

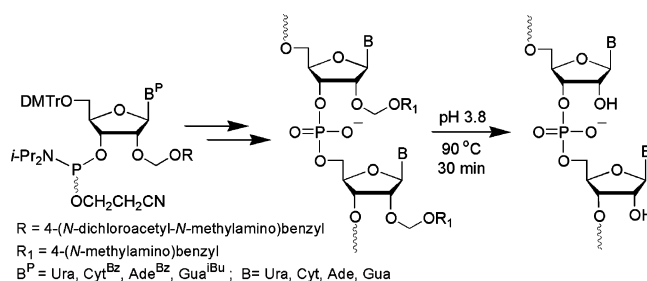
## The 4-(*N*-Dichloroacetyl-*N*-methylamino)benzyloxymethyl Group for 2'-Hydroxyl Protection of Ribonucleosides in the Solid-Phase Synthesis of Oligoribonucleotides

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Emerging RNA-based technologies for controlling gene expression have triggered a high demand for synthetic oligoribonucleotides and have motivated the development of ribonucleoside phosphoramidites that would exhibit coupling kinetics and coupling efficiencies comparable to those of deoxyribonucleoside phosphoramidites. To fulfill these needs, the novel 4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxymethyl group for 2'-hydroxyl protection of ribonucleoside phosphoramidites **9a–d** has been implemented (Schemes 1 and 2). The solid-phase synthesis of AUCCGUAGCUAACGUCAUGG was then carried out employing **9a–d** as 0.2 M solutions in dry MeCN and 5-benzylthio-1*H*-tetrazole as an activator. The coupling efficiency of **9a–d** averaged 99% within a coupling time of 180 s. Following removal of all base-sensitive protecting groups, cleavage of the remaining 2'-[4-(*N*-methylamino)benzyl] acetals from the RNA oligonucleotide was effected in buffered 0.1 M AcOH (pH 3.8) within 30 min at 90 °C. RP-HPLC and PAGE analyses of the fully deprotected AUCCGUAGCUAACGUCAUGG were comparable to those of a commercial RNA oligonucleotide sharing an identical sequence. Enzymatic digestion of the RNA oligomer catalyzed by bovine spleen phosphodiesterase and bacterial alkaline phosphatase revealed no significant amounts of RNA fragments containing (2'→5')-internucleotidic phosphodiester linkages or noteworthy nucleobase modifications.

### Introduction

With the discovery of RNA interference (RNAi) as a cellular process for silencing the expression of specific genes,<sup>1</sup> small-interfering double-stranded RNAs (siRNAs) of 21–25 base pairs in length have been efficient in targeting and cleaving mRNAs

in mammalian cells.<sup>2,3</sup> Typically, this process entails the incorporation of the siRNA into a protein complex including the endonuclease Argonaute 2, which mediates the cleavage of the sense strand of the siRNA during activation of the RNA-induced-silencing complex (RISC).<sup>4</sup> The antisense strand of the siRNA guides the RISC to complementary sequences in target mRNAs; RISC then cleaves the targeted mRNA at a single site

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between nucleobases 10 and 11 relative to the 5'-end of the siRNA antisense strand,<sup>4a,c,d</sup> thereby resulting in a decreased expression level of the encoded protein. The RNAi machinery also serves as an effector for endogenous, noncoding RNAs known as microRNAs (miRNAs).<sup>5</sup> These miRNAs are abundant and are believed to be important in many biological processes through regulation of gene expression by controlling the efficiency of mRNA translation. Unlike an mRNA, a pre-miRNA is embedded in a ~70-nucleotide (nt) hairpin structure, which is cleaved by the RNase III enzyme Dicer to produce a "mature" miRNA as a ~21-nt double-stranded RNA.<sup>4d,6</sup> Like siRNAs, miRNAs are assembled into the RISC and guide the complex to partially complementary mRNA sites by Watson-Crick base-pairing. The expression of targeted mRNAs is therefore blocked either by translational repression or by degradation.<sup>7</sup> Although miRNAs do not encode proteins and the precise molecular function of these biomolecules in mammals is largely unknown, each miRNA has the potential to regulate hundreds of mRNAs. In order to better understand the function of miRNAs in vivo, chemically engineered single-stranded RNA oligonucleotides, termed "antagomirs", were developed.<sup>8</sup> The silencing of endogenous miRNAs with antagomirs in mice was specific, efficient, and long-lasting. Thus, the use of antagomirs may lead to a viable therapeutic strategy in the treatment of cancer, hepatitis, and diabetes considering the involvement of miRNAs in these diseases has been demonstrated.<sup>8</sup> These emerging RNA-based strategies in the control of gene expression have created a high demand for synthetic oligoribonucleotides and has spurred a "renaissance" in the development of rapid and efficient methods for solid-phase RNA synthesis. The design and implementation of 2'-hydroxyl protecting groups that would provide ribonucleoside phosphoramidites with coupling kinetics and coupling efficiencies comparable to those of deoxyribonucleoside phosphoramidites are key to the production of RNA oligonucleotides in sufficient quantity and purity for pharmaceutical applications. Technically, a 2'-hydroxyl protecting group must be stable to the reagents and conditions used during solid-phase RNA synthesis, and to the conditions employed for nucleobase and phosphate deprotection. The 2'-hydroxyl protecting group must, ultimately, be cleaved from the oligonucleotides under conditions that would not induce formation of (2'→5')-internucleotidic phosphodiester linkages and/or RNA chain cleavage. The search for an optimal 2'-hydroxyl protecting group for ribonucleosides has been ongoing for decades.<sup>9</sup> Particularly noteworthy is the progress made in the synthesis of RNA oligonucleotides over

the past decade through ribonucleoside phosphoramidites functionalized with the 2-nitrobenzyloxymethyl (1),<sup>10</sup> 4-nitrobenzyloxymethyl (2),<sup>11</sup> 1-[2-(trimethylsilyl)ethoxy]methyl,<sup>12</sup> 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl,<sup>13</sup> triisopropylsilyloxymethyl (3),<sup>14</sup> substituted 1-(benzyloxy)ethyl,<sup>15</sup> 1-(2-cyanoethoxy)ethyl,<sup>16</sup> (2-cyanoethoxy)methyl (4),<sup>17</sup> levulinyl (5),<sup>18</sup> 2-*tert*-butyldithiomethyl (6),<sup>19</sup> acyloxymethyl,<sup>20</sup> acylthiomethyl,<sup>20</sup> bis-(2-acetoxyethoxy)methyl (7),<sup>21</sup> 2-(4-tolylsulfonyl)ethoxymethyl (8),<sup>22</sup> and allyl<sup>23</sup> groups for 2'-hydroxyl protection. Although ribonucleoside phosphoramidites 1–8 exhibit reaction kinetics and coupling efficiencies in solid-phase RNA synthesis<sup>10,11,14,17–19,21,22</sup> comparable to those of DNA phosphoramidites in typical solid-phase DNA synthesis, many ribonucleoside phosphoramidites do not, presumably because of steric hindrance created by bulky 2'-*O*-protecting groups in the vicinity of the activated phosphoramidite entities. It should also be emphasized that the implementation of a number of 2'-*O*-protected ribonucleoside phosphoramidites in solid-phase RNA synthesis required nucleobase, 5'-hydroxyl, and phosphate protecting groups different from those typically employed in standard solid-phase DNA synthesis. These requirements demanded the development of laborious and often costly strategies for the preparation of RNA phosphoramidite building blocks. Furthermore, the stability of some 2'-*O*-protecting groups during solid-phase synthesis of RNA oligonucleotides is questionable and certain 2'-*O*-protecting groups generated side products upon deprotection, which had to be deactivated to prevent nucleobase alkylation. In an attempt to circumvent these drawbacks and

