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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Streamlined Process for the Chemical Synthesis of RNA Using 2′-*O*-Thionocarbamate-Protected Nucleoside Phosphoramidites in the Solid Phase

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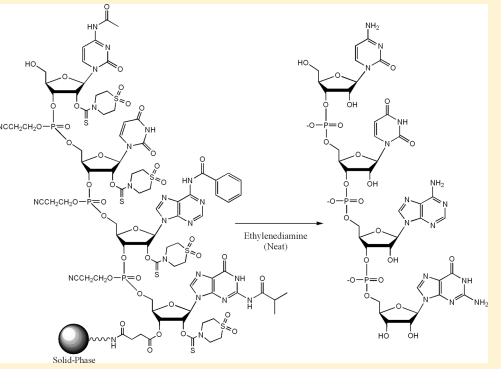
ABSTRACT: An improved method for the chemical synthesis of RNA was developed utilizing a streamlined method for the preparation of phosphoramidite monomers and a single-step deprotection of the resulting oligoribonucleotide product using 1,2-diamines under anhydrous conditions. The process is compatible with most standard heterobase protection and employs a 2′-*O*-(1,1-dioxo-1λ^6-thiomorpholine-4-carbothioate) as a unique 2′-hydroxyl protective group. Using this approach, it was demonstrated that the chemical synthesis of RNA can be as simple and robust as the chemical synthesis of DNA.



INTRODUCTION

Recent revelations about the large and expanding roles that noncoding RNAs have in the biology of cell differentiation and maintenance have inspired a significant expansion in both research and drug discovery on small RNAs.[1−4] In turn, there has been a renewed interest in finding a cheaper and less cumbersome method for the chemical synthesis of small RNAs and complex RNA sequences containing a variety of chemical modifications.[5,6] Several new protective groups, synthesis protocols, and deprotection strategies have been proposed and demonstrated.[7−14] Akin to previous protocols, none of these recent developments combine streamlined, high-yield, and inexpensive monomer synthesis with simple and inexpensive isolation of the desired RNA products.

For many years, it had been acknowledged that the most effective method for making sequence-defined DNA oligonucleotides is based upon the solid-phase phosphoramidite method.[15,16] Modifications to the original protocols gave rise to streamlined, high-yield, inexpensive monomer synthesis, followed by rapid, high-yielding, solid-phase oligonucleotide synthesis, and finally simple, inexpensive deprotection and isolation of the desired DNA products.[17] By comparison, application of the phosphoramidite method to RNA synthesis has historically reported monomer preparations that are cumbersome, low-yielding, and expensive. The lower coupling efficiency of typical RNA monomers also severely limits the length of RNA attainable by solid-phase oligonucleotide synthesis, and the postsynthetic processing needed to deprotect the RNA products can be unwieldy, time-consuming, difficult to scale, and expensive.

Achieving high-yield synthesis of phosphoramidite monomers necessitates that protection of each of the reactive groups on the carbohydrate residues be accomplished regiospecifically in nearly quantitative yield. For DNA synthesis monomers, this is illustrated by the preparation of 5′-(4,4′-dimethoxytrityl)-*N*-acetyl-2′-deoxycytidine 3′-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (Scheme 1).

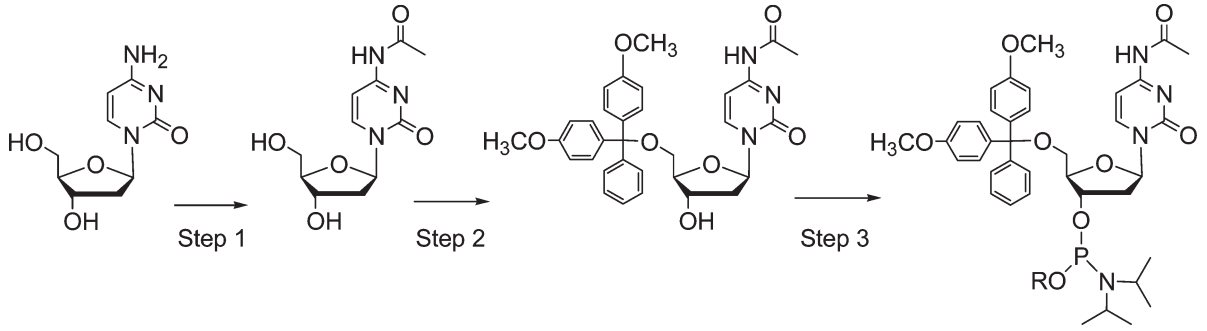
2′-Deoxynucleosides are converted in three steps to the desired phosphoramidite monomer: (1) The heterobase amine is regiospecifically converted to an amide using transient silylation of the hydroxyl residues with trimethylsilyl chloride and acylation. (2) The primary hydroxyl (5′-OH) is regioselectively protected with 4,4′-dimethoxytriphenylmethyl chloride (DMT-Cl). (3) The remaining secondary hydroxyl is phosphitylated using (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite or (2-cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite. The overall scheme gives ∼70% isolated yield of the desired DNA synthesis monomer. This high-yield regiospecific scheme results in low-cost, high-purity DNA monomers.

The typical deprotection and isolation protocol for DNA products is also simple and efficient. Once the DNA oligonucleotide

Received: February 18, 2011
Published: June 20, 2011

dx.doi.org/10.1021/ja201561z | *J. Am. Chem. Soc.* 2011, 133, 11540–11556

Scheme 1. Three-Step Procedure for the Preparation of DNA Synthesis Monomers



has been prepared in the solid phase, the phosphorus protective groups and solid-support linkage are cleaved concurrently with the heterobase protective groups using ammonium hydroxide. For large-scale synthesis or to obtain higher integrity DNA products, the phosphorus protective group is removed first with a hindered primary or secondary amine such as *tert*-butylamine or diethylamine.[18] The amine traps the acrylonitrile byproduct of the β-cyanoethyl protective group[19] and prevents modification to the heterobases. The solid-support linkage is then cleaved along with the heterobase amide protective groups using a more nucleophilic amine, typically ammonia or methylamine under aqueous conditions. The DNA dissolves in this aqueous amine solution and is isolated by filtration from the solid support. The aqueous amine solution containing the DNA is finally evaporated and the product purified by reverse-phase or ion-exchange chromatography.

Monomers for RNA synthesis have conventionally been much more difficult to prepare than DNA monomers, the additional hydroxyl in the 2′-position compelling the presence of an extra protective group. For a streamlined synthesis, this protective group needs to be efficiently placed on the nucleoside in a regiospecific manner. The vicinal *cis*-diol motif of the 2′- and 3′-hydroxyls on the RNA nucleoside is notoriously difficult to regiospecifically protect due to protective group migration between the two hydroxyls. Typical esters are eliminated as a potential choice for 2′-hydroxyl protection because the rate of acyl migration is such that it is difficult to isolate the 2′- from the 3′-isomer.[20] A number of quite elegant attempts at producing RNA synthesis monomers have been reported, but often the reaction of the protective group reagent with the secondary hydroxyl proceeds in low yield, and the reagent itself is difficult or expensive to synthesize.[21] In several examples, low reactivity of the protective group reagent necessitates conversion of the 2′-hydroxyl to an alkoxide or metal complex,[22] which invariably results in low yields due to side reactions with the heterobases.[23]

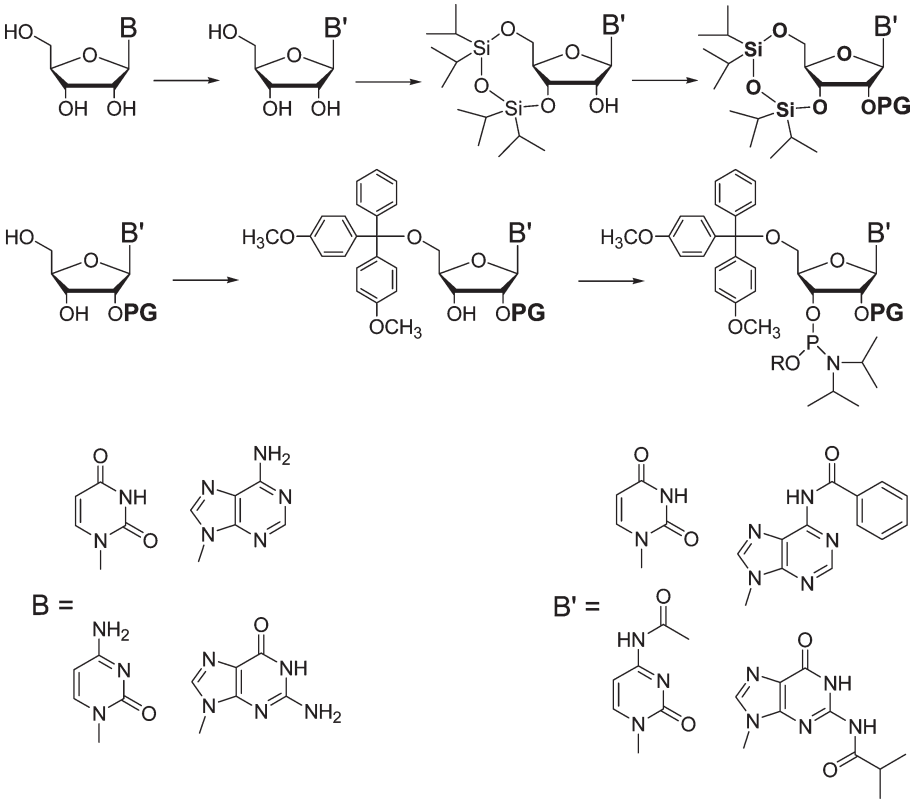
The most frequently used 2′-protective group for RNA synthesis is the fluoride-labile *tert*-butyldimethylsilyl (TBDMS) ether.[24] This protective group is commonly placed upon the nucleoside non-regiospecifically, and chromatographic separation is used to isolate the 2′-protected nucleoside intermediate from the 3′-protected regioisomer, giving low yields of the final RNA monomers.[25] The use of silicon-containing protective groups for the 2′-hydroxyl, removed from the RNA product with fluoride ion, has produced robust methods for the chemical synthesis of RNA but necessitates removal of the synthetic RNA from glass surfaces prior to fluoride ion exposure. This technique is not practical in applications such as beads or microarrays[26] and, together with the extra processing and purification required to remove the fluoride reagent, greatly increases the complexity of performing high-throughput RNA synthesis.

In contrast, RNA monomers that do not require fluoride ion to remove the 2′-hydroxyl protective group can utilize the convenient regiospecific synthesis methods originally developed by Markiewicz (Scheme 2).[27] This approach was shown to be an effective way to produce 2′-orthoester-protected RNA monomers in high yield.[28] A similar strategy has been described to produce TBDMS-protected RNA monomers in higher yields by eliminating the need for chromatographic separation of the 2′- and 3′-isomers using 3′−5′-cyclic di-*tert*-butylsilane-protected nucleosides.[29] The TBDMS group is regiospecifically attached to the 2′-position, and then the 3′−5′-cyclic silane is orthogonally removed using HF/pyridine solutions in dichloromethane (DCM) at low temperature.[30]

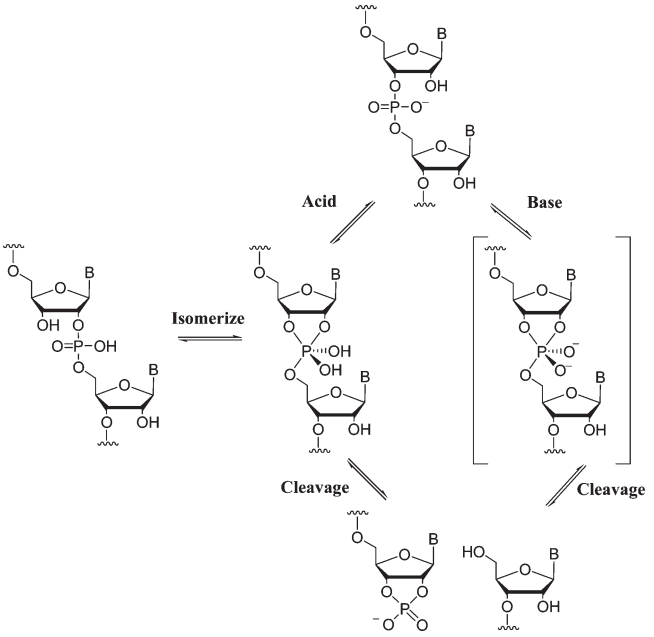
It has been well accepted in the literature that the protective group for the 2′-hydroxyl should be removed from the final RNA product under neutral to mildly acidic conditions due to the lability of the RNA internucleotide linkage at pH extremes[31] (Scheme 3). In both aqueous acid and aqueous base, the 3′−5′-phosphodiester linkage of the RNA molecule entropically favors the formation of a nucleotide 2′−3′-cyclic phosphate, resulting in strand cleavage. The ease with which these reactions can occur has limited the choice of functional groups appropriate for protection of the 2′-hydroxyl and significantly constrains the reaction conditions suitable for removal. Under acidic conditions, the internucleotide bond of RNA can cleave or isomerize, giving rise to 2′−5′-linked oligonucleotides, while under basic conditions RNA readily undergoes cleavage via a transesterification reaction involving the deprotonated 2′-oxyanion group.[32] Under basic conditions, 2′→3′ migration does not occur, apparently because the lifetime of the dianionic phosphorane intermediate is too short for pseudorotation to occur.[33] The pK_a of the RNA 2′-hydroxyl in aqueous solution can vary depending on salt concentration and base sequence, but it is typically reported to be <13.[34,35] The pK_a of (protonated) ammonia is ∼9.2; the concentrated aqueous ammonium hydroxide solution most often used for removing protective groups from synthetically prepared oligonucleotides thus has a pH > 12. At this high pH, the 2′-hydroxyl will be at least 10% ionized, and base-catalyzed transesterification results in backbone cleavage.

