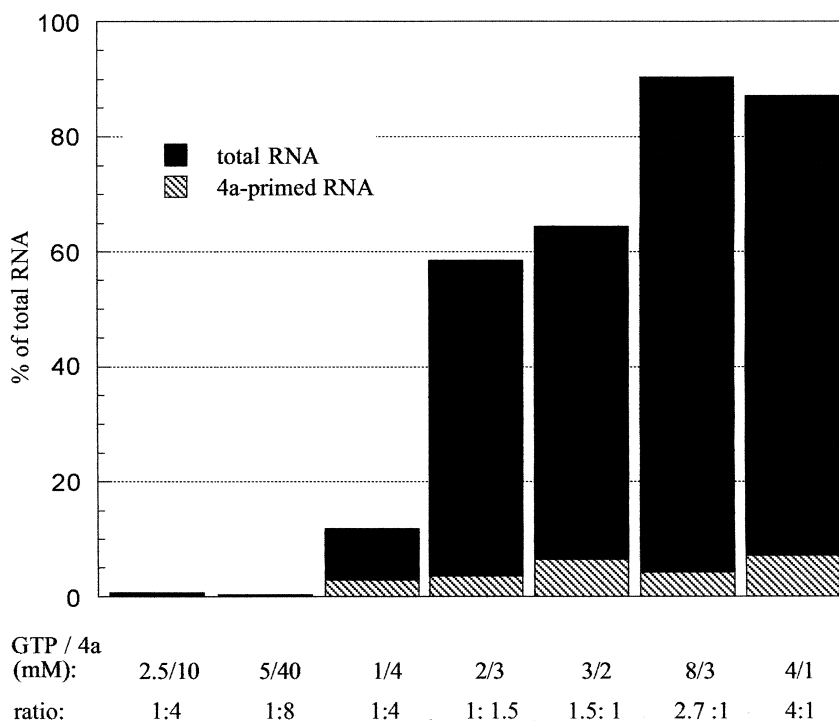


Figure 4. Quantitative analysis of transcription yields and priming efficiencies using **4a** in the *incorporation-method*. The height of the bars indicate the total yield of RNA after transcription in the presence of various ratios of GTP/**4a** (mM) at 2.4 μM DNA and 12 mM MgCl_2 . The shaded portions represent the fraction primed with **4a**. Percentage values are normalized to an *in vitro* transcription reaction that contained each NTP at 5.0 mM without **4a**.

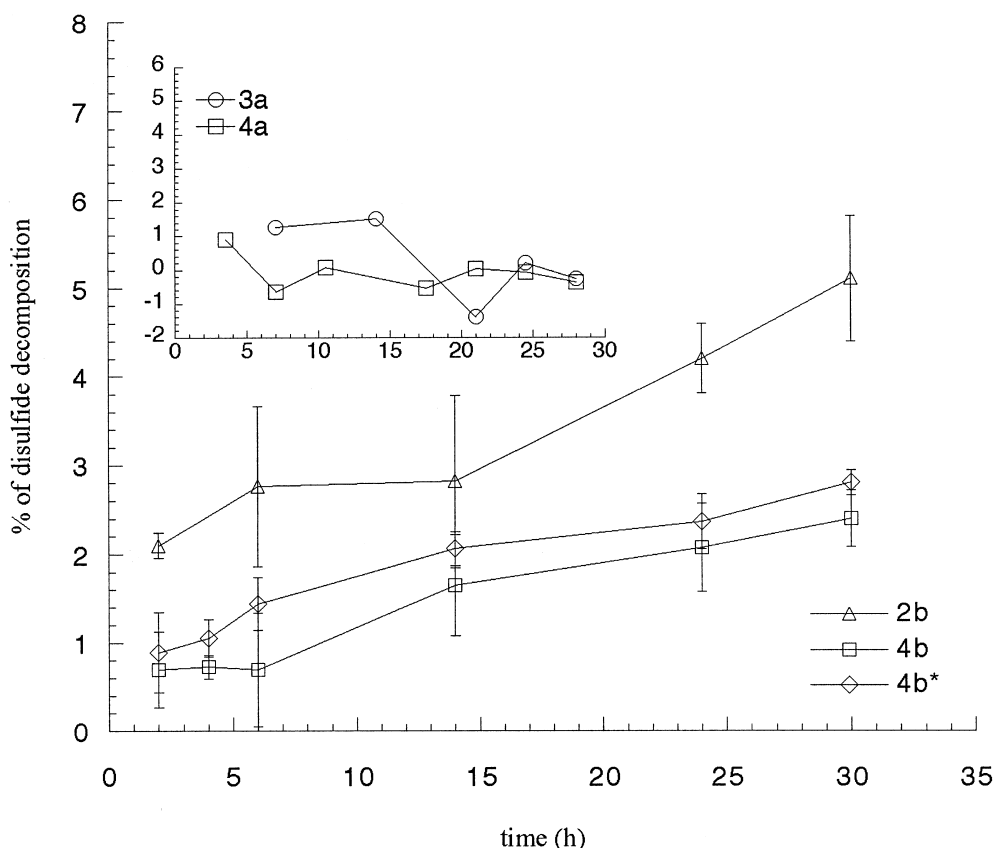


Figure 5. Decomposition of RNA coupled substrates **2b**, **4b** and **4b*** (obtained from **4b** after disulfide-bond cleavage with 0.2 M DTT) in buffered solution (50 mM K-MOPS, 200 mM NaCl, pH 7.4). Analysis was performed by RNA affinity chromatography in duplicate (see Methods). The compounds were analyzed for up to 30 h and the percentage of decomposition was plotted versus time. Inset: Decomposition of the model compounds **3a**, **4a** under the same conditions was analyzed by reversed phase HPLC (see Methods). Almost no decomposition of **1a** was detected after 72 h incubation in aqueous buffer (50 mM TRIS, 5 mM MgCl₂, 500 mM NaCl, pH 7.4) (data not shown). Compound was also stable after incubation at 95 °C for 5 min in the same buffer; only upon treatment with 2 M mercaptoethanol, **1a** clearly reacted to afford *N*-Bz-cysteine and GMPS.