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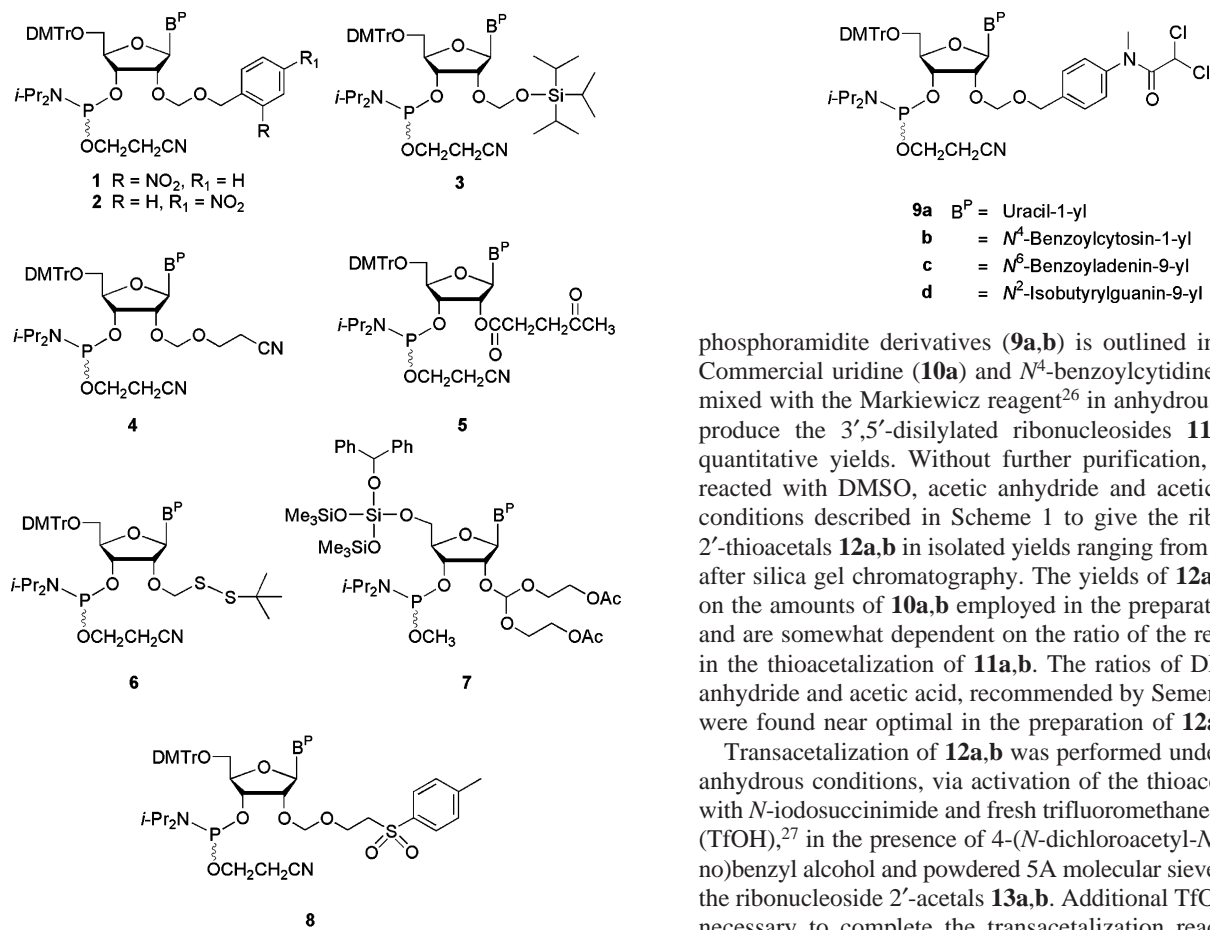
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DMTr = 4,4'-dimethoxytrityl; B<sup>P</sup> = uracil-1-yl or N-protected nucleobases

inspired by the work of Gough et al.<sup>10,11</sup> and Pfliegerer et al.<sup>15,24</sup> on the use of benzyl acetals as 2'-hydroxyl protecting groups in RNA synthesis, we recently reported the 4-(N-dichloroacetyl-N-methylamino)benzyloxymethyl group for 2'-hydroxyl protection of ribonucleoside phosphoramidite **9a**, which was employed in the solid-phase synthesis of the chimeric polyuridylic acid U<sub>19</sub>dT.<sup>25</sup> RP-HPLC analysis of unpurified [2'-O-[4-(N-methylamino)benzyloxy]methyl U]<sub>19</sub>dT revealed a high quality product. Thus, activation of **9a** with 5-ethylthio-1H-tetrazole produced within 180 s coupling efficiencies comparable to those obtained with deoxyribonucleoside phosphoramidites. Furthermore, RP-HPLC analysis of unpurified U<sub>19</sub>dT indicated that hydrolysis of the 2'-acetals from the oligoribonucleotide proceeded smoothly under mild acidic conditions without significant internucleotidic chain cleavage. These encouraging results prompted us to synthesize the ribonucleoside phosphoramidites **9b–d** and assess the coupling kinetics and coupling efficiencies of these monomers in the solid-phase synthesis of AUC-GUAGCUAACGUCAUGG. The results of our investigations will be the focus of this report.

## Results and Discussion

The synthesis of 2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyloxy]methyl ribonucleosides (**14a,b** and **15a,b**) and their

phosphoramidite derivatives (**9a,b**) is outlined in Scheme 1. Commercial uridine (**10a**) and N<sup>4</sup>-benzoylcytidine (**10b**) were mixed with the Markiewicz reagent<sup>26</sup> in anhydrous pyridine to produce the 3',5'-disilylated ribonucleosides **11a,b** in near quantitative yields. Without further purification, **11a,b** were reacted with DMSO, acetic anhydride and acetic acid under conditions described in Scheme 1 to give the ribonucleoside 2'-thioacetals **12a,b** in isolated yields ranging from 73% to 85% after silica gel chromatography. The yields of **12a,b** are based on the amounts of **10a,b** employed in the preparation of **11a,b** and are somewhat dependent on the ratio of the reactants used in the thioacetalization of **11a,b**. The ratios of DMSO, acetic anhydride and acetic acid, recommended by Semenyuk et al.,<sup>19</sup> were found near optimal in the preparation of **12a,b**.