Commonly used nucleobase protecting groups for DNA synthesis are removed with aqueous solutions of amines such as ammonia or methylamine. For such groups to be used in RNA synthesis, it is essential that the 2′-hydroxyl protection remain intact during any aqueous amine treatment, hence the common

Scheme 2. Regiospecific Placement of a 2′-Hydroxyl Protective Group (PG) Using the Markiewicz Method



Scheme 3. RNA Internucleotide Bond Cleavage under Acidic or Basic Conditions



use of the amine-stable, fluoride-labile TBDMS 2′-hydroxyl protecting group. If the 2′-hydroxyl is unintentionally uncovered during the aqueous amine nucleobase deprotection, the result is hydroxyl deprotonation and RNA backbone cleavage. This

Scheme 4. Effect of Solvent on Charge Stabilization and Acid/Base Equilibrium Constants

cleavage is observed during extended treatment at elevated temperatures of amide nucleobase-protected RNA oligonucleotides, due to hydroxide-mediated cleavage of the 2′-TBDMS silicon−oxygen bond,[36] and can be minimized by using aqueous methylamine at ambient temperature for short exposure times.[37]

Under basic conditions, the rate of RNA 2′−3′-cyclophosphate formation and internucleotide bond cleavage is known to be first-order in 2′-oxyanion concentration. From pH 9 to ∼13, specific base catalysis generates a 10-fold increase in the number of reactive 2′-oxyanion groups for each unit increase in pH, and a 10-fold increase in the rate of cleavage.[38] However, it is also known that the ionization constants of weak acids and bases may be substantially altered in the presence of organic solvents.[39] Under certain nonaqueous conditions, amine bases will be less prone to deprotonate alcohols due to the properties of acid/base equilibria in such systems. In general, for a neutral compound ionizing to a charged anionic species, such as a hydroxyl group ionizing to an alkoxide, decreasing the dielectric of the solvent results in a decrease in the acid equilibrium constant (increase in pK_a) for the equilibrium shown in Scheme 4a.

This effect is due to the less polar solvent being less able to stabilize the two new charges formed, resulting in a shift of the

dx.doi.org/10.1021/ja201561z |J. Am. Chem. Soc. 2011, 133, 11540–11556

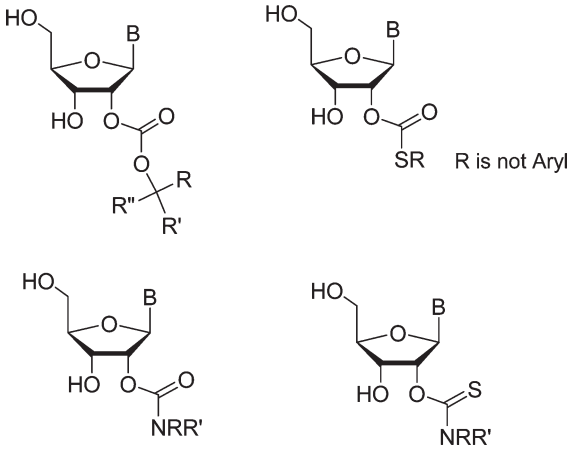


Figure 1. Carbonyl and thionyl 2′-hydroxyl protective group motifs shown to be resistant to 2′→3′ migration.

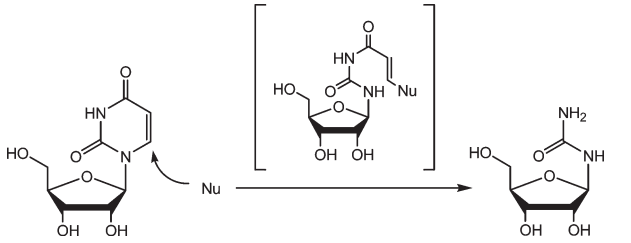
equilibrium to the left. For a charged compound dissociating to a charged plus a neutral compound (Scheme 4b), such as the dissociation of a protonated amine, decreasing the dielectric of the solvent in general results in only relatively small changes in pK_a. The dissociation results in no net change in charge, and the less polar solvent destabilizes both sides of the equilibria, with the resulting changes in pK_a tending to be significantly less. This observation is demonstrated by measuring the change in pK_a of the acid/base pair of phenol and ammonia in water versus acetonitrile (ACN). The pK_a of phenol is reported to be ∼10 in water (dielectric constant, 78), while in ACN (dielectric constant, 36) phenol is a much weaker acid, with pK_a ≈ 27 (ΔpK_a ≈ 17).[40] Ammonia does become a stronger base in ACN; the pK_a of the conjugate acid increases from 9.2 to 16.5 when moving from water to ACN, but the ΔpK_a is only ∼7;[41] the acid/base pair of phenol and ammonia, which in water has a pK_a difference of <1 pK_a unit, has a pK_a difference of ∼10 pK_a units in ACN.

This phenomenon could be used advantageously in a deprotection scheme for synthetic oligoribonucleotides, wherein a hydroxyl becomes significantly less acidic while a conjugate amine base becomes only slightly more basic. The acid/base pair of such an oligoribonucleotide 2′-hydroxyl and an anhydrous amine should show a trend similar to that demonstrated with phenol/ammonia. Based upon reported pK_a values, it should be possible to find an amine whose nucleophilic properties could be utilized to simultaneously remove the heterobase protective group, the 2′-hydroxyl protective group, and the solid-support attachment while not giving base-catalyzed backbone cleavage. This approach would avoid the complications introduced by the use of fluoride and completely avert any possible acid-catalyzed isomerization of the internucleotide bond, resulting in higher integrity RNA synthesis products obtained under simplified conditions analogous to those of DNA synthesis.

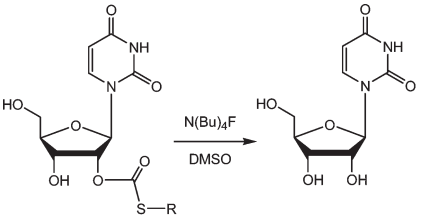
RESULTS

The desire for an efficient monomer synthesis led us to initiate our investigation of nucleophile-labile 2′-protective groups by exhaustively screening possible carbonyl and thionyl groups for two qualities: first, their ability to react with a 3′−5′-tetraisopropyldisiloxane-protected nucleoside to produce the desired 2′-hydroxyl-protected products in high yield, and second, their ability to resist protective group migration during fluoride-mediated removal of the siloxane. We found that rapid 2′→3′ migration limited our choices to non-aryl thiocarbonates, tertiary carbonates, carbamates, and thionocarbamates (Figure 1); all other carbonyl or thionyl protective groups examined quickly isomerized from the 2′- to the 3′-hydroxyl during removal of the disiloxane, using a range of HF/triethylamine (TEA) or HF/pyridine compositions, under conditions of varying concentrations, pH, and temperatures.

Scheme 5. Depyrimidination of Uridine Nucleosides with Peroxyanions



Scheme 6. Cleavage of Thiocarbonate Protective Groups from Uridine Nucleosides with TBAF in DMSO



R	$T_{1/2}$
Methyl	3 min
Benzyl	5 min
Ethyl	10 min
Isopropyl	70 min
t-Butyl	60 min

Tertiary carbonates were previously described as 2′-hydroxyl protective groups for RNA synthesis by Losse et al.[42] Although stable to migration, this protective group motif was eliminated from our study due to our inability to find a high-yield method for its removal with nucleophiles.

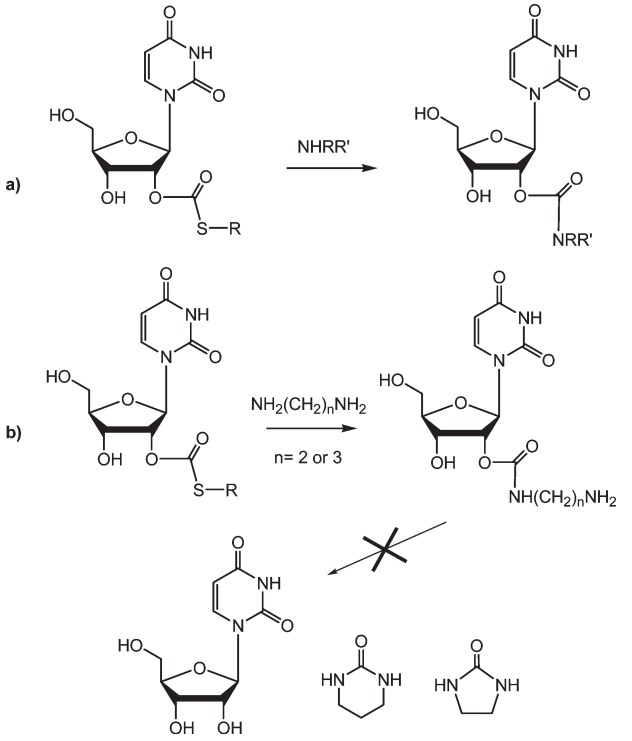
We then evaluated removal of the remaining carbonyl and thionyl protective groups using the mildly basic, highly nucleophilic deprotection conditions containing peroxyanions as developed by Sierzchala et al.[43] Under these conditions, both thionocarbamates and thiocarbonates were removed effectively while carbamates were left intact.

During our studies with peroxyanions, we discovered that uridine heterobase, unlike thymine, is susceptible to cleavage, resulting in a uridine byproduct with a loss of 52 amu. The formation of this compound is consistent with the conversion of the heterobase to urea (Scheme 5) and appears to be analogous to the described depyrimidination of 2′-fluorouridine with alkyl amines.[44] Thus, peroxyanion-containing solutions were abandoned as potential cleavage reagents.

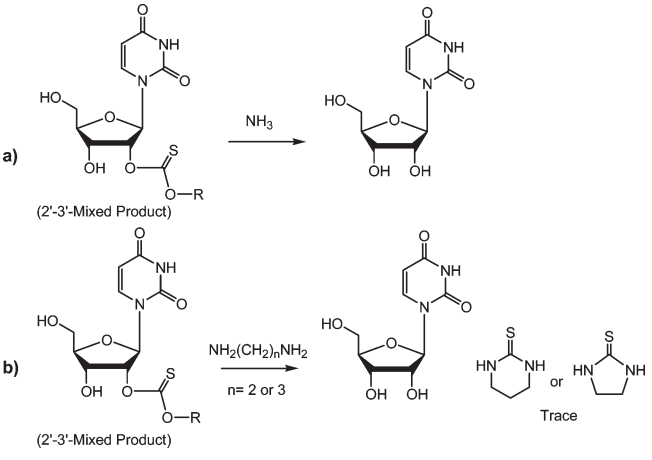
Thiocarbonates could be readily removed from the 2′-hydroxyl using tetrabutylammonium fluoride (TBAF) in dimethyl sulfoxide (DMSO) under conditions that should leave the internucleotide bond intact (Scheme 6). Although effective, the use of fluoride ion for the removal of the 2′-hydroxyl protective group would not allow for a one-step final deprotection and as a result this approach was also abandoned.