immobilized RNA under various salt-, buffer-, and temperature-conditions. At various time points, unbound RNA resulting from disulfide bond cleavage was eluted and quantified by scintillation counting. The results of these experiments are shown in Figures 6 and 7.

Omission of any salts or buffer results in a destabilized linkage (19% decomposition after 30 h at 23 °C, filled rhombus) compared to a K-MOPS solution adjusted to pH 7.4 (9% after 30 h at 23 °C, open square). The comparison of the reaction performed in plain water (19%, filled rhombus) with that in 100 mM Mg²⁺ (1%, filled triangle) shows that Mg²⁺-ions are stabilizing the linkage. The presence of 100 mM Mg²⁺ in the buffer (6%, open circle) had no influence on stability compared to the buffered solution without divalent metal ions (9%, open square). Increasing the temperature has a strong effect on the stability of this linkage. Cleavage in buffered solution in the absence of Mg²⁺ at 37 °C reaches about 25% after 30 h of incubation (open triangle) compared to only 9% at 23 °C under otherwise identical conditions (open square). No degradation of the eluted RNA molecules was detected as analyzed by polyacrylamide electrophoresis of eluted RNA (data not shown).

Figure 7a shows a systematic investigation of the effect of pH on the stability of the two linkages. Whereas the

alkyl-flanked disulfide bond remains stable without any detectable influence of pH the phosphorothioate sulfide-linkage clearly is much more susceptible to basic pH. Most likely the alkyl-S-S-PO₃-RNA bridge is reduced to GMPS-primed RNA and the corresponding thiol as the eluted RNA fractions could be almost quantitatively re-immobilized on thiopropyl sepharose (data not shown). Figure 7b confirms that the high amount of eluted RNA at high values of pH is not due to increased RNA degradation. The RNA itself is equally stable under the various elution conditions used.

Conclusion

It is critical for the success of an in vitro selection experiment that (i) the substrate gets effectively attached to RNA, (ii) the attached substrate remains bound to the RNA during the course of the selection, and (iii) the immobilized active RNA molecules can be easily removed from the solid support.

We have compared two different strategies allowing the incorporation of functional groups into RNA molecules at the 5'-ends. The two strategies involve direct priming of T7 transcripts with a highly functionalized initiator nucleotide in a one-step protocol, the

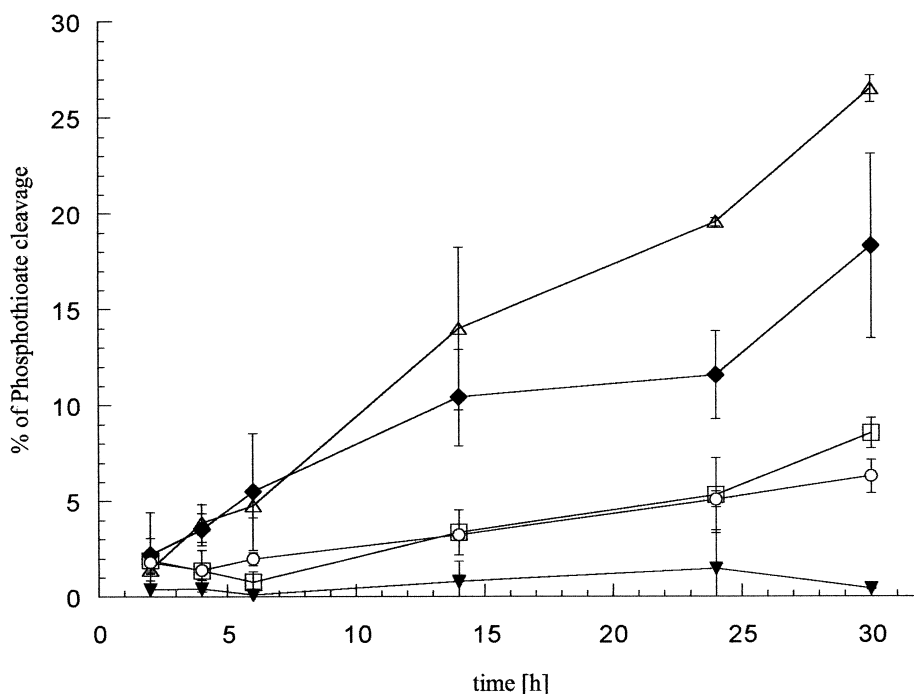


Figure 6. Stability of the phosphorothioate sulfide-linkage under various conditions. Radiolabeled GMPS-primed RNA was immobilized on thiopropyl-sepharose and incubated at room temperature. Aliquots were taken at various time points and analyzed. Percentage of decomposition as detected by scintillation counting is plotted versus time. (▼) 100 mM MgCl₂ in distilled water, (□) in 50 mM K-MOPS, pH 7.4, 200 mM NaCl, at 23 °C, (◆) in distilled water, (△) in 50 mM K-MOPS, pH 7.4, 200 mM NaCl at 37 °C, (○) in 50 mM K-MOPS, pH 7.4, 200 mM NaCl, 100 mM MgCl₂.