Transacetalization of **12a,b** was performed under rigorously anhydrous conditions, via activation of the thioacetal function with N-iodosuccinimide and fresh trifluoromethanesulfonic acid (TfOH),<sup>27</sup> in the presence of 4-(N-dichloroacetyl-N-methylamino)benzyl alcohol and powdered 5A molecular sieves to produce the ribonucleoside 2'-acetals **13a,b**. Additional TfOH was often necessary to complete the transacetalization reaction. Crude **13a,b** were completely desilylated within 16 h by treatment with ammonium fluoride<sup>19,28</sup> in methanol. The acetals **14a,b** were purified by silica gel chromatography and isolated as pure amorphous solids in yields averaging 65% relative to the amounts of **12a,b** utilized in the preparation of **13a,b**. 5'-O-Dimethoxytritylation of **14a,b** was carried out in anhydrous pyridine using a slight molar excess (1.2 equiv) of 4,4'-dimethoxytrityl chloride. The reaction was monitored by TLC and was found complete within 2 h. After routine workup, crude **15a,b** were purified by chromatography on silica gel and were isolated as lyophilized powders in yields of ca. 90%. The phosphorylation of **15a,b** was effected within 2 h when employing 2-cyanoethyl N,N-diisopropylchlorophosphoramidite and dry Et<sub>3</sub>N in anhydrous CH<sub>2</sub>Cl<sub>2</sub>. After an aqueous workup, the crude ribonucleoside phosphoramidites **9a,b** were purified by silica gel chromatography using C<sub>6</sub>H<sub>6</sub>:Et<sub>3</sub>N (9:1 v/v) as the eluent. The presence of Et<sub>3</sub>N in the eluent is necessary to neutralize the inherent acidity of silica gel and thus prevent decomposition of **9a,b** during chromatography. Purified **9a,b** were freed from residual Et<sub>3</sub>N by precipitation in cold (−78 °C) hexane. The white precipitates were dissolved in dry C<sub>6</sub>H<sub>6</sub> and the resulting solutions were frozen and then lyophilized under high vacuum affording moisture and Et<sub>3</sub>N-free **9a,b** in yields averaging 80%. However, depending upon the original amount of residual moisture and Et<sub>3</sub>N contaminating **9a,b**, an additional round of precipitation in cold hexane and lyophilization from dry benzene may be

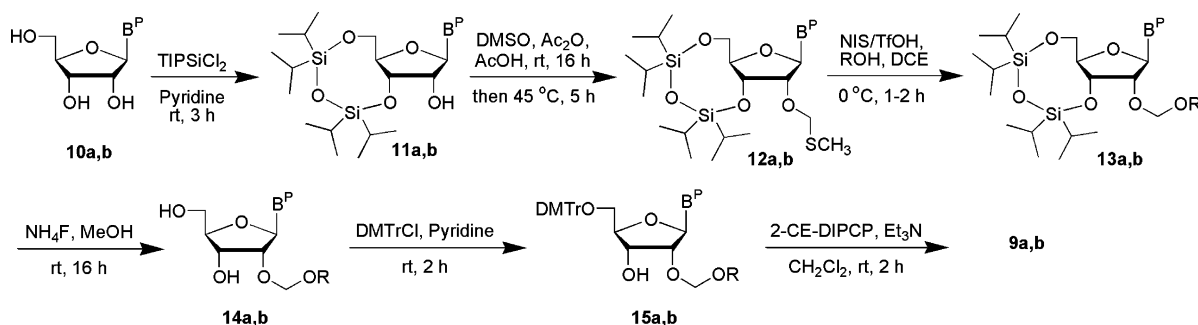
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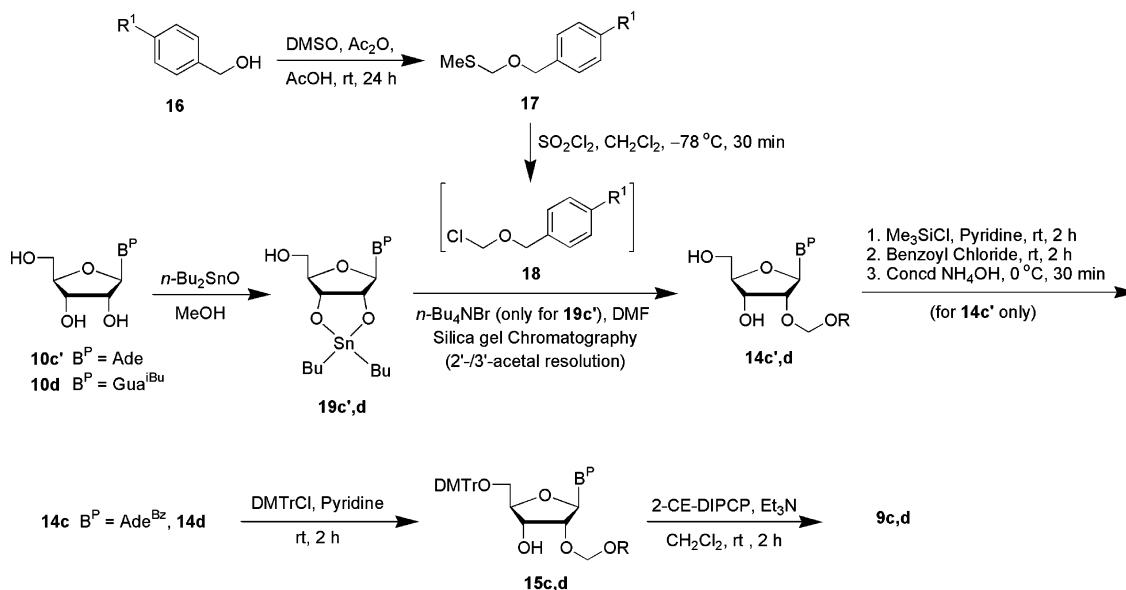
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SCHEME 1. Synthesis of 2'-O-Protected Ribonucleosides 14a,b and 15a,b and Ribonucleoside Phosphoramidites 9a,b<sup>a</sup>

<sup>a</sup> Keys: B<sup>P</sup>, uracil-1-yl (a) and N<sup>4</sup>-benzoylcytosin-1-yl (b); R, 4-(*N*-dichloroacetyl-*N*-methylamino)benzyl; TIPSiCl<sub>2</sub>, 1,3-dichloro-1,1,3,3-tetraisopropylsilyloxane; NIS, *N*-iodosuccinimide; TfOH, trifluoromethanesulfonic acid; DCE, 1,2-dichloroethane; DMTrCl, 4,4'-dimethoxytrityl chloride; 2-CE-DIPCP, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite.

SCHEME 2. Synthesis of 2'-O-Protected Ribonucleosides 14c,d and 15c,d and Ribonucleoside Phosphoramidites 9c,d<sup>a</sup>

<sup>a</sup> Keys: Ade, adenin-9-yl; Ade<sup>Bz</sup>, N<sup>6</sup>-benzoyladenin-9-yl; Gua<sup>iBu</sup>, N<sup>2</sup>-isobutyrylguanin-9-yl; R<sup>1</sup>, *N*-dichloroacetyl-*N*-methylamino; R, 4-(*N*-dichloroacetyl-*N*-methylamino)benzyl.

required to rigorously ensure the absence of moisture and base contaminants, and guarantee high coupling efficiency of phosphoramidite monomers.