Use of strong nucleophiles such as ammonia or methylamine demonstrated that most 2′-carbamates remained completely

Scheme 7. Complete Conversion of Thiocarbonates to Carbamates by Exposure to Nucleophilic Amines with No Detectable Formation of Ureas or Cyclic Ureas



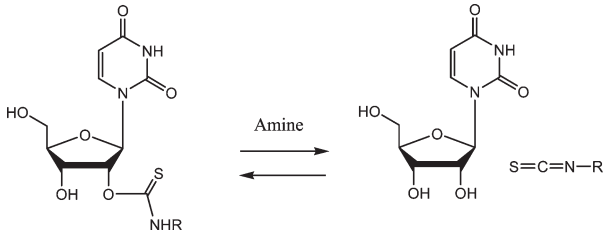
Scheme 8. Complete Cleavage of Thionocarbonates by Exposure to Nucleophilic Amines with Detectable Formation of Ureas or Cyclic Ureas



intact after several hours of treatment at elevated temperatures, and when 2′-thiocarbonates were treated with alkylamines, they were shown to undergo facile transformation into highly stable 2′-carbamate products (Scheme 7a). The use of 1,2- or 1,3-diamines gave only the monosubstituted product and did not result in any detectable formation of the cyclic urea and free 2′-hydroxyl products at temperatures <100 °C (Scheme 7b).

When the sulfur atom of the thiocarbonate linkage was moved to the double bond, forming the thionocarbonate, the reaction with ammonia gas gave exclusively the desired 2′-hydroxyl

Scheme 9. Elimination of Primary Thionocarbamates To Form Isothiocyanates



products (Scheme 8a), and when protected nucleosides were treated with 1,2- or 1,3-diamines at room temperature, along with product it was possible to detect formation of the cyclic thiourea products by LC/MS (Scheme 8b). However, due to rapid 2′→3′ migration, we were unable to isolate the 2′-thionocarbonate-protected nucleosides free of a significant contamination of the 3′-isomer.

These results led us to focus on thionocarbamates, which when treated with strong nucleophilic amines proved to be more labile than the corresponding carbamates. We evaluated the rate of removal of potential thionocarbamate 2′-hydroxyl protective groups with anhydrous gaseous ammonia by preparing a variety of uridine phosphoramidite monomers, each with a different 2′-hydroxyl thionocarbamate protective group, and synthesizing the corresponding protected UT dimers on controlled pore glass (CPG). Ammonia deprotection was performed in a pressure vessel at 80 psi, conditions previously used for deprotection and cleavage of oligonucleotides from a solid-phase resin.[45] While it has been reported that thionocarbamates formed from primary amines are readily cleaved by an E1cb mechanism through base-catalyzed isothiocyanate formation,[46] we found that, under anhydrous gaseous ammonia conditions, thionocarbamates derived from primary amines were cleaved quite slowly. Even the aniline thionocarbamate was slow to cleave, which via elimination should favorably form phenylisothiocyanate (Scheme 9).

The relative acidity of the conjugate acids of the primary amines was not consistently correlated with the rate of deprotection of the thionocarbamate, and most notably the thionocarbamate of morpholine, a secondary amine, was significantly more rapidly removed by gaseous ammonia than any of the tested primary amine thionocarbamates (Table 1).

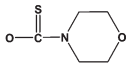
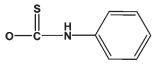
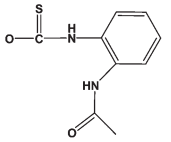
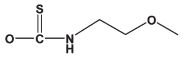
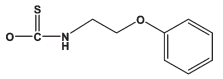
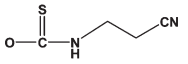
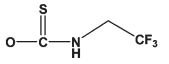
We then screened cleavage rates with gaseous ammonia for a variety of thionocarbamates made with secondary amines that spanned a range of pK_a values. Thiomorpholine-1,1-dioxide in particular became the leading candidate, as the cleavage rate of this group was comparable to the reported rate of deprotection of heterobase-protected *N*-2-isobutyrylguanosine by gaseous ammonia[45] (Table 2).

With this encouraging result, the uridine phosphoramidite monomer, 2′-hydroxyl-protected as the thionocarbamate of thiomorpholine-1,1-dioxide, was used for solid-phase synthesis of U_4T. Deprotection with gaseous ammonia at room temperature for 6 h gave RNA products without detectable amounts of internucleotide bond cleavage, as analyzed by ion-exchange HPLC or LC/MS (Figure 2).

However, for the longer U_9T oligoribonucleotide, 6 h of exposure to ammonia gas at room temperature gave a typical internucleotide bond cleavage profile (Figure 3) and diminished amounts of full-length product. The use of gaseous methylamine for a shorter time[45] gave even more internucleotide bond

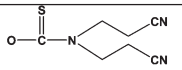
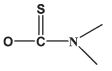
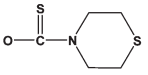
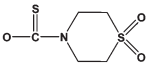
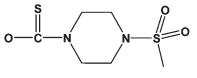
dx.doi.org/10.1021/ja201561z |*J. Am. Chem. Soc.* 2011, 133, 11540–11556

Table 1. Cleavage Rates of Primary Amine 2′-*O*-Thionocarbamate Protective Groups from UT Dimers on CPG Using Ammonia Gas in a Pressure Vessel at 80 psi

Compound Number	2′-Thionocarbamate Structure (Uridine)	% Deprotection		Predicted* pKa (amine conjugate acid)
		1hr	16hr	
6e		4.3	96	9.0
6f		22.4	71.2	4.6
6g			57.6	
6h			43.7	8.8
6i			58.4	8.2
6j			81.3	7.1
6k			26.2	5.5

* Calculated using Advanced Chemistry Development Software V11.02 (1994−2011, ACD/Labs).

Table 2. Cleavage Rates of Secondary Amine 2′-*O*-Thionocarbamate Protective Groups from UT Dimers on CPG Using Ammonia Gas in a Pressure Vessel at 80 psi

Compound Number	2′-Thionocarbamate Structure	% of UT Deprotection				Predicted* pKa
		1h	6h	16h	24h	
6l		3.6	24.5	63.1	80.6	6.1
6m		0	0	8.5	14.5	10.7
6n		0	7.6	35.7	57	9.1
6a		38.1	100	100	100	6.5
6o		9.5	63.9	94	100	6.4

* Calculated using Advanced Chemistry Development Software V11.02 (1994−2011, ACD/Labs).

cleavage. Apparently the rate of removal of the thiomorpholine-1,1-dioxide thionocarbamate group by the nucleophilic gaseous amine was too slow to avoid the gaseous amine base-catalyzed internucleotide bond cleavage.

As discussed above, we believed that it should be possible to find anhydrous amine treatment conditions that do not cause cleavage of the RNA internucleotide bond. This was supported by a report by Goldsborough, in which full-length native RNA

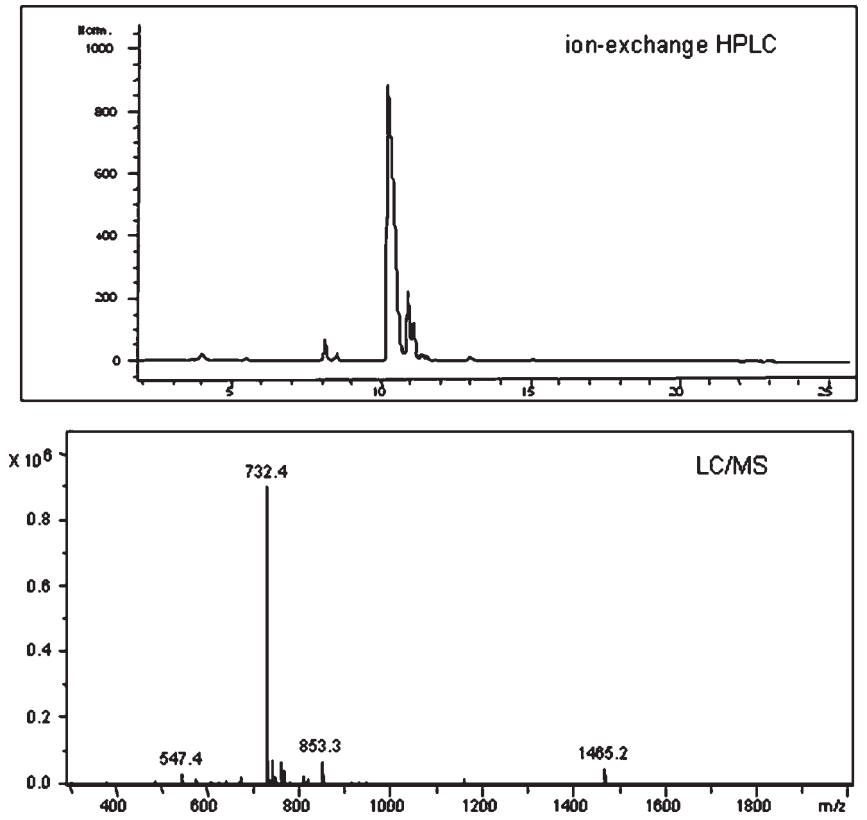


Figure 2. Ion-exchange HPLC and LC/MS evaluation of U_4T synthesized on thymidine containing CPG using 2′-*O*-TC-protected uridine phosphoramidite and deprotected for 6 h with gaseous ammonia.

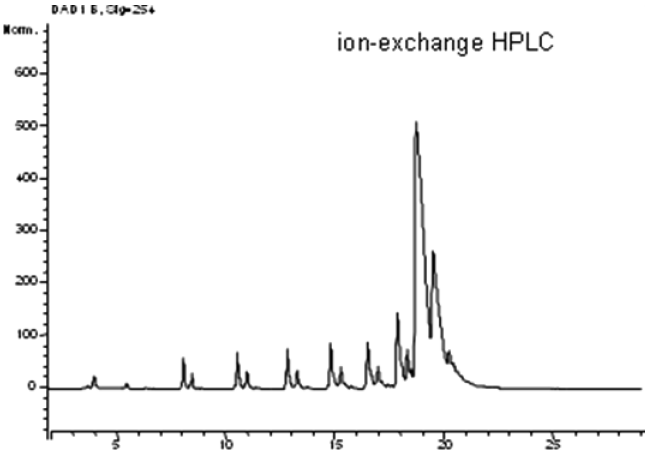


Figure 3. Ion-exchange HPLC evaluation of U_9T synthesized on thymidine containing CPG using 2′-*O*-TC-protected uridine phosphoramidite and deprotected for 6 h with gaseous ammonia.

was recovered from 2′-hydroxyl acetylated transcripts after exposure to alkylamines under conditions containing minimal amounts of water.[47] To explore the stability of RNA under conditions pertinent for removal of a thionocarbamate protecting group, a synthetic, fully deprotected, mixed-sequence 21-mer oligoribonucleotide was treated with a variety of neat, anhydrous alkyl amines. From this study, ethylenediamine (EDA) was shown to be particularly advantageous, since no significant internucleotide cleavage was detected after 17 h at room temperature (Figure 4).

Ethylenediamine was then examined for its ability to remove a variety of thionocarbamate protecting groups from CPG-bound UT_{15} oligonucleotides. These model oligoribonucleotides were synthesized by reacting CPG-bound T_{15} with our previously prepared uridine phosphoramidites containing a series of different thionocarbamates in the 2′-position. The degree of thionocarbamate protective group removal from the products after a 2 h exposure to neat, anhydrous EDA was evaluated by HPLC and LC/MS. The 2′-thionocarbamate of thiomorpholine-1,1-dioxide was shown to be completely removed after 2 h, which was faster than for any of the other tested thionocarbamates (Table 3). Importantly, this rate was once again similar to that reported for removal of the *N*-2-isobutyryl group from guanosine using EDA,[48] suggesting that all protective groups could be removed simultaneously from an oligoribonucleotide without increasing reaction exposure time or resorting to alternative heterobase protective groups.