incorporation-method, and a two-step strategy, the *GMPS-coupling-method*, in which the RNA transcripts are first primed with GMPS and then functionalized at the thiophosphate group with thiopyridine activated substrates. We also determined the stability of the resulting linkages and found that the 5'-modifications introduced via the *GMPS-coupling-method* in which a phosphorothioate sulfide is obtained exhibited slightly reduced stability compared to a linkage in which the disulfide groups are flanked by alkyl chains. We also found that several parameters, such as buffer composition, temperature and pH play a very critical role for stability of the various linkages studied. The phosphorothioate sulfide linkage is significantly more sensitive when exposed pH values above 7.5 compared to the alkyl disulfide linkage which might argue for the *incorporation-method*. Interestingly, high concentrations of the initiator nucleotide **4a** in the T7 in vitro transcription reaction resulted in a markedly reduced overall yield of RNA. This suggests that **4a** has a strong inhibitory effect on T7 RNA polymerase at concentrations exceeding 3 mM. The addition of GMP is known to preferentially initiate transcription¹¹ which could explain the improved yield- up to 3.5-fold over standard in vitro transcription reactions.

In summary, the incorporation efficiency of complex initiator nucleotides such as **4a** that are introduced by the *incorporation-method* is markedly reduced when compared to the *GMPS-coupling-method* that takes advantage of the much more efficient incorporation of GMPS followed by subsequent derivatization with activated thiols that occurs almost quantitatively. However, this conclusion cannot be generalized. In previous studies

it was shown that another initiator nucleotide, anthracene-polyethylene glycol-GMP did not show any inhibitory effect on transcription by T7 RNA polymerase.^{13,22}

The *incorporation-method* certainly requires time consuming optimization of conditions for incorporation.^{13,22} The higher yield obtained by the *GMPS-coupling-method*, however, is somewhat compromised by the slightly reduced stability observed for the phosphorothioate-disulfide linkage in comparison to the more inert character of the disulfide group embedded between alkyl groups introduced into RNA by **4**. Another advantage of the *incorporation-method* is that it produces 'ready to use' RNA conjugates directly after transcription reaction.

Our results suggest that following the *GMPS-incorporation-method* might be advantageous if large amounts of derivatized RNA are desired and if the RNA is not exposed to values of pH exceeding 7.5, temperatures higher than 23 °C, and low-salt conditions. In cases where maximum stability of the disulfide linkage is desired but the adherence to these conditions cannot be guaranteed it is advantageous to follow the *incorporation-method* or the two-step process (c) in Figure 1.

Experimental

General materials and methods

MALDI-TOF mass spectra were obtained on a Bruker Reflex spectrometer. Electron ionization spray (ESI) mass spectroscopy was performed on a Finnigan MAT