Although the preparation of ribonucleosides **14a,b** proceeded satisfactorily according to the reaction pathway shown in Scheme 1, we experienced difficulties in obtaining reproducible results in the synthesis of **14c,d** (Scheme 2). Despite our attempts at improving the synthetic process, reproducibility issues were reoccurring and forced us to adopt a different approach to the preparation of **14c,d**. We opted for the method of Gough et al.,<sup>10b</sup> which was reported for the synthesis of ribonucleoside precursors leading to the preparation of phosphoramidites **1** and **2**. As shown in Scheme 2, thioacetalization of 4-(*N*-dichloroacetyl-*N*-methylaminobenzyl) alcohol (**16**)<sup>25</sup> in DMSO, acetic anhydride and acetic acid was carried out affording the thioacetal **17** in 65% yield within 24 h. Stannylation of adenosine (**10c'**) and *N*<sup>2</sup>-isobutyryl guanosine (**10d**) upon reaction with dibutyltin oxide in methanol produced the respective stannylidene derivatives **19c',d**, which were used in the next synthetic step without further purification. The thioacetal **17** was treated with sulfonyl chloride in CH<sub>2</sub>Cl<sub>2</sub> to generate the chloromethyl ether intermediate **18**, which was

condensed with **19c',d** to produce a regioisomeric mixture of ribonucleoside 3'- and 2'-acetals. The ribonucleoside 2'-acetals **14c',d** were separated by silica gel chromatography<sup>29</sup> and were isolated in yields of 30–35%. Both 3'- and 5'-hydroxyls of **14c'** were transiently protected within 2 h upon reaction with trimethylsilyl chloride in dry pyridine.<sup>30</sup> Benzoylation of the exocyclic amino group of adenine was then achieved by adding benzoyl chloride to the reaction mixture. After 2 h, the crude product was subjected to aqueous workup and the material was desilylated by treatment with conc. NH<sub>4</sub>OH at 0 °C for 30 min to afford the ribonucleoside 2'-acetal **14c**. Regioselective 5'-*O*-dimethoxytritylation of **14c,d** was accomplished in 2 h when employing a slight molar excess (1.2 equiv) of dimethoxytrityl chloride in pyridine; 5'-*O*-protected ribonucleoside 2'-acetals **15c,d** were isolated in yields of 90–95% after purification

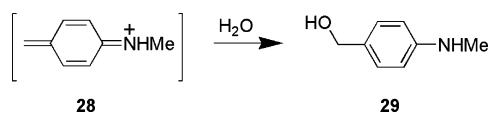
(29) This purification step is critically important. One must ensure that the desired ribonucleoside 2'-acetal is not contaminated with any 3'-acetal to prevent subsequent incorporation of (2'→5')-internucleotidic phosphodiester linkages into the oligoribonucleotide during solid-phase synthesis.

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on silica gel. Phosphinylation of **15c,d** was carried out under conditions similar to those employed for **15a,b**. The purification and post-purification processing of the resulting ribonucleoside phosphoramidites **9c,d** were performed under near identical conditions to those used for the production of **9a,b**. The phosphinylation of **15a,b** and **15c,d** produced phosphoramidites **9a–d** in yields varying from 62 to 85%. The key ribonucleoside 2'-acetals **14a–d** were characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopies and by high-resolution time-of-flight mass spectrometry using atmospheric pressure electron spray ionization (APESI-HRMS), whereas phosphoramidites **9a–d** were characterized by  $^{31}\text{P}$  NMR spectroscopy and APESI-HRMS (data shown in the Experimental Section and in the Supporting Information).

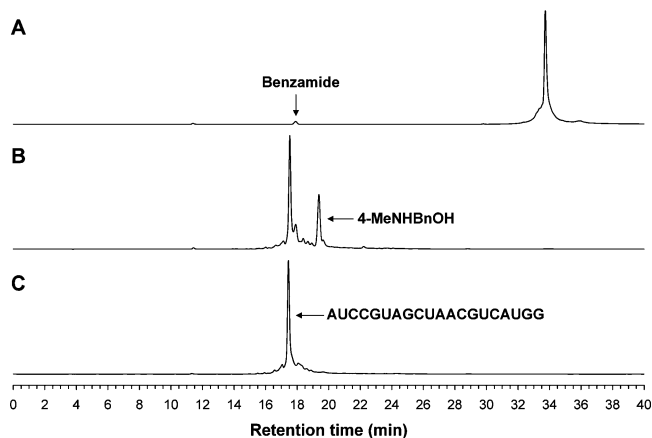
The 2'-*O*-[4-(*N*-methylamino)benzyloxy]methyl ribonucleoside phosphoramidites **9a–d** were then employed in the solid-phase synthesis of AUCCGUAGCUAACGUCAUGG as a model oligoribonucleotide to evaluate the coupling kinetics and efficiencies of these phosphoramidites. Solid-phase synthesis of the 20-mer RNA oligonucleotide was carried out on a 0.2  $\mu\text{mol}$  scale using **9a–d** as 0.2 M solutions in anhydrous MeCN. It should be emphasized that the effective concentration of activated **9a–d** in the synthesis column was  $\sim 0.1$  M. Phosphoramidite activators, such as 1*H*-tetrazole, 5-ethylthio-1*H*-tetrazole, and 5-benzylthio-1*H*-tetrazole were tested in the preparation of the RNA oligonucleotide; 0.25 M 5-benzylthio-1*H*-tetrazole in MeCN produced the best result in terms of phosphoramidite coupling efficiency, which averaged 99% within a coupling time of 180 s. The stepwise coupling yields were determined spectrophotometrically at 498 nm through the standard dimethoxytrityl cation assay. After completion of the RNA oligonucleotide chain assembly, the solid-phase-linked 20-mer was exposed to concd  $\text{NH}_4\text{OH}$  for 10 h at 55  $^\circ\text{C}$  to cleave nucleobase and phosphate protecting groups concurrently with the dichloroacetyl group of each 2'-acetal function, and release the 2'-*O*-protected oligoribonucleotide from the solid support. The RP-HPLC profile of the unpurified 2'-*O*-[4-(*N*-methylamino)benzyloxy]methyl (4-MABOM) RNA oligomer, shown in Figure 1A, is reflective of the high coupling efficiency of phosphoramidites **9a–d** on the basis of the relatively low level of failure sequences clustered at the base of the single major peak.

Removal of the 2'-*O*-(4-MABOM) groups from the RNA oligomer was effected by dissolving the oligonucleotide in 0.1 M AcOH adjusted to pH 3.8 with *N,N,N',N'*-tetramethylethylenediamine (TEMED)<sup>21b</sup> and heating the solution at 90  $^\circ\text{C}$  for 30 min. The RP-HPLC profile of the deprotection reaction is presented in Figure 1B. Aside from benzamide, which was generated during nucleobase deprotection, 4-(*N*-methylamino)benzyl alcohol (**29**)<sup>31</sup> was produced through hydrolysis of the *N*-methyliminoquinone methide intermediate **28**<sup>32</sup> that is presumably generated during the cleavage of the 2'-acetals.



(31) The RP-HPLC retention time of **29** ( $t_R = 19.6$  min) is identical to that of an authentic sample of 4-(*N*-methylamino)benzyl alcohol that was prepared from the reduction of 4-(*N*-methylamino)benzoic acid (see ref 25).