All four typical nucleoside phosphoramidites were then prepared with the 2′-hydroxyls protected as the thionocarbamate of thiomorpholine-1,1-dioxide, forming 2′-*O*-(1,1-dioxo-1λ^6-thiomorpholine-4-carbothioate), the TC protective group (Figure 5). A mixed-sequence oligoribonucleotide was synthesized on an ABI 394 DNA/RNA synthesizer using a thymidine containing CPG and the standard 1.0 μmol RNA synthesis cycle.

After treatment of the resin with EDA for 2 h, the amine was removed by filtration, and the CPG was washed with ACN. The oligonucleotide remained adsorbed to the CPG during this process, after which it was eluted from the resin with water. This crude product was compared to the same oligoribonucleotide synthesized from commercially obtained 2′-TBDMS-protected

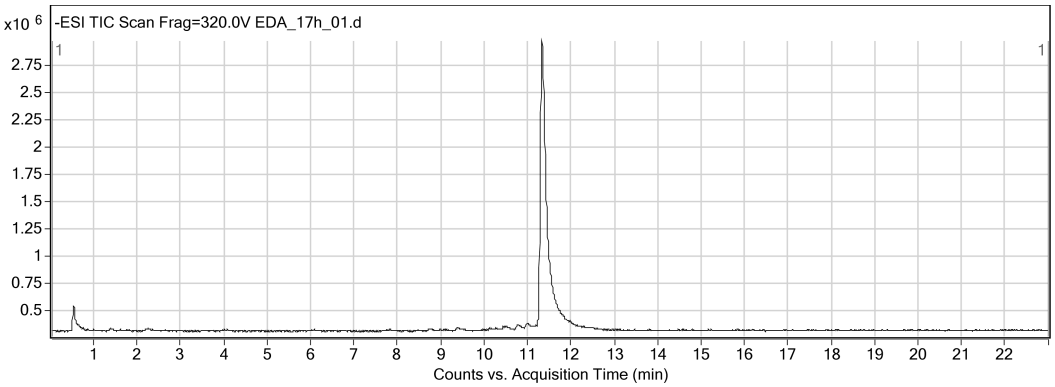
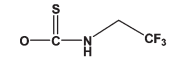
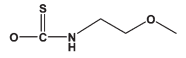
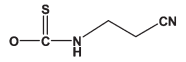
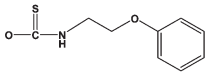
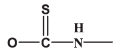
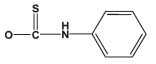
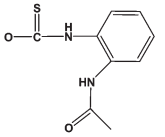
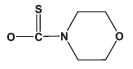
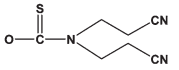
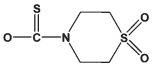


Figure 4. LC/MS of mixed-sequence RNA 21-mer adsorbed to CPG, treated with neat EDA for 17 h, washed with ACN to remove EDA, dried under vacuum, eluted from CPG with water, and injected into the LC/MS instrument.

Table 3. Cleavage Rates of Primary and Secondary Amine 2′-*O*-Thionocarbamate Protective Groups from UT_{15} on CPG Using Neat EDA for 2 h at Room Temperature

Compound Number	2′-Thionocarbamate Structure (Uridine)	% Deprotection of UT_{15} after 2h
6k		23.3
6h		20.1
6j		24.1
6i		22.3
6p		13.8
6f		71.6
6g		78.4
6e		51.5
6l		42.2
6a		100

RNA phosphoramidites using the recommended conditions for synthesis, deprotection, and isolation.[49] Analysis by ion-exchange HPLC (Figure 6) and LC/MS (Figure 7) indicated that the product made with the new RNA phosphoramidite monomers was of a quality comparable to that of the product made with TBDMS-protected monomers.

To further demonstrate the utility of this approach, we prepared a single-strand 54-nucleotide (nt) minimal ribozyme hammerhead[50] (Figure 8) on 3′-*O*-acetylguanosine containing CPG. The oligoribonucleotide should self-cleave in the presence of divalent metal ions to produce a 46-nt fragment and an 8-nt fragment with a 2′−3′ cyclic phosphate.

The oligoribonucleotide was deprotected using neat EDA for 2 h at room temperature, the CPG was washed with ACN and dried with a stream of argon, the desired RNA was washed from the column using 100 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, and the crude product was analyzed by ion-exchange HPLC (Figure 9a). The EDTA was removed using Sepadex G-25 in 15 mM Tris buffer, pH 7.5, and the product was analyzed by ion-exchange HPLC, showing partial cleavage of the oligonucleotide resulting in three distinct products (Figure 9b), and by LC/MS, confirming the identity of the three products as the full-length 54-mer, a 46-mer, and an 8-mer cyclic phosphate (Figures 10 and 11). Magnesium chloride was added to the desalted oligoribonucleotide to a final concentration of 15 mM, and the mixture was allowed to sit at room temperature for 24 h, resulting in complete conversion of the material to the 46-mer and 8-mer cyclic phosphate (Figure 9c). The 46-nt cleavage product was synthesized as an ion-exchange HPLC standard using the same method, confirming the ion-exchange HLPC retention time (Figure 9d).

■ DISCUSSION

The synthesis of RNA phosphoramidite monomers containing a 2′-*O*-(1,1-dioxo-1λ^6-thiomorpholine-4-carbothioate) (TC) protective group can be accomplished regiospecifically in very high overall yield with inexpensive, commercially available reagents. These monomers exploit the standard 5′-DMT protection as well as the amide heterobase and cyanoethyl−phosphorus protections found in routine DNA synthesis. This simple, streamlined monomer synthesis is illustrated in Scheme 10, resulting in a 60−70% overall isolated yield of the phosphoramidite monomer from the tetraisopropyldisiloxane-protected nucleoside for all four common nucleosides.

The observation that RNA with a free 2′-hydroxyl is remarkably resistant to degradation during treatment with certain amine bases under nonaqueous conditions has allowed us to employ 2′-TC-protected RNA monomers for the high-yield synthesis and isolation of mixed-sequence 21-mer oligoribonucleotides, which

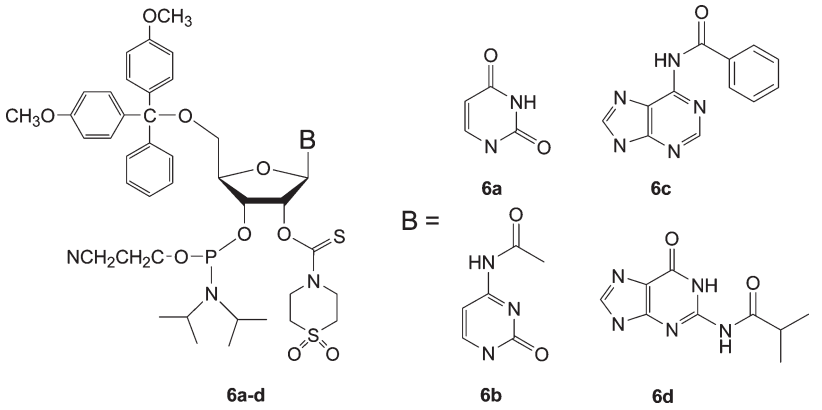


Figure 5. 2′-*O*-TC-protected phosphoramidite monomers used for mixed-sequence RNA synthesis.

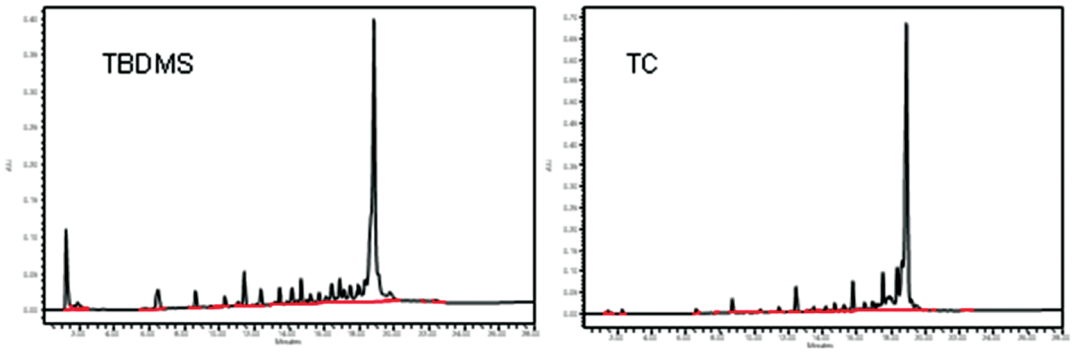


Figure 6. Comparison of ion-exchange chromatograms of crude RNA using TBDMS- and TC-protected monomers synthesized on thymidine containing CPG (5′-GUG UCA GUA CAG AUG AGG CCT-3′): TBDMS, 40.8% full-length; TC, 45.9% full-length by peak integration.

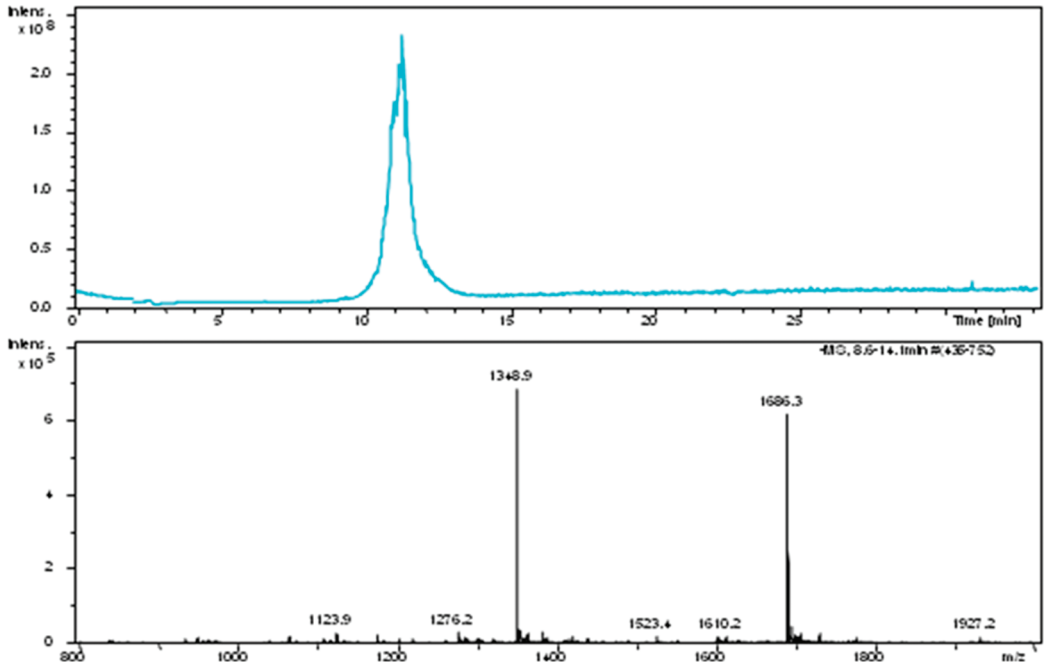


Figure 7. LC/MS evaluation of crude RNA using TC-protected monomers synthesized on thymidine containing CPG (5′-GUG UCA GUA CAG AUG AGG CCT-3′).

dx.doi.org/10.1021/ja201561z |*J. Am. Chem. Soc.* 2011, 133, 11540–11556

are deprotected in a single step with neat EDA. The process flow for the use of neat EDA is further advantageous in that the cleaved and deprotected RNA 21-mer remains insoluble and adsorbed to the solid-phase matrix. To obtain the fully deprotected RNA, the solid support is simply washed with a few drops of water or an aqueous buffer. For syntheses of oligoribonucleotides smaller than ~10 residues, recovery of the desired RNA product is improved if toluene is used as a cosolvent during the deprotection, since very short oligoribonucleotides can be partially soluble in neat EDA. The rate of deprotection appears to be proportional to the amount of EDA present: a 50% solution of EDA in toluene gives complete deprotection in 4 h rather than 2 h, but with no detectable decrease in RNA quality. In our screening of various amines, only anhydrous 1,2-diamines were found to provide effective RNA deprotection without detectable internucleotide bond cleavage.