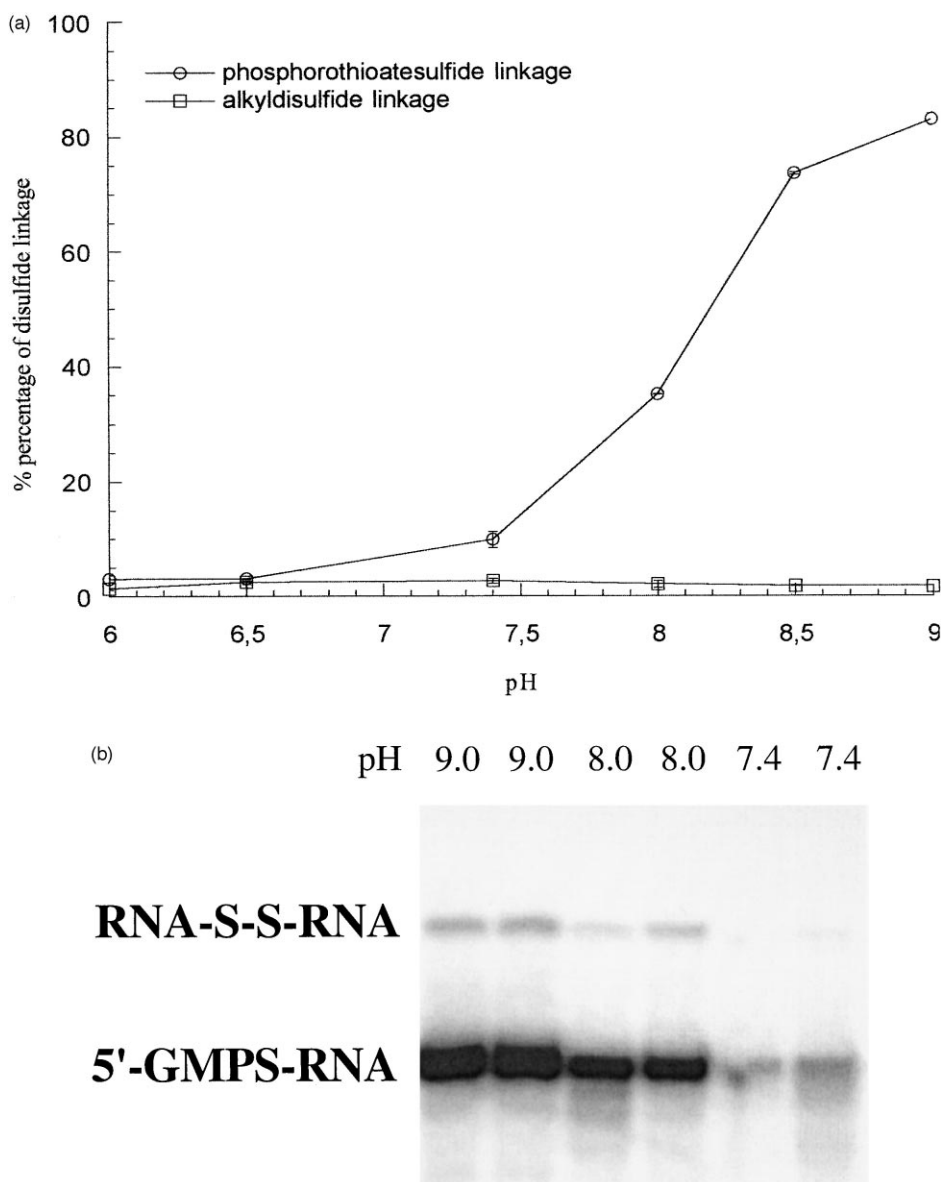


Figure 7. (A) Decomposition of phosphorothioatesulfides and alkylidissulfides at various pHs. Percentage of cleavage from the matrix is plotted versus pH. GMPS-primed RNA was coupled to thiopropyl-sepharose and incubated for 24 h at room temperature in 50 mM K-MOPS, 200 mM NaCl, and the pH value given in the x-axis. **4b** was immobilized on a streptavidin-agarose column and incubated at the same conditions. (B) PAGE analysis of the eluted fractions at pH 9.0, 8.0, 7.4 obtained from the pH-studies shown in (7A) (double determinations). Lower bands correspond to GMPS-primed 94mer RNA, whereas shifted bands correspond to oxidized 94mer (RNA-S-S-RNA).

7M spectrometer. A Bruker AMX-400 spectrometer was used for 400 MHz ^1H NMR analyses. Polyacrylamide gel electrophoresis (PAGE) was carried out as described unless otherwise stated. Radioactivity was assayed on a Molecular Dynamics Phosphor Imager. HPLC was carried out on a Millipore, Millennium v2.10 or Beckman System Gold programmable solvent module 126 chromatograph using analytical and preparative C18 reversed-phase columns (Beckman). dNTPs, NTPs, DNase I were purchased from Boehringer Mannheim, radiochemicals from NEN. Taq polymerase was from Stratagene, RNasin from Promega, T7 RNA polymerase from various sources as indicated. Oligonucleotides were synthesized on a Millipore Expedite oligonucleotide synthesizer using standard phosphoramidite chemistry unless otherwise stated. Scintillation

counting was done in a Beckman LS 6000 Sc liquid scintillation counter. *N* $^{\alpha}$ -Boc-citrulline-*p*-nitrophenyl-ester **11** and *S*-Trityl-cysteine **12** were purchased from Bachem, *N*-Monomethoxytrityl-aminoethyl-cyanoethyl-*N,N*-diisopropyl phosphoramidite from PerSeptive Biosystems. [(*N*-acryloylamino)phenyl]-mercuric chloride (APM) was synthesized¹⁸ and mercurio-polyacrylamide gels were prepared with this material as described.¹⁸ Guanosine monophosphorothioate (GMPS) was synthesized and purified as described.¹²

Preparation of DNA oligonucleotides

The 222mer DNA library used for transcription studies with GMPS, GMP and AMP to generate a 196mer RNA-library was synthesized as described previously.⁹

The 113mer DNA (5'-TCT AAT ACG ACT CAC TAT AGG GAG AGA CAA GCT TGG GTC TGG TTT GAA CGG GGG CGC ATC AGC CAT GCT ATA AAC TCC AAA GAA GAG AAA GAG AAG TTA ATT AAG GAT CCT CAG) was serving as a template to produce the 94mer RNA used for **2b**, **4b** and **4b***. The 213mer DNA-library M213.1 was synthesized as ssDNA the 111mer M111.1, 113mer M113.1 DNA-libraries²⁵ amplifying, ligating and finally amplifying again as previously described.²⁶

T7 Transcriptions

T7 transcriptions were performed essentially as described.²⁷ DNA templates for T7 transcription were synthesized by standard phosphoramidite chemistry. The transcription reactions with GMPS, GMP and AMP were performed in a typical 100 μ L transcription reaction mixture containing 40 mM TRIS-HCl, pH 8.0, 22 mM MgCl₂, 6.8 mM spermidine, 30 mM DTT, 0.1% Triton X 100, 5 mM of each NTP, 4–80 mM of NMP, 2.4 μ M dsDNA template, 20U ribonuclease inhibitor, 3 μ M α -[³²P]-GTP (10 μ Ci/ μ L) and 250 U of T7 RNA polymerase.