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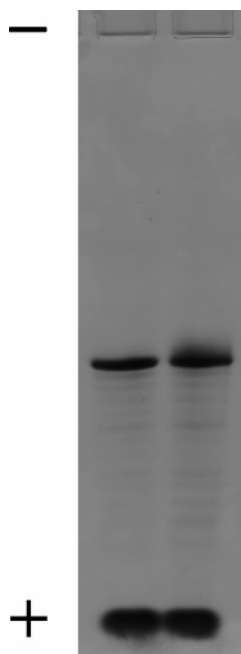
**FIGURE 1.** RP-HPLC profiles of unpurified 2'-*O*-protected/deprotected AUCCGUAGCUAACGUCAUGG. (A) AUCCGUAGCUAACGUCAUG[2'-*O*-(4-MABOM)]<sub>19</sub>G. (B) AUCCGUAGCUAACGUCAUG[2'-*O*-(4-MABOM)]<sub>19</sub>G in 0.1 M AcOH/TEMED, pH 3.8, 90  $^\circ\text{C}$ , 30 min. (C) Material of profile B that was desalted using a PD-10 (Sephadex G-25M) column. RP-HPLC analyses were performed employing a 5  $\mu\text{m}$  Supelcosil LC-18S column (25 cm  $\times$  4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min and was then held, isocratically, for 20 min. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit.

Desalting the fully deprotected RNA oligonucleotide through a size exclusion column gave a high quality product, the RP-HPLC profile of which, is shown in Figure 1C. The RP-HPLC profile is comparable to that of a commercial oligonucleotide of identical RNA sequence (data shown in the Supporting Information). Polyacrylamide gel electrophoresis analysis of the unpurified and desalted RNA oligonucleotide was also compared with that of the commercial RNA oligonucleotide reference. As shown in Figure 2, the electrophoretic mobility and the purity of the RNA oligonucleotide prepared from ribonucleoside phosphoramidites **9a–d** are comparable to those of the commercial RNA oligonucleotide reference.

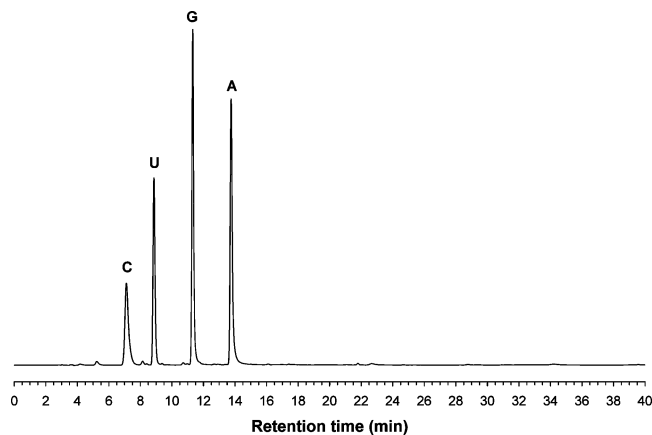
The purity of the fully deprotected oligoribonucleotide in terms of nucleobase modifications and integrity of the (3'→5')-internucleotidic phosphodiester linkages throughout the RNA sequence was also assessed through enzymatic hydrolysis. Specifically, the unpurified and desalted RNA 20-mer was subjected to snake venom phosphodiesterase (SVP) and bacterial alkaline phosphatase (BAP) for 16 h at 37  $^\circ\text{C}$ . As displayed in Figure 3, the RP-HPLC profile of the enzymatic digest indicates that the RNA oligonucleotide is completely degraded to the respective nucleosides without significant amounts of nucleobase modification.

While the cleavage of the 2'-*O*-(4-MABOM) group is performed under acidic conditions, vicinal migration of each (3'→5')-internucleotidic phosphodiester linkage may occur and may also lead to RNA chain cleavage.<sup>33</sup> To evaluate the extent of (2'→5')-internucleotidic phosphodiester linkage formation under these conditions, the crude fully deprotected RNA oligonucleotide was purified by RP-HPLC and then desalted by size-exclusion chromatography. The pure RNA oligomer was then treated with bovine spleen phosphodiesterase (BSP) and BAP for 16 h at 37  $^\circ\text{C}$ . BSP, unlike SVP, does not cleave

(33) Reese C.B. *Org. Biomol. Chem.* **2005**, *3*, 3851–3868 and references therein.



**FIGURE 2.** Polyacrylamide gel analysis (20% polyacrylamide-7 M urea, 1X Tris Borate EDTA buffer, pH 8.3) of unpurified and desalted AUCCGUAGCUAACGUCAUGG. Left lane: commercial RNA oligomer (0.25 OD<sub>260</sub>). Right lane: RNA oligomer (0.25 OD<sub>260</sub>) obtained from the deprotection of AUCCGUAGCUAACGUCAUG[2'-O-(4-MABOM)]<sub>10</sub>G under acidic conditions. Oligonucleotides are visualized as blue bands upon staining the gel with Stains-all. Bromophenol blue is used as a marker and shows as a large band, in each lane, at the bottom of the gel.



**FIGURE 3.** RP-HPLC chromatogram of unpurified and desalted AUCCGUAGCUAACGUCAUGG after treatment with snake venom phosphodiesterase and bacterial alkaline phosphatase. RP-HPLC analyses were performed under conditions identical to those described in the caption of Figure 1. Key: C, cytidine; U, uridine; G, guanosine; A, adenosine.

(2'→5')-internucleotidic phosphodiester linkages<sup>34</sup> and thus provides a means to assess the level of (2'→5')-internucleotidic phosphodiester bond formation among the native (3'→5')-internucleotidic phosphodiester linkages. Figure 4B shows near complete digestion of the RP-HPLC purified and desalted RNA

oligomer<sup>35</sup> that was exposed to acidic conditions (pH 3.8) at elevated temperature (90 °C) during the time allocated for complete 2'-O-deprotection (30 min). RNA fragments containing at least one or several (2'→5')-internucleotidic phosphodiester linkages are expected to exhibit longer retention times than those of individual ribonucleosides but shorter than the retention time of the full length 20-mer (17.5 min) under identical chromatographic conditions.

Amplification of the baseline corresponding to retention times in the range of 12 to 18 min (Figure 4B') revealed a number of peaks, the relative intensity of which, increased upon increasing exposure to acidic conditions by an additional 30 min at 90 °C (Figure 4A'). This trend is consistent with increased formation of (2'→5')-internucleotidic phosphodiester linkages with increasing exposure time to acidic conditions. The formation of (2'→5')-internucleotidic phosphodiester linkages under acidic conditions is not detected in the SVP/BAP digestion reaction since it has been demonstrated that synthetic (2'→5')UpU is completely hydrolyzed to uridine by the enzymes (data shown in the Supporting Information). One can nonetheless safely state that the amount of RNA fragments containing (2'→5')-internucleotidic phosphodiester linkages, produced under the deprotection conditions defined above for 2'-O-(4-MABOM) RNA oligonucleotides, is negligible.