Successful nucleophilic deprotection of the 2′-TC-hydroxyl using an amine requires that the thionocarbamate be sufficiently electrophilic and sterically accessible such that the rate of deprotection is fast relative to any subsequent base-catalyzed internucleotide bond cleavage. Thus, it proved necessary to design a thionocarbamate that was slightly electron-withdrawing. The desired reaction of a secondary amine thionocarbamate when treated with a nucleophilic amine under nonaqueous conditions is shown in Scheme 11. Amine R-NH_2 attacks the thiocarbonyl **I**, and the desired 2′-hydroxyl **II** is liberated along with the side product thiourea **III**. This reaction likely goes through transition state **IV** and may be concerted or go through tetrahedral intermediate **V**.[51,52]

In addition to the desired reaction is the possible formation of the transcarbamoylation products **VI** and **VII**, a process that may be favored by electron-withdrawing substituents on the amine leaving group. LC/MS analysis of the reaction of anhydrous monoamines with thionocarbamate **I** showed varying amounts of the transcarbamoylated product **VI**, along with the desired product **II**. Compound **VI** was shown to be slowly converted to the desired product **II**, presumably by further reaction with amine and formation of thiourea **VIII** as a byproduct. The time required for conversion to the desired product by this process can potentially result in undesirable amounts of RNA degradation, due to the extended exposure of the deprotected RNA to amine base, even in nonaqueous systems.

1,2-Diamines proved to be particularly advantageous nucleophiles for the deprotection of the thionocarbamates. Ethylenediamine, when used together with the TC protective group formed from thiomorpholine-1,1-dioxide, gave complete deprotection and formed only trace amounts of transcarbamoyated product (Scheme 12). TC-protected RNA **IX** gives the desired fully deprotected product **II**, and thiourea **X** that is formed may decompose to the cyclic ethylenethiourea **XI** and thiomorpholine-1,1-dioxide **XII**. In addition, the transcarbomylation product **XIII** is rapidly converted to the RNA product **II**, presumably via direct intramolecular formation of the cyclic thiourea **XI**, although a reversible addition/elimination mechanism going through the intermediate **XIV** to form thiourea **XV** has not been ruled out.

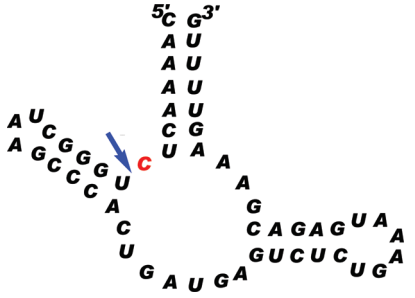


Figure 8. Single-strand 54-nt minimal ribozyme hammerhead.

CONCLUSION

In all cases, the RNA produced by the currently described method gave products that were comparable or superior to control oligoribonucleotides produced using TBDMS-protected

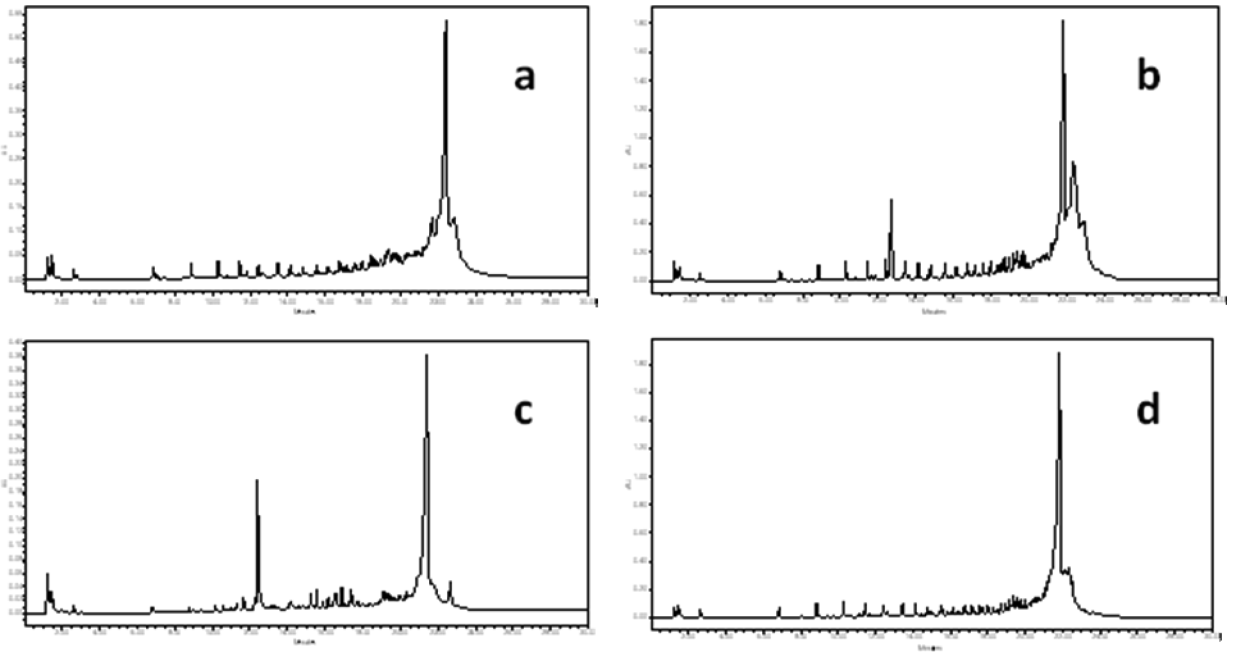


Figure 9. Ion-exchange HPLC of (a) crude 54-mer washed from CPG with 100 mM EDTA, (b) crude 54-mer desalted on G-25, (c) crude 54-mer incubated with 15 mM of $MgCl_2$ for 24 h at room temperature, and (d) crude 46-mer.

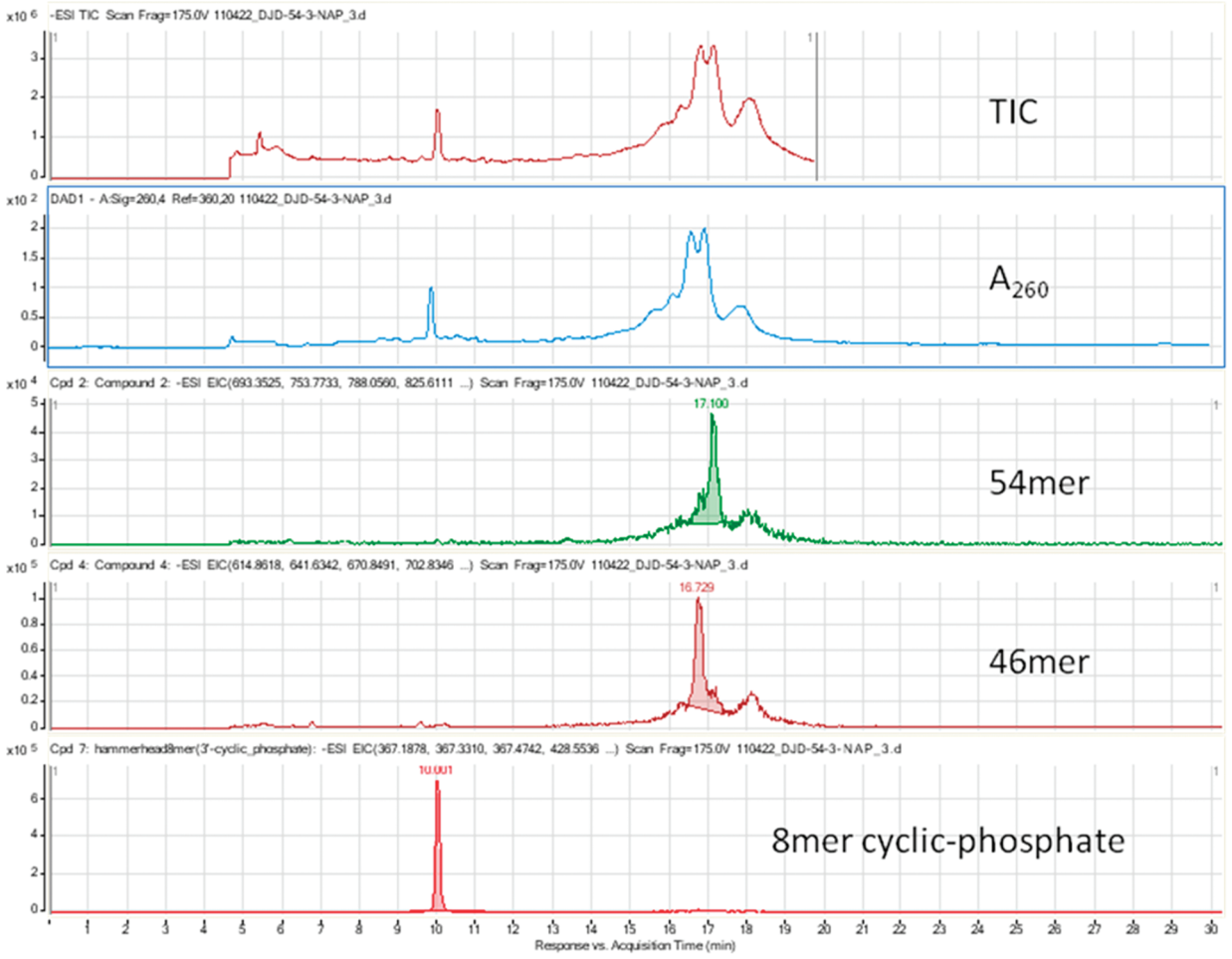


Figure 10. LC/MS of partially cleaved, desalted, 54-nt minimal hammerhead ribozyme showing the total ion chromatogram (TIC), UV absorbance chromatogram (A_{260}), and molecular-specific ion chromatograms for the 54-mer, 46-mer, and 8-mer cyclic phosphate.

monomers when evaluated by ion-exchange HPLC and LC/MS for percent full-length product and total amount of deprotected RNA recovered postsynthesis. This method appears to be broadly useful for the rapid and simplified construction of high-quality RNA sequences.

EXPERIMENTAL SECTION

General Procedures. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. All solvents were obtained from Sigma-Aldrich. Thiomorpholine-1,1-dioxide was obtained from TCI America. 1,1′-Thiocarbonyldiimidazole was obtained ≥95.0% pure from Sigma-Aldrich. (2-Cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite was obtained from either Sigma-Aldrich or ChemGenes. 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^2-acetylcytidine, 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine, 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-N^6-benzoyl-adenosine, and 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-N^2-isobutyryl-guanosine were obtained from ChemGenes. All other DNA synthesis reagents were obtained from Glen Research. Medium-pressure, preparative column chromatography was performed using 230−400 mesh silica gel from Sorbent Technologies. Thin-layer chromatography (TLC) was performed on aluminum-backed silica 60 F_{254} plates from EMD Chemicals. HPLC chromatography was performed on an Agilent Technologies 1200 HPLC instrument, reverse-phase using Zorbax C-18 columns from Agilent Technologies and ion-exchange using DNAPac-100 columns from Dionex. NMR data were recorded on a Bruker 400 MHz spectrometer. Tetramethylsilane was used as an internal reference for ^{1}H and ^{13}C NMR. An external capillary containing 85% H_3PO_4 was used as a reference for ^{31}P NMR. Downfield chemical shifts were recorded as positive values for ^{31}P NMR. LC/MS analyses were performed on Agilent Technologies ESI-TOF and ESI-ion trap mass spectrometers attached to Agilent Technologies 1100 or 1200 HPLC systems.