T7 transcriptions with the initiator nucleotide **4a** were carried out to obtain **4b** under the same conditions without DTT, but with various concentrations of Mg²⁺, DNA-template, GTP and **4a** as mentioned above. The reaction mixtures were incubated at 37°C overnight. After transcription the RNA was precipitated by adding ammonium acetate/ethanol, re-suspended in 9 M urea, 50 mM EDTA pH 8.0 and loaded on a preparative 8% (94 nt transcript), 5% (213/158 nt transcripts) denaturing polyacrylamide gel. Products were separated on a 24 cm vertical gel apparatus, the band was located by UV-shadowing, cut out, eluted for 1.5 h in 0.3 M NaOAc at 65°C, precipitated by adding NaOAc/ethanol and re-dissolved in distilled water. The 100 μ L reaction mixture containing **4a** yielded under the optimized conditions of 2.4 μ M of DNA template, 12 mM of MgCl₂ and a ratio between **4a** and GTP of 3 and 8 mM an average of 5.80 μ g of modified transcripts, which corresponds to 0.38 mol of modified RNA per mol of DNA template.

HPLC-stability studies

The stability of **1a**, **3a** and **4a** was determined by HPLC on a C₁₈ reversed-phase column using gradient elution with 0–40 min H₂O, 0.1% TFA, 40–50 min CH₃CN. The samples were incubated at 25°C and every 3 h a 10 μ L aliquot was automatically taken and immediately injected.

Cleavage studies on RNA-matrices

Coupling to the matrices thiopropyl-sepharose 6B (Pharmacia)/streptavidin-agarose (Pierce) was achieved by dissolving GMPS-primed ³²P-radiolabeled RNA respectively **2b**, **4b**, **4b*** in each coupling buffer (for thiopropyl-sepharose 6B: 25 mM HEPES, 1 mM EDTA, pH 7.4, for streptavidin-agarose: 25 mM Na₂HPO₄/NaH₂PO₄ 150 mM NaCl, pH 6.9) and then incubated for 30 min with the double volume of a 50% slurry of

each matrix, equilibrated in coupling buffer. The matrix was washed subsequently with 20 column volumes each of washing buffer (25 mM K-MOPS, 1 M NaCl, 5 mM EDTA, pH 7.4), denaturing buffer (4 M urea, 5 mM EDTA, adjusted with K-MOPS to pH 7.4) and finally with water. The RNA linked to the matrix was then transferred to a spin filter tube, resuspended in the buffer and incubated for the time given in the figure legends. After the time of incubation, the solution was centrifuged at 4900 g for 1 min. Each sample was additionally washed with 20 column volumes with incubation-solution and the amount of eluted RNA was detected by scintillation counting.

Syntheses

BzCys-S-Py (8). A solution of benzoic acid (0.071 g, 0.585 mmol), *N*-hydroxysuccinimide (NHS, 0.067 g, 0.585 mmol), and 1,3-dicyclohexylcarbodiimide (0.145 g, 0.702 mmol) in CH₂Cl₂ (5 mL) was stirred at room temperature. After 1 h, the precipitate was filtered off and the filtrate was concentrated in vacuo to yield a white solid. Recrystallization from 2-propanol afforded **6** (0.100 g, 78%). The NHS-activated benzoic acid **6** (0.100g, 0.456 mmol) was dissolved in triethylamine (0.762 mL, 5.47 mmol), and DMSO (3 mL). To the solution, *S*-(2-thiopyridyl)-L-cysteine **6** (0.147 g, 0.547 mmol) was added and the reaction mixture was stirred at room temperature. After 4 h, the reaction mixture was washed with 1 M hydrochloric acid, and the aqueous layer was extracted with CH₂Cl₂ (3×5 mL). The combined organic layers were washed with H₂O (5×5 mL) to remove DMSO, dried over MgSO₄, filtered, and concentrated in vacuo. Recrystallization from 2-propanol and ether afforded *S*-(2-thiopyridyl)-L-*N*-Bz-cysteine **8** (0.65 g, 43%) as a white solid. ¹H NMR (500 M Hz, CD₃OD) δ =8.34 (app d, *J*=5.0, 1H), 7.83 (d, *J*=7.1 Hz, 2H), 7.78 (d, *J*=8.1 Hz, 1H), 7.68 (t, *J*=8.5 Hz, 1H), 7.56 (t, *J*=7.5 Hz, 1H), 7.47 (t, *J*=7.5 Hz, 2H), 7.16 (dd, *J*=10.0, 4.5 Hz, 1H), 4.88 (dd, *J*=9.5, 4.2 Hz, 1H), 3.48 (dd, *J*=14.0, 4.2 Hz, 1H), 3.33 (dd, *J*=14.0, 9.5 Hz, 1H). ¹³C NMR (125 M Hz, CD₃OD) δ =173.5, 170.4, 160.8, 150.6, 139.2, 135.3, 133.1, 129.7, 128.7, 122.7, 121.7, 53.9, 41.9. HRMS (FAB) *m/e* for C₁₅H₁₅N₂O₃S₂ (M + H)⁺, calcd 335.0524, found 335.0525.