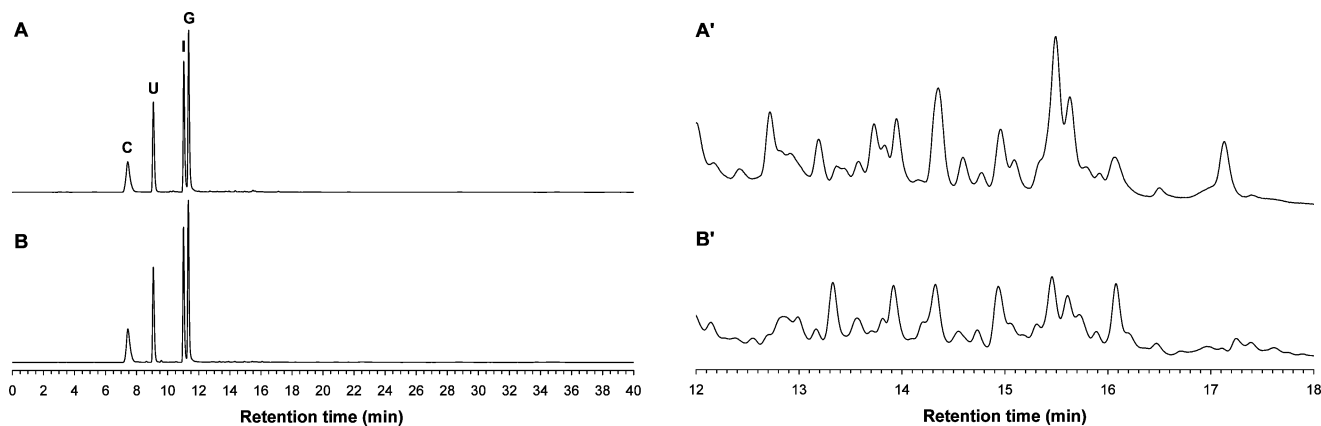
In summary, RNA phosphoramidites protected with a 4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxymethyl group for 2'-O-protection allow rapid and efficient solid-phase RNA synthesis without a mandatory requirement for nucleobase, 5'-hydroxyl, and phosphate protecting groups to differ from those traditionally employed in standard solid-phase DNA synthesis. Consequently, the conditions used for the cleavage of nucleobase and phosphate protecting groups are essentially identical to those used for standard DNA oligonucleotide deprotection, and resulted in the release of a 2'-O-(4-MABOM)-protected RNA oligonucleotide of high quality (Figure 1A). Removal of the 2'-O-(4-MABOM) group from the RNA oligonucleotide proceeded smoothly, in a buffered acetic acid solution kept at 90 °C, without producing significant amounts of (2'→5')-internucleotidic phosphodiester linkages and nucleobase modifications (Figures 3 and 4). The overall quality of the native unpurified RNA oligonucleotide after full deprotection was comparable to that of a commercial oligonucleotide of identical RNA sequence on the basis of chromatographic (data shown in the Supporting Information) and electrophoretic data (Figure 2). Although the 4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxymethyl group is perfectly suitable for 2'-hydroxyl protection in solid-phase RNA synthesis, an improved method for 2'-O-protection of ribonucleoside purines with this group is necessary for the commercialization of phosphoramidites **9a–d**. This objective is currently under development in our laboratories.

## Experimental Section

**3',5'-O-(1,1,3,3-Tetraisopropylidisiloxane-1,3-diyl)uridine (11a).** Uridine (**10a**, 15 mmol) was dried by coevaporation with anhydrous pyridine (3 × 20 mL) under reduced pressure. The dry ribonucleo-

(34) Synthetic (3'→5')UpU is a substrate for BSP but synthetic (2'→5')UpU is not. Data are presented in the Supporting Information. See also: (a) Giannaris, P. A.; Damha, M. J. *Nucleic Acids Res.* **1993**, *21*, 4742–4749. (b) Thayer, J. R.; Rao, S.; Puri, N.; Burnett, C. A.; Young, M. *Anal. Biochem.* **2007**, *361*, 132–139.

(35) Commercial bovine spleen phosphodiesterase contains some (1% w/w) adenosine deaminase (ADA) as a contaminant. Such a low concentration of ADA in the digestion reaction was sufficient to quantitatively convert adenosine to inosine. This conversion was unambiguously demonstrated by mixing adenosine with BSP/BAP under the conditions used for the digestion of the RNA oligomer. RP-HPLC analysis of the reaction indicated complete conversion of adenosine to inosine, which had a retention time identical to that of a commercial sample of inosine (data shown in the Supporting Information).



**FIGURE 4.** RP-HPLC profiles of purified and desalted AUCCGUAGCUAACGUCAUGG after treatment with bovine spleen phosphodiesterase and bacterial alkaline phosphatase. (A) The RP-HPLC-purified RNA oligomer had been exposed to pH 3.8 at 90 °C for a period of time of 30 min after 2'-*O*-deprotection and RP-HPLC purification. (A') 100-Fold baseline amplification of chromatogram A corresponding to retention times in the range of 12–18 min. (B) The RNA oligomer had been exposed to pH 3.8 at 90 °C for 30 min during 2'-*O*-deprotection prior to purification. (B') 100-Fold baseline amplification of chromatogram B corresponding to retention times in the range of 12–18 min. RP-HPLC analyses were performed under conditions identical to those described in the caption of Figure 1. Key: C, cytidine; U, uridine; I, inosine; G, guanosine.

side was redissolved in anhydrous pyridine (70 mL), and 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane<sup>26</sup> (5.76 mL, 18 mmol) was added, dropwise, over a period of 30 min. The reaction mixture was then stirred at ~25 °C until **10a** had completely converted to a new product (~3 h) as indicated by TLC (CHCl<sub>3</sub>/MeOH (9:1 v/v)). The reaction mixture was concentrated to an oil, which was then dissolved in CH<sub>2</sub>Cl<sub>2</sub> (200 mL). The solution was extracted with satd NaHCO<sub>3</sub> (150 mL). The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and rotoevaporated to dryness under vacuum. The product was dried further by coevaporation with toluene (3 × 20 mL). Crude **11a**<sup>19</sup> was isolated as a white foam and used without purification in the next synthetic step.

**3',5'-*O*-(1,1,3,3-Tetraisopropylidisiloxane-1,3-diyl)-2'-*O*-methylthiomethyluridine (12a).** To a solution of crude **11a** in DMSO (15 mL) were added glacial AcOH (23 mL) and Ac<sub>2</sub>O (15 mL). The solution was stirred at ~25 °C for 16 h and then at 45 °C until completion of the reaction (~5 h), which was monitored by TLC (CHCl<sub>3</sub>/MeOH (95:5 v/v)). The solution was transferred to a 2 L Erlenmeyer flask to which was added, under vigorous stirring, a solution of K<sub>2</sub>CO<sub>3</sub> (31 g) in water (200 mL). The precipitated material was isolated either by filtration or decantation and was redissolved in a minimum volume of THF (~15–20 mL). The resulting solution was then poured into water (250 mL) to give the crude product as a gummy material. Most of the water was decanted; the crude product was carefully dried by consecutive coevaporation with pyridine (30 mL), toluene (3 × 30 mL) and dichloromethane (30 mL). The crude ribonucleoside **12a** was purified by chromatography on silica gel (150 g in a 6.5 cm i.d. column) using a gradient of MeOH (0 → 3%) in CH<sub>2</sub>Cl<sub>2</sub>. Fractions containing pure **12a** (TLC) were collected, rotoevaporated to a foam under low pressure, and dissolved in dry C<sub>6</sub>H<sub>6</sub> (~20 mL); the solution was frozen and then lyophilized under high vacuum to produce a white powder (7 g, 12.8 mmol, 85%). Characterization data obtained from <sup>1</sup>H and <sup>13</sup>C NMR analysis of **12a** are in agreement with those reported by Semenyuk et al.<sup>19</sup>

**N<sup>4</sup>-Benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)-cytidine (11b).** This compound was prepared under conditions similar to those employed for the synthesis of **11a**. Crude **11b**<sup>19</sup> was also used without purification in the next synthetic step.