General Procedure for the Preparation of 2′-*O*-Thionocarbamate-Protected Nucleoside Phosphoramidites 6a−p. *Step 1.* 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl) nucleosides of uridine, adenosine, cytidine, and guanosine (**1**, N-protected as shown in Figure 5) were dissolved in anhydrous ACN at concentrations of 0.2−0.3 M in a round-bottom flask. Next, 1.1 equiv of 1,1′-thiocarbonyldiimidazole and 0.1 equiv of 4-(dimethylamino)pyridine (DMAP) were added to the flask. The reaction was stirred or shaken for 2−3 h and the reaction progress monitored by TLC, showing spot-to-spot conversion. If necessary, the flask was heated to keep the reaction soluble. The reaction products (**2**), the 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-thiocarbonylimidazole nucleosides of uridine and cytidine, usually crystallized after the reaction mixture was left to sit at room temperature and were collected by filtration, while the nucleoside products (**2**) of adenosine and guanosine typically remained in solution.

Step 2. Nucleoside products **2** that remained soluble in ACN were used in solution without isolation or purification. Crystalline nucleoside products **2** were dissolved at a concentration of 0.2 M in ACN with heating and used for the next reaction while still warm to keep the

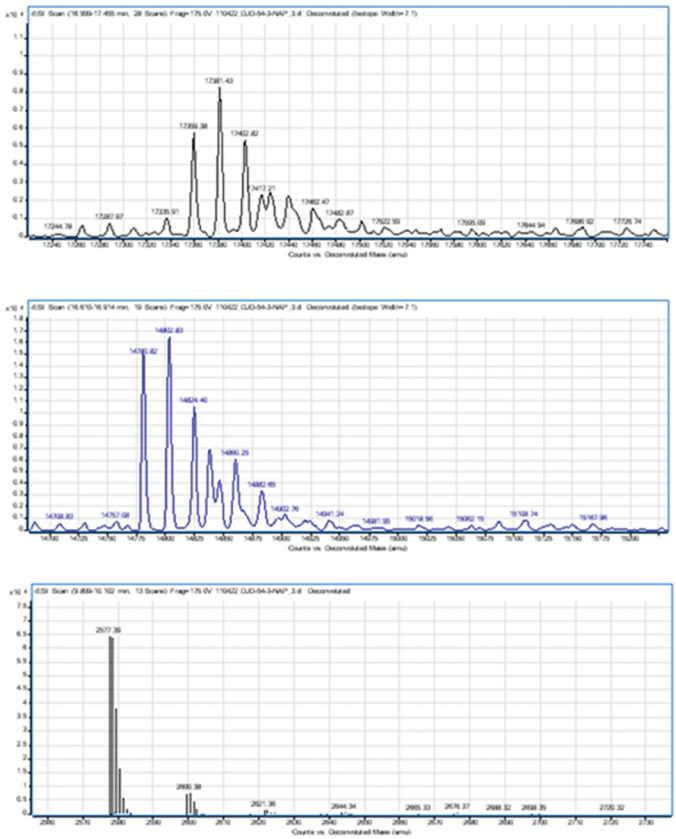


Figure 11. Deconvoluted LC/MS of partially cleaved, desalted, 54-nt minimal hammerhead ribozyme showing the calculated and observed exact masses for the 54-mer, 46-mer, and 8-mer cyclic phosphate products.

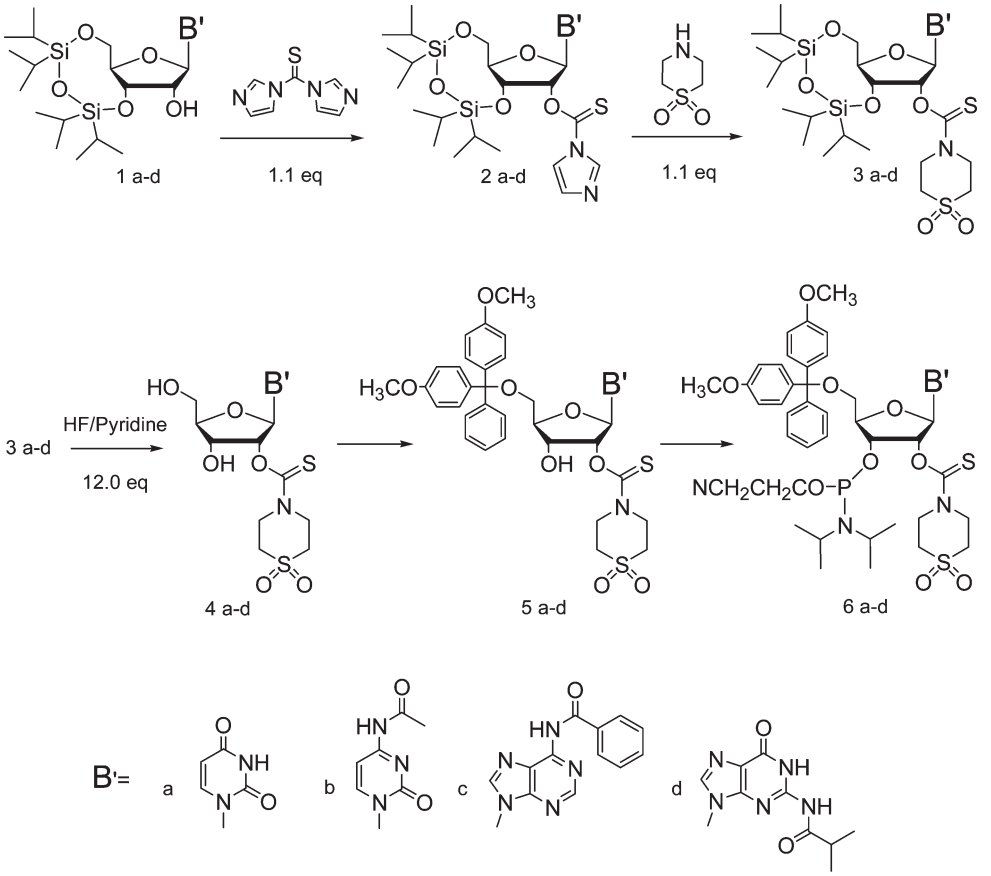
starting material in solution. To each 2′-*O*-thiocarbonylimidazole nucleoside **2** in solution was added 1.2 equiv of a primary or secondary amine, and the reaction mixture was stirred or shaken and monitored by TLC for completion. Most 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-thionocarbamate nucleoside products (**3**) spontaneously crystallized after this step, and any remaining reactants were removed by filtration. Those which did not readily crystallize at this stage were purified by medium-pressure silica gel chromatography. For non-aniline derivatives, the reactions were typically shown to be complete by TLC in 2 h without further heating. To prepare aniline derivatives of cytidine, adenosine, and guanosine, the thiocarbonylimidazole nucleosides **2** were heated to reflux in the presence of a catalytic amount of DMAP. The reaction was typically complete in 3 h, and the products (**3**) were isolated by filtration or silica gel chromatography. Under these reaction conditions, the thiocarbonylimidazole nucleoside (**2**) derived from uridine was preferentially converted to the 2,2′-anhydro product. The 2′-*O*-thionocarbamate aniline derivatives of uridine (**3**) were successfully prepared by displacement of the 2′-*O*-thiocarbonylimidazole in the presence of *p*-toluenesulfonic acid monohydrate (pTsOH). To the dissolved uridine nucleoside **2** in ACN was added 2.0 equiv of the aniline compound and 1.0 equiv of pTsOH. The reaction was heated to reflux and allowed to proceed for 12 h, after which the reaction was ∼50% complete as assayed by TLC, and products were purified by silica gel chromatography.

Step 3. All isolated 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-thionocarbamate nucleoside products (**3**) were dissolved in 2-methyltetrahydrofuran (2-MeTHF) at a concentration of 0.2 M, and 6.0 equiv of anhydrous pyridine was added. The mixture was cooled to 0 °C in an ice/water bath, and 12.0 equiv of hydrogen fluoride was added dropwise as a solution of HF/pyridine (70% HF). After addition, the flask was removed from the ice/water bath, and the reaction was allowed to proceed for 2 h at room temperature. The 2-MeTHF solution was extracted twice with water and then dried over anhydrous sodium sulfate. The sodium sulfate was removed by filtration and the solvent evaporated under vacuum on a rotary evaporator, keeping the water bath temperature <40 °C.

Step 4. The crude desilylated 2′-*O*-thionocarbamate nucleoside product **4** was dissolved in DCM at a concentration of 0.1 M, and 1.05 equiv of *N*-methylmorpholine (NMM) was added, followed by 1.0 equiv of DMT-Cl. The reaction was followed by TLC, and if after 30 min the reaction was not shown to be complete, additional portions of 0.1 equiv each of NMM and DMT-Cl were added sequentially until complete consumption of the starting material.

Step 5. An additional 1.05 equiv of NMM was then added to the reaction mixture, and the flask was cooled to 0 °C in an ice/water bath. To the unisolated 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-thionocarbamate nucleoside product (**5**) was added (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (1.0 equiv) dropwise with stirring, and the reaction was allowed to proceed for 2 h. The reaction was followed by TLC, and if after 2 h the reaction was not shown to be complete, additional portions of 0.1 equiv each of NMM and (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite were added sequentially until complete consumption of the starting material. The reaction mixture was then extracted twice with a saturated aqueous solution of sodium bicarbonate. Bulk water was removed from the organic layer by extraction with brine, and the DCM solution was dried over anhydrous sodium sulfate. The solvent was evaporated on a rotary evaporator, and the resulting 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-thionocarbamate nucleoside-3′-*O*-(β-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite product (**6**) was purified by silica gel chromatography in hexanes using the appropriate ethyl acetate gradients from 50% to 100% or acetone gradients from 10%

Scheme 10. Streamlined Synthesis of 2′-*O*-TC-Protected Phosphoramidite Monomers



to 50%, determined by product resolution on TLC. The identity of each compound was determined by ^{31}P and ^{1}H NMR and mass spectrometry.

Important Experimental Notes. It is important to crystallize or silica gel purify the protected nucleoside products prior to removing the 3′,5′-*O*-tetraisopropyldisiloxane group with HF/pyridine in order to produce high-quality final phosphoramidite products; this removes reaction products from the first two steps of the reaction that can contaminate the final products.

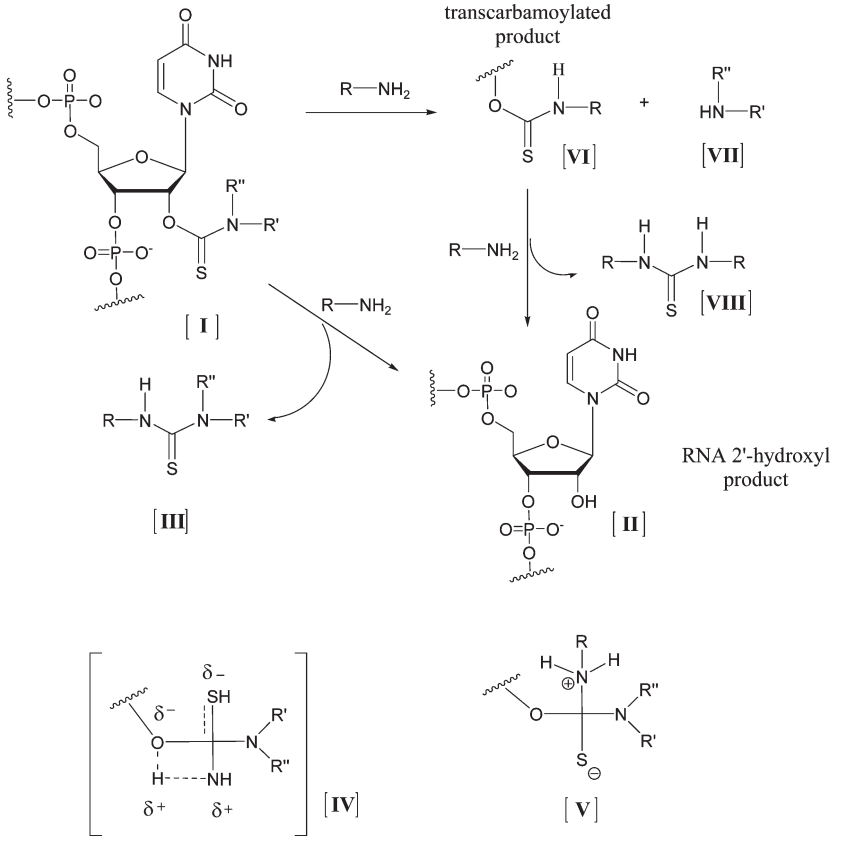
It is important to remove residual HF/pyridine, pyridine, and ACN from the protected nucleosides prior to reacting with DMT-Cl; this is done by extraction with water followed by evaporation under high vacuum. Failure to remove these reagents will result in inhibition of the tritylation and phosphitylation reactions.