BzCys-GMPS (1a). A solution of guanosine-5'-phosphorothioate **9** (GMPS, 5.0 mg, 0.012 mmol) and *S*-(2-thiopyridyl)-L-benzoylcysteine **8** (25.0 mg, 0.072 mmol) in 50 mM TRIS-HCl (1 mL, pH = 7.4) was incubated at room temperature. After 1 h, the reaction mixture was injected into an HPLC apparatus equipped with a UV detector (C₁₈ reversed phase column, 17:83, methanol: 0.1% trifluoroacetic acid in H₂O, 25 mL/min). The peak with the retention time of 13.4 min was isolated and concentrated in vacuo to afford 8 mg (62%) of **1a** as a white solid. ¹H NMR (500 M Hz, CD₃CN:D₂O, 10:90) δ =8.86 (d, *J*=5.3 Hz, 1H), 7.82 (d, *J*=8.0 Hz, 2H), 7.66 (t, *J*=7.4, 1H), 7.55 (t, *J*=7.7 Hz, 2H), 5.96 (d, *J*=5.2 Hz, 1H), 4.91 (dd, *J*=8.5, 5.0 Hz, 1H), 4.78–4.76 (m, 1H), 4.52 (t, *J*=4.6 Hz, 1H), 4.40 (app t, *J*=3.2 Hz, 1H), 4.30–4.28 (m, 1H), 4.25–4.22 (m, 1H), 3.49 (dd, *J*=14.0, 5.0 Hz, 1H), 3.32 (dd, *J*=14.2, 8.5 Hz, 1H).

LRMS (FAB) m/e for $C_{20}H_{24}N_6O_{10}PS_2$ ($M+H$)⁺, calcd 603, found 603.

Biotin-Cys-GMPS-primed RNA (2b). A 10 mM solution of thiopyridine-activated biotinylated cysteine **10** in 10% DMF/50 mM TRIS (pH 7.4) was incubated with 33 μ M GMPS primed ^{32}P labeled 94mer RNA (TRIS buffer: 50 mM TRIS, 5 mM $MgCl_2$, 250 mM NaCl). The amount of disulfide formation was determined as quantitative by measuring the UV-absorbance of the leaving-group thiopyridine at 343 nm after 30 min. The reacted mixture RNA was precipitated, redissolved in streptavidin-binding buffer and added to column containing 250 μ L streptavidin-agarose. After an additional 30 min, the unbound RNA is washed off the column with TRIS buffer. Approximately 70% of the RNA remained bound to streptavidin-agarose could be detected by scintillation counting. This result implies, that about 70% of the 94mer RNA was primed with GMPS during in vitro transcription.

Biotin-Cys-S-Py (9). A solution of D-biotin-*N*-hydroxy-succinimide ester (0.165 g, 0.484 mmol) in DMF (2 mL) was added to a solution containing *S*-(2-thiopyridyl)-L-cysteine **6** (0.100g, 0.376 mmol), triethylamine (1.04 mL, 7.50 mmol), H_2O (1 mL), and CH_3CN (2 mL). The resulting mixture was stirred for 4 h and then concentrated by rotary evaporation to obtain a pale white solid. Recrystallization from methanol and ether afforded 0.138g (80%) of thiopyridyl-biotin **10** as a white solid. 1H NMR (500 M Hz, $D_2O:CD_3OD$, 10:90) δ =8.44–8.43 (m, 1H); 7.87–7.82 (m, 2H); 7.30–7.27 (m, 1H); 4.71 (dd, J =9.1, 4.2 Hz, 1H); 4.54–4.51 (m, 1H); 4.35–4.32 (m, 1H); 3.34 (dd, J =13.0, 4.3 Hz, 1H); 3.25–3.21 (m, 1H); 3.18 (dd, J =14.0, 9.0 Hz, 1H); 2.94 (dd, J =12.8, 5.0 Hz, 1H); 2.73–2.70 (m, 1H); 2.33–2.24 (m, 2H); 1.76–1.59 (m, 4H); 1.50–1.46 (m, 2H). ^{13}C NMR (100 M Hz, $D_2O:CD_3OD$, 10:90) δ =176.6, 173.9, 166.3, 160.5, 150.6, 139.6, 123.0, 121.8, 63.4, 61.7, 57.0, 53.1, 41.3, 41.2, 36.5, 29.6, 29.4, 26.8. HRMS (FAB) m/e for $C_{18}H_{25}N_4O_4S_3$ ($M+H$)⁺, calcd 457.1038, found 457.1049.

Aminohexyl-guanosine-5'-monophosphate (17). 220 mg (0.44 mmol) 2'-*O*, 3'-*O*, N^2 -triisobutrylguanosine **15** were co-evaporated three times from pyridine, dried in vacuo over P_4O_{10} , and dissolved in 3.0 mL dry CH_3CN under argon atmosphere. To this, a solution of 150 mg (0.22 mmol) *N*-monomethoxytrityl-aminohexyl-cyanoethyl-*N,N*-diisopropyl phosphoramidite in 3.0 mL dry CH_3CN , and, subsequently, 1.9 mL of a 0.47 M solution of tetrazol in pyridine were added under argon atmosphere. After 20 min, 3.0 mL of 0.1 M I_2 , dissolved in THF:pyridine: H_2O (2:2:1 v/v) was added. Excess I_2 was reduced by adding 10.0 mL of a saturated $Na_2S_2O_3$ solution, the product extracted with 50.0 mL CH_2Cl_2 . The organic phase was washed with 40 mL of saturated $NaHCO_3$, dried with Na_2SO_4 , and concentrated in vacuo. Purification was achieved by flash chromatography (silica gel 0.063–0.200 mm, $CH_2Cl_2:CH_3OH$ (95:5 v/v)). The product was dissolved in 0.3 mL ethanol and incubated overnight in 1.0 mL 33% NH_3 at 55 °C. After removal of excess NH_3 in vacuo the product was