**N<sup>4</sup>-Benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)-2'-*O*-methylthiomethylcytidine (12b).** To a solution of crude **11b** in DMSO (15 mL) were added glacial AcOH (15 mL) and Ac<sub>2</sub>O (10 mL). The solution was stirred at ~25 °C for 16 h and then at 45 °C until completion of the reaction (~5 h), which was monitored by TLC (CHCl<sub>3</sub>/MeOH (95:5 v/v)). The solution was then

transferred to a 2 L Erlenmeyer flask and was vigorously stirred while adding slowly a solution of K<sub>2</sub>CO<sub>3</sub> (35 g) in water (270 mL). The precipitated material was purified and processed as described for the preparation of **12a**. Purified **12b** was isolated as a white powder (7.1 g, 11 mmol, 73%). Characterization data obtained from <sup>1</sup>H and <sup>13</sup>C NMR analysis of **12b** are in agreement with those reported by Semenyuk et al.<sup>19</sup>

**3',5'-*O*-(1,1,3,3-Tetraisopropylidisiloxane-1,3-diyl)-2'-*O*-[4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxy]methyluridine (13a).** To a cooled (0 °C) suspension of thoroughly dried **12a** (5.00 mmol), 4-(*N*-dichloroacetyl-*N*-methylamino)benzyl alcohol<sup>25</sup> (**16**, 766 mg, 5.00 mmol), and powdered 5A molecular sieves (~200 mg) in anhydrous 1,2-dichloroethane (DCE, 50 mL) was added a freshly prepared solution of *N*-iodosuccinimide (NIS, 1.12 g, 5.00 mmol) and fresh trifluoromethanesulfonic acid (TfOH, 64 μL, 0.72 mmol) in DCE/Et<sub>2</sub>O ((1:1 v/v), 25 mL). Progress of the reaction was assessed by monitoring the disappearance of **12a** by TLC (CHCl<sub>3</sub>/MeOH (93:7 v/v)). The addition of more TfOH (2 up to 4 × 0.72 mmol) may be necessary to complete the reaction. The suspension was filtered; the filtrate was diluted with CH<sub>2</sub>Cl<sub>2</sub> (150 mL) and washed, consecutively, with aqueous 1 M sodium bisulfite (100 mL) and satd NaHCO<sub>3</sub> (100 mL). The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and, then, evaporated to a foam under reduced pressure. Crude **13a** was used in the next synthetic step without further purification.

**N<sup>4</sup>-Benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)-2'-*O*-[4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxy]methylcytidine (13b).** This compound was prepared from **12b** under conditions similar to those employed for the synthesis of **13a**. Crude **13b** was used without purification in the next synthetic step.<sup>36</sup>

**2'-*O*-[4-(*N*-Dichloroacetyl-*N*-methylamino)benzyloxy]methyluridine (14a).** Crude **13a** (5.00 mmol) was dissolved in methanol (30 mL), and ammonium fluoride<sup>19,28</sup> (741 mg, 20.0 mmol) was added. The reaction mixture was stirred at ~25 °C until cleavage of the disiloxy group was complete (~16 h) as indicated by TLC (CHCl<sub>3</sub>/MeOH (9:1 v/v)). The reaction mixture was concentrated in vacuo, and then partitioned between CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and satd NaHCO<sub>3</sub> (50 mL). The aqueous layer was extracted further with CH<sub>2</sub>Cl<sub>2</sub> (2 × 100 mL). The organic extracts were pooled together and were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated

(36) Silica gel chromatography may occasionally be necessary to separate **13b** from unreacted **12b** to avoid subsequent purification problems. Typically, **13b** is adequately resolved from **12b** when a gradient of MeOH (0–2%) in CH<sub>2</sub>Cl<sub>2</sub> is employed as an eluent.

to dryness under reduced pressure. Crude **14a** was purified by chromatography on silica gel (20 g in a 2.5 cm i.d. column) using a gradient of MeOH (0 → 5%) in CH<sub>2</sub>Cl<sub>2</sub>. Fractions containing pure **14a** (TLC) were collected and rotoevaporated to a foam under low pressure. The yield of pure ribonucleoside **14a** was 70% (1.75 g, 3.5 mmol) relative to the amount of **12a** that was employed in the preparation of **13a**. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 11.31 (s, 1H), 7.94 (d, *J* = 8.0 Hz, 1H), 7.41–7.33 (m, 4H), 6.22 (s, 1H), 5.95 (d, *J* = 5.8 Hz, 1H), 5.60 (d, *J* = 8.0 Hz, 1H), 5.26 (d, *J* = 5.8 Hz, 1H), 5.17 (t, *J* = 5.1 Hz, 1H), 4.83 (s, 2H), 4.61 (d, <sup>2</sup>*J* = 12.4 Hz, 1H), 4.55 (d, <sup>2</sup>*J* = 12.4 Hz, 1H), 4.24 (t, *J* = 5.1 Hz, 1H), 4.14 (dt, *J* = 5.1, 4.4 Hz, 1H), 3.91 (m, 1H), 3.70–3.55 (m, 2H), 3.23 (s, 3H). <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ 162.9, 162.8, 150.5, 140.3, 140.2, 138.4, 128.9, 126.9, 101.9, 93.4, 86.1, 85.2, 78.2, 68.5, 67.9, 64.7, 60.5, 38.2. ESI/TOF-HRMS: calcd for C<sub>20</sub>H<sub>22</sub>Cl<sub>2</sub>N<sub>3</sub>O<sub>8</sub> (M – H)<sup>–</sup> 502.0784, found 502.0780.

**N<sup>4</sup>-Benzoyl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyl-oxymethyl]cytidine (14b)**. This compound was prepared on a 5 mmol scale and purified under conditions similar to those employed for the synthesis and purification of **14a**. Pure ribonucleoside **14b** was isolated in a yield of 60% (1.8 g, 3.0 mmol) relative to the amount of **12b** that was employed in the preparation of **13b**. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 11.30 (s, 1H), 8.57 (d, *J* = 7.4 Hz, 1H), 8.01 (d, *J* = 7.4 Hz, 2H), 7.6 (m, 1H), 7.52 (d, *J* = 8.0 Hz, 2H), 7.46 (d, *J* = 9.1 Hz, 2H), 7.35 (m, 3H), 6.19 (s, 1H), 5.97 (d, *J* = 2.5 Hz, 1H), 5.03 (d, *J* = 6.6 Hz, 1H), 4.92 (d, *J* = 6.6 Hz, 1H), 4.66 (m, 2H), 4.24 (dd, *J* = 3.0, 2.7 Hz, 1H), 4.17 (m, 1H), 3.99 (m, 1H), 3.83 (dd, *J* = 12.3, 2.2 Hz, 1H), 3.67 (dd, *J* = 12.3, 2.6 Hz, 1H), 3.19 (s, 3H). <sup>13</sup>C NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 167.3, 163.1, 162.7, 154.4, 145.0, 140.4, 138.6, 133.0, 132.7, 129.5, 128.4, 126.9, 96.1, 93.3, 88.6, 84.3, 78.7, 68.1, 67.5, 74.7, 59.4, 38.2. APESI-HRMS: calcd for C<sub>27</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>8</sub> (M)<sup>+</sup> 606.1284, found 606.1277.