It is important not to heat the protected nucleoside products above 40 °C during the evaporation process after removing the 3′,5′-*O*-tetraisopropyldisiloxane group with HF/pyridine; excess heating or exposure to acids or bases can result in detectable 2′→3′ migration of the thionocarbamate protective group.

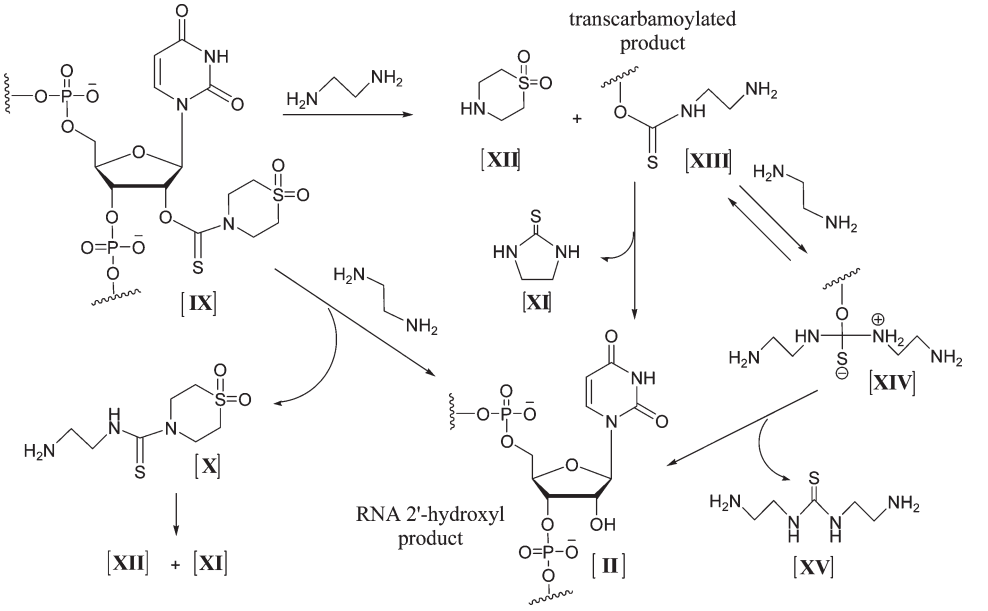
Optimized Protocols for the Preparation of 2′-*O*-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-Protected Nucleoside Phosphoramidites (6a–d). *Synthesis of 2′-O-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-5′-O-(4,4′-dimethoxytrityl)-uridine-3′-O-(β-cyanoethyl)-N,N-diisopropylphosphoramidite (**6a**).* 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-uridine (**1a**, 10 g, 20.5 mmol) was dissolved in ACN (100 mL). 1,1′-Thiocarbonyldiimidazole (4.13 g, 22.6 mmol, 1.1 equiv) and a catalytic amount of DMAP were added, and the mixture was briefly heated with a heat gun to dissolve. The reaction was stirred for 4 h at 30 °C and then placed in a freezer at −20 °C to cool. After 1 h the product had crystallized to a white solid and was isolated by filtration and dried under vacuum, giving 11.5 g of the 2′-thiocarbonylimidazole product (**2a**). The crystallized product was dissolved in ACN (100 mL) by heating with a heat gun, and while still hot, thiomorpholine-1,1-dioxide (2.90 g, 22 mmol, 1.1 equiv) was added to the mixture and the reaction stirred for 2 h at 30 °C. If the reaction mixture crystallized before the first hour of stirring, it was dissolved by additional heating and allowed to react for the final hour. The reaction was evaporated to a solid on a rotary evaporator, and the product (**3a**) was dissolved in 2-MeTHF (100 mL) with 10 mL of anhydrous pyridine (6.0 equiv). The mixture was then cooled to 0 °C in an ice/water bath, and HF/pyridine (3.1 mL, 12 equiv) was added dropwise with stirring. After addition, the flask was removed from the ice/water bath, and the reaction was allowed to proceed for 2 h at room temperature. The 2-MeTHF solution was extracted with water twice (50 mL), and the aqueous layers were combined and extracted twice with 25 mL portions of 2-MeTHF. The organic layers were combined and dried over anhydrous sodium sulfate. The sodium sulfate was removed by filtration and the solvent evaporated under vacuum on a rotary evaporator, keeping the water bath temperature <40 °C. The resulting foam was placed under high vacuum overnight to remove any residual solvent. This crude product (**4a**) was then dissolved in 200 mL of DCM (0.1 M) along with 1.6 mL of NMM (14.7 mmol). DMT-Cl (4.75 g, 14 mmol) was added to the solution with stirring. The reaction progress was followed by HPLC on an Agilent Eclipse XDB-C8 column (3.5 μm, 3.0 × 100 mm column) using a water/ACN gradient from 35% to 95% ACN. After 1 h, the reaction was shown to be ∼75% complete, and another 0.6 mL of NMM was added to the reaction mixture, followed by 1.5 g (4.5 mmol) of DMT-Cl. The reaction was stirred for 2 h, and analysis by HPLC demonstrated that the reaction was complete. To the crude reaction mixture containing **5a**, in the same flask was then added 2.1 mL of NMM (19.5 mmol), followed by 4.1 mL (18.5 mmol) of

dx.doi.org/10.1021/ja201561z |*J. Am. Chem. Soc.* 2011, 133, 11540–11556

Scheme 11. Cleavage of 2′-Thionocarbamates with Alkyl Amines



Scheme 12. Cleavage of 2′-Thionocarbamates with 1,2-Diamines



(2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite. After 15 min, a precipitate formed and was dissolved by the addition of 25 mL of DCM with stirring. After 2 h, the reaction was shown to be complete by HPLC, and 100 mL of DCM was added, followed by 100 mL of saturated $NaHCO_3$. The reaction was stirred until neutralization was complete and then transferred to a separatory funnel. The DCM layer was isolated, extracted with saturated NaCl, and finally transferred to an Erlenmeyer flask containing anhydrous Na_2SO_4. The dried DCM layer was filtered and evaporated on a rotary evaporator. The resulting oil was purified by medium-pressure silica gel chromatography on a column

dx.doi.org/10.1021/ja201561z |*J. Am. Chem. Soc.* 2011, 133, 11540–11556

pre-equilibrated with 10% acetone in hexanes and neutralized with 1% TEA; the excess TEA was removed by flushing the column with a volume of 10% acetone in hexanes. The evaporated product was dissolved in a minimum volume of DCM and placed on top of the silica gel column. The product **6a** was eluted using a gradient of acetone from 10% to 30% and evaporated to a foam, giving 13.5 g of product, a 71% overall yield. The compound was identified by ^{31}P and ^{1}H NMR and mass spectroscopy. ^{31}P NMR (ACN-d_3) δ (ppm): 150.31, 149.40 (diastereomers). ^{1}H NMR (ACN-d_3) δ: 9.2 (bs, 1H), 7.65 (dd, 1H), 7.5, 7.35, 6.9 (m, 13H), 6.18 (dd, 1H), 6.04, 5.97 (m, 1H), 5.47 (dd, 1H), 4.93 (m, 1H), 4.83 (m, 1H), 4.65 (m, 1H), 4.4, 4.32 (m, 1H), 4.1, 4.0 (m, 1H), 3.9 (m, 1H), 3.78 (d, 6H), 3.8−3.7 (m, 2H), 3.6 (m, 2H), 3.49−3.17 (m, 4H), 3.07 (m, 2H), 2.67, 2.52 (m, 2H), 1.17 (m, 12H). ESI-MS: m/z 946.2911 $[M+Na]^+$.

*Synthesis of 2′-O-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-5′-O-(4,4′-dimethoxytrityl)-N^2-acetylcytidine-3′-O-(β-cyanoethyl)-N,N-diisopropylphosphoramidite (**6b**).* 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^2-acetylcytidine (**1b**, 10 g, 19 mmol) was dissolved in DCM (28 mL, 0.7M). 1,1′-Thiocarbonyldiimidazole (3.74 g, 21 mmol, 1.1 equiv) was added with a catalytic amount of DMAP, and the mixture was briefly heated with a heat gun to dissolve. The reaction was stirred for 2.5 h at ambient temperature, after which time the reaction mixture became cloudy from the formation of a white precipitate. Acetonitrile (45 mL) was added to the reaction, followed by thiomorpholine-1,1-dioxide (2.84 g, 21 mmol, 1.1 equiv). The reaction was heated with a heat gun to dissolve the precipitate, and then the solution was stirred for 2 h at ambient temperature. The reaction mixture was evaporated to dryness, dissolved in a minimum amount of ACN with mild heating, and then refrigerated overnight at 5 °C. The product crystallized from this mixture and was isolated by filtration, giving 13.4 g of **3b** after drying under vacuum. The isolated crystals were dissolved in 95 mL of 2-MeTHF, and 9.2 mL of pyridine was added. The mixture was cooled to 0 °C in an ice bath, and HF/pyridine (3.0 mL, 114 mmol, 12 equiv) was added dropwise with stirring. The reaction was stirred at 0 °C for 1 h, transferred to a separatory funnel, and extracted with two 40 mL volumes of water. The water was back-extracted with 2-MeTHF, and the organic layers were combined and evaporated on a rotary evaporator, keeping the water bath at 35 °C, resulting in a glassy solid of 2′-*O*-(1,1-dioxo-1λ^6-thiomorpholine-4-carbothioate)-N^2-acetylcytidine (**4b**). The solid was dissolved in 175 mL of DCM, and 1.6 mL of NMM (15 mmol) was added to the solution, followed by 5.1 g of 4,4′-dimethoxytrityl chloride (15 mmol). The reaction progress was followed by HPLC. After 1 h, the starting material reaction was shown to be 85% converted to product. A second addition of NMM (0.33 mL, 3 mmol) and dimethoxytrityl chloride (1 g, 3 mmol) resulted in complete conversion of the starting material, as assayed by HPLC. The crude reaction mixture containing **5b** was then converted to the final product by addition of 1.6 mL of NMM (14.6 mmol), followed by 3.1 mL of (2-cyanoethyl)-*N,N*-diisopropylchlorophosphoramidite (13.3 mmol). The reaction mixture was stirred for 2 h at room temperature, and then 100 mL of DCM was added, followed by 100 mL of saturated $NaHCO_3$. The reaction was stirred until neutralization was complete and then transferred to a separatory funnel. The DCM layer was isolated, extracted with saturated NaCl, and finally transferred to an Erlenmeyer flask containing anhydrous Na_2SO_4. The dried DCM layer was filtered and evaporated on a rotary evaporator. The resulting oil was purified by medium-pressure silica gel chromatography on a column pre-equilibrated with 10% acetone in hexanes and neutralized with 1% TEA; the excess TEA was removed by flushing the column with a volume of 10% acetone in hexanes. The evaporated product was dissolved in a minimum volume of DCM and placed on top of the silica gel column. The product **6b** was eluted using a gradient of acetone from 10% to 30% and evaporated to a foam, giving 12.3 g of product, a 67% overall yield. The compound was identified by ^{31}P and ^{1}H NMR and mass spectrometry. ^{31}P NMR (ACN-d_3) δ (ppm): 150.08, 149.35 (diastereomers). ^{1}H NMR (ACN-d_3) δ: 8.88 (s, 1H, NH), 8.09−8.02 (dd, 1H, C5 or 6), 7.5, 7.35, 6.89 (m, 13H, DMT), 7.1 (dd, 1H, C5 or 6), 6.2 (m, 1H, 1′), 6.11, 6.08 (m, 1H, 2′), 4.8, 4.71 (m, 1H, 3′), 4.9, 4.65 (m, 2H, thiomorpholine), 4.4, 4.35 (m, 1H, 4′), 4.05, 3.87 (m, 2H, thiomorpholine), 3.49 (m, 2H, iPr), 3.48, 3.41 (m, 2H, 5′), 2.77 (m, 2H, thiomorpholine), 2.66, 2.53 (m, 2H, thiomorpholine), 2.18 (s, 6H, DMT), 2.14 (s, 3H, CH_3), 1.25 (m, 12H, iPr). ESI-MS: m/z 987.3179 $[M+Na]^+$.