incubated in 1.0 mL 80% acetic acid for 45 min at 23 °C. Excess acetic acid was evaporated in vacuo, the product suspended in 1.0 mL water and then dried in vacuo for 20 h. Final purification was achieved by dissolving the colourless powder in 2.0 mL buffer A (100 mM triethylammonium acetate, pH 7.0) and preparative reversed phase HPLC under the following conditions: 0–15 min: 85% buffer A (100 mM triethylammonium acetate, pH 7.0), 15% buffer B (100 mM triethylammonium acetate pH 7.0 in 80% CH_3CN). Combined fractions were lyophilized in vacuo to yield 24.2 mg of a colourless powder (24%). MS (ESI⁺): m/e calcd for $C_{16}H_{26}O_8N_6P$: 463.3, found: 463.3 MH; HPLC (analytical): 10.69 min (7.98 min for triisobutrylguanosine); UV (H_2O): λ_{max} =252.2 nm, 272 nm (shoulder).

Initiator nucleotide (4a). 24.2 mg (52.4 μ mol) amino-hexyl-guanosine-5'-monophosphate **17** and 194 mg (340 μ mol) sulfosuccinimidyl-(biotinamido)-ethyl-1,3-dithiopropionate **18** were dissolved in 35 mL 0.02 M KH_2PO_4 pH 8.1 and stirred at 23 °C overnight. The solution was concentrated to 7.7 mL in vacuo and purified by preparative reversed-phase HPLC as described for amino-hexyl-guanosine-5'-monophosphate. Combined fractions were lyophilized in vacuo to yield 26.4 mg of a white solid (31 μ mol, 50%). HABA-Test (SIGMA): 27.3 mg; 1H NMR (400 M Hz, D_2O): δ =8.07 (s, 1H), 5.88 (d, J =6 Hz, 1H), 4.82 (m, 1H), 4.55 (m, 1H), 4.48 (t, J =4 Hz, 1H), 4.36 (m, 1H), 4.29 (m, 1H), 4.04 (s, 2H), 3.69 (t, J =6.5 Hz, 2H), 3.47 (t, J =6.5 Hz, 2H), 3.36 (m, 1H), 3.07 (t, J =7 Hz, 2H), 2.91 (m, 3H), 2.82 (m, 2H), 2.74 (m, 1H), 2.60 (t, J =6.5 Hz, 2H), 2.29 (t, J =7.75 Hz, 2H), 1.70–1.30 (m, 10H), 1.12 (m, 4H); ^{13}C NMR (500 M Hz, D_2O): δ =177.2, 174.2, 165.6, 159.2, 154.3, 152.3, 137.8, 116.6, 87.2, 84.1 (d, J_{CP} =9 Hz), 73.8, 70.8, 66.6 (d, J_{CP} =5.5 Hz), 65.2 (d, J_{CP} =5 Hz), 62.4, 60.6, 55.8, 40.1, 39.6, 38.2, 37.2, 35.8, 35.4, 33.8, 30.0 (d, J_{CP} =7 Hz), 28.5, 28.2, 28.0, 26.1, 25.5, 24.9; ^{31}P NMR (121.5 M Hz, D_2O): δ =−0.1 (s, 1P); MS (MALDI): m/z calcd for $C_{31}H_{50}O_{11}S_3N_9P$: 853.6, found: 853 MH; HPLC (preparative): 17.21 min (10.51 min for amino-hexyl-guanosine-5'-monophosphate); UV (H_2O): λ_{max} =252.2 nm, 270 nm (shoulder).

Alkylsulfide-primed RNA (4b*). 4a-initiated 94mer RNA was incubated with the same volume of a solution containing 200 mM DTT, pH 7.4 overnight at 23 °C, afterwards precipitated by addition of NaOAc/ethanol, washed several times with 70% ethanol and redissolved in the required buffer.

L-Citrullyl-cysteine-trifluoroacetate (13). To a solution of 1.98 g (5.00 mmol) of the activated ester N^{α} -Boc-citrulline-*p*-nitrophenylester **11** in 10 mL DMF a suspension of 1.82 g (5.00 mmol) *S*-Tritylcysteine **12** in 20 mL of triethylamin:dimethylformamid (1:1 v/v) was added dropwise. The solution was stirred at ambient temperature for 16 h, concentrated in vacuo, and the product was purified by flash chromatography $CHCl_3:CH_3OH$ (1:9 v/v). The product was dissolved in a mixture of 5 mL of trifluoroacetic acid and 5 mL of ethylmercaptane. After 2 h of stirring, the product was

precipitated by adding 50 mL of diethylether, subsequently filtered and washed with diethylether. Recrystallization from methanol/diethylether afforded 0.73 g (61%) of a white solid. ^1H NMR (300 M Hz, D_2O): δ = 4.48 (dd, 3J = 5 Hz, 3J = 5 Hz, 1H, $\text{CH}_2\text{CH}_2\text{CH}$), 4.11 (dd, 3J = 7 Hz, 3J = 7 Hz, 1H, NDCHCH_2), 3.14 (t, 3J = 7 Hz, 2H, NDCH_2CH_2), 3.03–2.91 (m, 2H, NDCHCH_2), 1.98–1.90 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}$), 1.66–1.55 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$).

L-Citrullyl-S-(2-thiopyridyl)-cysteine trifluoroacetate (14). α To 0.78 g (2.00 mmol) of the dipeptide **13** in 15 mL methanol:water (1:1 v/v) were added 2.2 g (10.0 mmol) 2,2'-dipyridyl disulfide. The solution was stirred at ambient temperature for 16 h, concentrated in vacuo to 2–3 mL and then extracted water (5×10 mL). The aqueous phases were combined, washed with three 30 mL portions of CH_2Cl_2 and then lyophilized. The impurities in the crude product were removed by flash chromatography $\text{CHCl}_3:\text{CH}_3\text{OH}$ (1:9 v/v) and the purified product was eluted with water. After lyophilization of the combined aqueous phases 0.79 g (79.2%) of the product were obtained as a pale yellow powder. ^1H NMR (400 M Hz, D_2O): δ = 8.67 (d, 3J = 7 Hz, 1H, heteroaryl-H), 8.42 (dd, 3J = 9 Hz, 3J = 8 Hz, 1H, heteroaryl-H), 8.25 (d, 3J = 9 Hz, 1H, heteroaryl-H), 7.83 (dd, 3J = 7 Hz, 3J = 8 Hz, 1H, heteroaryl-H), 4.11 (dd, 3J = 8 Hz, 3J = 5 Hz, 1H, NDCHCH_2), 3.50 (dd, 2J = 15 Hz, 3J = 5 Hz, 1H, CH_2S), 3.33 (dd, 2J = 15 Hz, 3J = 8 Hz, 1H, CH_2S), 3.14 (t, 3J = 9 Hz, 2H, NDCH_2), 1.99–1.93 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}$), 1.66–1.55 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$); MS (FD): m/z calcd for $\text{C}_{14}\text{H}_{22}\text{O}_4\text{S}_2\text{N}_5$ [$\text{M}^+ - \text{CF}_3\text{COO}^-$] = 388, found 388.

Acknowledgements

This study was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to M.F. and by a NATO travel grant to M. F. and J. S. N. We thank Nicole Rubner for operating HPLC-stability studies, Carol Zehetmeier for excellent technical assistance, Michael Blind, Günter Mayer, Felix Hausch, Barbara Gatto, Nikolai Raffler and Oliver Thum for helpful discussions and Wolfgang Steglich and Ernst-L. Winnacker for support. A. J. and B. S. express their gratitude to the DFG for support. J. S. N. thanks the following agencies for support in the

form of awards: the Camille and Henry Dreyfus Foundation (Teacher-Scholar Award), the Alfred P. Sloan Foundation (Alfred P. Sloan Research Fellowship), and the American Chemical Society (Arthur C. Cope Scholar Award).

References

- Dewey, T. M.; Zyzniewski, M. C.; Eaton, B. E. *Nucleosides Nucleotides* **1996**, *15*, 1611.
- Aurup, H.; Williams, D. M.; Eckstein, F. *Biochemistry* **1992**, *31*, 9636.
- Burgin, A. B.; Pace, N. R. *EMBO J.* **1990**, *9*, 4111.
- Burgstaller, P.; Famulok, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1189.
- Breaker, R. R. *Chem. Rev.* **1997**, *97*, 371.
- Pan, T. *Curr. Opin. Chem. Biol.* **1997**, *1*, 17.
- Jäschke, A. In *The Many Faces of RNA*; Eggleston, D. S.; Prescott, C. D.; Pearson, N. D., Eds.; Academic Press: London, 1998; pp 179–190.
- Hausch, F.; Jäschke, A. *Bioconjug. Chem.* **1997**, *8*, 885.
- Zhang, B.; Cech, T. R. *Nature* **1997**, *390*, 96.
- Zhang, B.; Cech, T. R. *Chem. Biol.* **1998**, *5*, 539.
- Sampson, J. R.; Uhlenbeck, O. C. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 1033.
- Connolly, B. A.; Romaniuk, P. J.; Eckstein, F. *Biochemistry* **1982**, *21*, 1983.
- Seelig, B.; Jäschke, A. *Tetrahedron Lett.* **1997**, *38*, 7729.
- Pitulle, C.; Kleineidam, R. G.; Sproat, B.; Krupp, G. *Gene* **1992**, *112*, 101.
- Eisele, K.; Hoppe Seylers, Z. *Physiol. Chem.* **1975**, *356*, 845.
- Hnatowich, D. N.; Virzi, F.; Ruschkowski, M. *J. Nucl. Med.* **1987**, *28*, 1294.
- Green, N. M. *Meth. Enzymol.* **1970**, *18A*, 418.
- Igloi, G. L. *Biochemistry* **1988**, *27*, 3842.
- Wecker, M.; Smith, D.; Gold, L. *RNA* **1996**, *2*, 982.
- Vaish, N. K.; Heaton, P. A.; Fedorova, O.; Eckstein, F. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2158.
- Axelrod, V. D.; Kramer, F. D. *Biochemistry* **1985**, *24*, 5716.
- Seelig, B.; Jäschke, A. *Bioconjug. Chem.* **1999**, *10*, 371.
- Unrau, P. J.; Bartel, D. P. *Nature* **1998**, *395*, 260.
- Seelig, B.; Jäschke, A. *Chem. Biol.* **1999**, *6*, 167.
- Geiger, A.; Burgstaller, P.; von der Eltz, H.; Roeder, A.; Famulok, M. *Nucleic Acids Res.* **1996**, *24*, 1029.
- Bartel, D. P.; Szostak, J. W. *Science* **1993**, *261*, 1411.
- Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, *15*, 8783.
- Milligan, J. F.; Uhlenbeck, O. C. *Meth. Enzymol.* **1989**, *180*, 51.