*Synthesis of 2′-O-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-5′-O-(4,4′-dimethoxytrityl)-N^6-benzoyladenosine-3′-O-(β-cyanoethyl)-N,N-diisopropylphosphoramidite (**6c**).* 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^6-benzoyladenosine (**1c**, 6.14 g, 10 mmol) was dissolved in DCM (14 mL, 0.7 M). 1,1′-Thiocarbonyldiimidazole (1.88 g, 10.5 mmol) was added with a catalytic amount of DMAP. The mixture was briefly heated with a heat gun to dissolve and then stirred for 3.5 h at ambient temperature. Thiomorpholine-1,1-dioxide (1.49 g, 11 mmol) was added and stirred for 2 h at ambient temperature. An additional 30 mL of ACN was then added to the reaction mixture, followed by 5 mL of anhydrous pyridine. The mixture was cooled to 0 °C in an ice bath, and HF/pyridine (3.1 mL, 119 mmol) was added dropwise with stirring. After the addition was complete, the reaction mixture was stirred for 2 h at ambient temperature. Water (350 mL) was added to the stirring mixture, the resulting emulsion was transferred to a separatory funnel, and a small organic layer was isolated upon standing. The cloudy aqueous layer was then extracted with 2-MeTHF (250 mL) to remove the suspended organic material, and the two organic portions were combined. The organic layer was then extracted again with water (300 mL) to remove residual HF and pyridine, followed by brine (300 mL) to remove bulk water. The organics were finally dried over anhydrous Na_2SO_4. The Na_2SO_4 was removed by filtration, and the solvents were evaporated. The resulting oily residue was dissolved in 2-MeTHF (150 mL) and the solvent evaporated, yielding 5.6 g of a crude solid (**4c**) after drying under high vacuum. The solid was suspended in a mixture of 2-MeTHF/DCM (1:1, 200 mL, 0.05 M), and then 1.2 mL of NMM (11 mmol, 1.1 equiv) and 3.38 g of DMT-Cl (10 mmol, 1 equiv) were added in small portions over 30 min with stirring. Once the reaction was demonstrated to be complete by HPLC, an additional 1.4 mL of NMM (13 mmol, 1.3 equiv) was added to the crude reaction mixture containing **5c**, followed by 2.67 mL of (2-cyanoethyl)-*N,N*-diisopropylchlorophosphoramidite (12 mmol, 1.2 equiv), and the reaction was stirred at ambient temperature for 3.5 h. Saturated $NaHCO_3$ (100 mL) was added with stirring, and the mixture was transferred to a separatory funnel. The organic layer was removed and the aqueous layer extracted with 100 mL of DCM. The organic layers were combined and dried over anhydrous Na_2SO_4. The solvent solution was separated from the Na_2SO_4 by filtration, and the filtrate was precipitated into hexanes (400 mL). The mixture was placed in a freezer at −20 °C overnight, after which time the suspended precipitate had settled and adhered to the walls of the glass flask. Purification was performed by medium-pressure silica gel chromatography on a column pre-equilibrated with 10% acetone in hexanes and neutralized with 1% TEA; the excess TEA was removed by flushing the column with a volume of 10% acetone in hexanes. The cold solvent was decanted, and the crude product was immediately dissolved in dry DCM and loaded onto the top of the silica gel column. The product **6c** was eluted using a gradient of acetone from 10% to 40% and evaporated to a foam, giving 6.72 g of product, a 64% overall yield. The compound was identified by ^{31}P and ^{1}H NMR and mass spectrometry. ^{31}P NMR (ACN-d_3) δ (ppm): 149.98, 149.50 (diastereomers). ^{1}H NMR (ACN-d_3) δ (ppm): 9.52 (bs, 1H, NH), 8.62 (s, 1H, C2 or 8), 8.33 (s, 1H, C2 or 8), 7.98 (m, 2H, Bz), 7.66 (m, 1H, Bz), 7.53 (m, 2H, Bz), 7.43 (m, 2H, DMT), 7.33−7.15 (m, 6H, DMT), 6.85−6.8 (m, 4H, DMT), 6.43 (m, 1H, 1′), 5.25 (m, 1H, 2′), 4.43 (m, 1H, 3′), 4.78 (m, 1H, thiomorpholine), 4.62

(m, 2H, thiomorpholine), 4.44 (m, 1H, thiomorpholine), 4.26 (m, 1H, 4′), 3.8, 3.67 (m, 2H, CE), 3.75 (s, 6H, DMT), 3.59 (m, 2H, iPr), 3.5−3.36 (m, 2H, 5′), 3.14 (m, 4H, thiomorpholine), 2.67, 2.52 (m, 2H, CE), 1.18 (m, 12H, iPr). ESI-MS: m/z 1051.3601 $[M+H]^+$, 1073.3429 $[M+Na]^+$.

*Synthesis of 2′-O-(1,1-Dioxo-1λ⁶-thiomorpholine-4-carbothioate)-5′-O-(4,4′-dimethoxytrityl)-N²-isobutyrylguanosine-3′-O-(β-cyanoethyl)-N,N-diisopropylphosphoramidite (**6d**).* 2′-*O*-(1,1-dioxo-1λ⁶-thiomorpholine-4-carbothioate)-*N*²-isobutyrylguanosine (**1d**, 12.2 g, 20.5 mmol) was dissolved in DCM (40 mL, 0.5 M). 1,1′-Thiocarbonyldiimidazole (4.13 g, 22.6 mmol, 1.1 equiv) and a catalytic amount of DMAP were added, and the mixture was stirred to dissolve. The reaction was stirred for 2 h at ambient temperature. Thiomorpholine-1,1-dioxide (2.90 g, 22 mmol, 1.1 equiv) was added to the mixture, along with 20 mL of ACN. The reaction flask was fitted with a condenser, heated to 50 °C for 30 min, and then stirred for 1.5 h at ambient temperature, during which time the reaction product crystallized. The crystallized product was isolated by filtration and dried under vacuum at room temperature, yielding 15.9 g of dried solid (**3d**). The product was dissolved in 2-MeTHF (100 mL) with 10 mL of anhydrous pyridine (6.0 equiv). The mixture was then cooled to 0 °C in an ice/water bath, and HF/pyridine (3.1 mL, 12 equiv) was added dropwise with stirring. After addition, the flask was removed from the ice/water bath, and the reaction was allowed to proceed for 2 h at room temperature. The 2-MeTHF solution was extracted with water twice (50 mL). The aqueous layers were combined and extracted twice with 25 mL portions of 2-MeTHF. The organic layers were combined and then dried over anhydrous sodium sulfate. The sodium sulfate was removed by filtration and the solvent evaporated under vacuum on a rotary evaporator, keeping the water bath temperature <40 °C. The resulting foam was placed under high vacuum overnight to remove any residual solvent. This crude product (**4d**) was then dissolved in 200 mL of DCM (0.1 M), along with 1.6 mL of NMM (14.7 mmol). DMT-Cl (4.75 g, 14 mmol) was added to the solution with stirring. The reaction progress was followed by HPLC. After 1 h, the reaction was shown to be ∼70% complete, and another 0.7 mL of NMM was added to the reaction mixture, followed by 2.1 g (6.2 mmol) of DMT-Cl. The reaction was stirred for 2 h, and analysis by HPLC demonstrated that the reaction was complete. To the crude reaction mixture containing **5d** in the same flask was then added 2.4 mL of NMM (21.5 mmol), followed by 4.6 mL (20.5 mmol) of (2-cyanoethyl)-*N,N*-diisopropylchlorophosphoramidite, and the reaction was stirred for 2 h at ambient temperature. After 2 h, the reaction was shown to be complete by HPLC, and 100 mL of DCM added, followed by 100 mL of saturated $NaHCO_3$. The reaction was stirred until neutralization was complete, and then it was transferred to a separatory funnel. The DCM layer was isolated, extracted with saturated NaCl, and finally transferred to an Erlenmeyer flask containing anhydrous Na_2SO_4. The dried DCM layer was filtered and evaporated on a rotary evaporator. The resulting oil was purified by medium-pressure silica gel chromatography on a column pre-equilibrated with 10% acetone in hexanes and neutralized with 1% TEA; the excess TEA was removed by flushing the column with a volume of 10% acetone in hexanes. The evaporated product was dissolved in a minimum volume of DCM and placed on top of the silica gel column. The product **6d** was eluted using a gradient of acetone from 10% to 30% and evaporated to a foam, giving 14.4 g of product, a 68% overall yield. The compound was identified by ^{31}P and ^{1}H NMR and mass spectrometry. ^{31}P NMR (ACN-d_3) δ (ppm): 150.96, 149.29 (diastereomers). ^{1}H NMR (ACN-d_3) δ (ppm): 9.03 (bs, 1H, NH), 7.91 (s, 1H, C8), 7.51 (m, 1H, DMT), 7.42−7.20 (m, 8H, DMT), 6.87−6.75 (m, 4H, DMT), 6.39, 6.31 (t, 1H, J = 6 Hz, 2′), 6.22 (q, 1H, J = 5 Hz, 1′), 4.98−4.88 (m, 1H, 3′), 4.88−4.71 (m, 1H, thiomorpholine), 4.58−4.47 (m, 1H, thiomorpholine), 4.45, 4.38 (m, 1H, 4′), 4.20 (m, 1H, thiomorpholine), 4.00 (m, 1H, thiomorpholine), 3.87−3.65 (m, 1H, CE), 3.76 (m, 6H, MeO), 3.59 (m, 2H, iPr), 3.59 (m, 1H, CE), 3.47, 3.4 (m, 2H, 5′), 3.18−2.99 (m, 4H, thiomorpholine), 2.67 (t, 1H, J = 6 Hz, CE), 2.45 (m, 1H, CE), 2.44 (m, 1H, iBu), 1.12 (m, 6H, iBu), 1.18, 1.05 (m, 12H, iPr). ESI-MS: m/z 1033.3741 $[M+H]^+$, 1055.3546 $[M+Na]^+$.

Synthesis and Deprotection of Oligonucleotides. Oligonucleotide synthesis was performed on an ABI 394 automated DNA/RNA synthesizer using 1.0 μmol columns obtained from Glen Research. The standard 1.0 μmol RNA synthesis cycle (10 min coupling) was utilized without modification. The TC phosphoramidites were dissolved in anhydrous ACN at concentration of 0.1 M, and a 0.25 M solution of 5-(ethylthio)-1*H*-tetrazole in anhydrous ACN was used as an activator. Post synthesis, the column containing the CPG was briefly dried with a stream of argon gas, followed by filling the column with neat EDA and allowing the column to sit at ambient temperature for 2 h. The EDA was rinsed from the column by flowing 1 mL of anhydrous ACN through the column, followed by a stream of dry argon. A syringe containing 0.5 mL of water was then attached to the column, and the RNA was washed from the column into a 1.5 mL Eppendorf tube. The RNA was evaporated in a Speed-Vac concentrator and analyzed by ion-exchange HPLC and LC/MS (Figures 6 and 7). It was discovered that the TC phosphoramidites of U and C (**6a** and **6b**) could precipitate from the ACN solution upon standing for several days, especially if the laboratory temperature dropped below 20 °C. The phosphoramidites of U and C were then demonstrated to be stable to precipitation in a 50:50 mixture of anhydrous ACN and anhydrous toluene (v/v), and the use of this solvent system was shown to have no deleterious effect on the phosphoramidite coupling or resulting RNA products.

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ACKNOWLEDGMENT

We thank Richard Shoemaker, Albert Meyer, Victor Molker, Margaret G. Readio, Michael Jones, David Eidenmeuller, and Steve Simon for technical assistance; Andrew Goldsborough, Brian Sproat, and Douglas Picken for helpful discussions; Steven Laderman, Neil Cook, Darlene Solomon, and Agilent Technologies for support.

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