**Preparation of 2',3'-O-(Dibutylstannylene) Ribonucleosides 19c',d**. Stannylation of adenosine (**10c'**) or *N*<sup>2</sup>-isobutrylguanosine (**10d**) was performed according to a method described in the literature.<sup>10b</sup> To a solution of **10c'** or **10d** (20 mmol) in MeOH (1 L) was added dibutyltin oxide (20 mmol). The reaction mixture was refluxed for 1 h and then allowed to cool to room temperature. The solvent was removed by rotoevaporation under reduced pressure. The crystalline material was left over phosphorus pentoxide for 16 h in a desiccator, under high vacuum, prior to be used without purification in the next synthetic step.

**4-(N-Dichloroacetyl-N-methylamino)benzyl Methylthiomethyl Ether (17)**. A modified literature procedure<sup>10b</sup> was employed for the preparation of **17**. Specifically, 4-(*N*-dichloroacetyl-*N*-methylamino)benzyl alcohol (**16**,<sup>25</sup> 10 g, 40 mmol) was dissolved in dry DMSO (40 mL). Ac<sub>2</sub>O (30 mL) and AcOH (20 mL) were added to the solution, which was left standing in the dark for 24 h at ~25 °C. The yellow solution was then added dropwise during 2 h to a vigorously stirred slurry of NaHCO<sub>3</sub> (88 g) in H<sub>2</sub>O (133 mL) to control the release of carbon dioxide. The reaction mixture was left stirring in the dark for an additional 24 h. The oily material was allowed to settle and the aqueous supernatant was carefully decanted. The crude oil was dissolved in EtOAc/hexane (1:1 v/v, 100 mL) and the solution was extracted with satd NaHCO<sub>3</sub> (3 × 100 mL) and once with 2 M NaCl (100 mL). The organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the solvent was removed by rotoevaporation under reduced pressure. The material left was purified by chromatography on silica gel (150 g in a 6.5 cm I.D. column) using a gradient of AcCN (0% → 15%) in CH<sub>2</sub>Cl<sub>2</sub> as the eluent. Fractions containing the desired product (TLC) were pooled together and rotoevaporated under low pressure to give **17** (8 g, 26 mmol, 65%) as a yellow oil. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 7.45 (d, *J* = 8.0 Hz, 2H), 7.38 (d, *J* = 8.0 Hz, 2H), 6.21 (s, 1H), 4.75 (s, 2H), 4.60 (s, 2H), 3.23 (s, 3H), 2.14 (s, 3H). <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ 162.7, 140.4, 138.5, 129.1, 127.0, 74.1, 68.1, 64.6, 38.1, 13.4.

**N<sup>6</sup>-Benzoyl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyl-oxymethyl]adenosine (14c)**. To a cold (–78 °C) stirred solution of **17** (4.9 g, 16 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> was added dropwise, within 5 min, 1 M SO<sub>2</sub>Cl<sub>2</sub> (16 mL, 16 mmol) in CH<sub>2</sub>Cl<sub>2</sub>.<sup>10b</sup> The reaction mixture was stirred at –78 °C for an additional 30 min and was then rotoevaporated under reduced pressure. The residue (**18**) was coevaporated with dry CH<sub>2</sub>Cl<sub>2</sub> (3 × 30 mL) under low pressure, and anhydrous DMF (20 mL) was added. The clear solution of **18** was immediately added, dropwise over a period of 5 min, to a stirred solution of **19c'** (8 g, 16 mmol) and dry tetra-*n*-butylammonium bromide (7.7 g, 16 mmol) in dry DMF (65 mL) kept at 60 °C. The reaction mixture was allowed to stir for 60 min at 60 °C, whereupon pyridine:H<sub>2</sub>O (3:2 v/v, 20 mL) was added. The solution was stirred for 25 min at ~25 °C and was then rotoevaporated under high vacuum to remove DMF. The crude material was dissolved in a minimum volume of pyridine/H<sub>2</sub>O (3:2 v/v); the solution was dispersed in silica gel (20 g) and allowed to dry overnight inside a fume hood. The coated silica gel was pulverized and layered on the top of a chromatography column (6.5 cm i.d.) packed with silica gel (150 g). The desired 2'-acetal **14c'** eluted faster than the 3'-acetal when a gradient of MeOH (0 → 5%) in CH<sub>2</sub>Cl<sub>2</sub> was used as the eluent.<sup>29</sup> Fractions containing **14c'** were pooled together and the solvents were removed by rotoevaporation under reduced pressure to give pure **14c'** (2.65 g, 5.00 mmol, 31%). Trimethylchlorosilane (3.2 mL, 25 mmol) was added to a solution of **14c'** (2.65 g, 5.00 mmol) in anhydrous pyridine (20 mL). The reaction mixture was stirred at ~25 °C for 2 h, upon which, benzoyl chloride (2.9 mL, 25 mmol) was added. After 2 h, the reaction mixture was cooled to ~0 °C; water (5 mL) was added and was followed, 10 min later, by the addition of concd NH<sub>4</sub>OH (10 mL). The open-flask reaction was left stirring at ~25 °C for 30 min. Solvents and excess NH<sub>4</sub>OH were removed by rotoevaporation under low pressure. The crude product (**14c**) was dissolved in EtOAc (250 mL) and the solution was extracted with 1 M NaHCO<sub>3</sub> (3 × 200 mL) and then with 2 M NaCl (3 × 200 mL). The organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and rotoevaporated to dryness under reduced pressure. The material left was purified by chromatography on silica gel (150 g in a 6.5 cm I.D. column) using a gradient of MeOH (0% → 2%) in CH<sub>2</sub>Cl<sub>2</sub> as the eluent. Fractions containing pure **14c** were collected and rotoevaporated to dryness affording a white solid (2.8 g, 4.4 mmol, 88%). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 11.21 (s, 1H), 8.77 (s, 1H), 8.71 (s, 1H), 8.05 (d, *J* = 7.1 Hz, 2H), 7.67–7.47 (m, 3H), 7.29 (d, *J* = 8.1 Hz, 2H), 7.18 (d, *J* = 8.1 Hz, 2H), 6.26 (d, *J* = 6.0 Hz, 1H), 6.20 (s, 1H), 4.90 (t, *J* = 5.5 Hz, 1H), 4.84 (2d <sup>2</sup>*J* = 12.3 Hz, 2H), 4.47 (d, <sup>2</sup>*J* = 11.9 Hz, 1H), 4.40 (dd, *J* = 4.1, 3.8 Hz, 1H), 4.32 (d, <sup>2</sup>*J* = 11.9 Hz, 1H), 4.05 (dt, *J* = 3.8, 3.6 Hz, 1H), 3.74 (dd, *J* = 11.9, 4.1 Hz, 1H), 3.61 (dd, *J* = 11.9, 3.8 Hz, 1H), 3.20 (s, 3H). <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ 165.5, 162.7, 152.0, 151.6, 150.4, 143.1, 140.3, 138.2, 133.2, 132.4, 128.9, 128.4, 126.9, 125.8, 93.8, 86.3, 86.1, 78.4, 69.1, 68.1, 64.8, 61.1, 38.2. APESI-HRMS: calcd for C<sub>28</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>6</sub>O<sub>7</sub> (M)<sup>+</sup> 630.1397, found 630.1388.

**N<sup>2</sup>-Isobutryl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyl-oxymethyl]guanosine (14d)**. The preparation of this compound is essentially identical to that of **14c'** with the exception of the following modification: the stannylated ribonucleoside **19d** (6.5 g, 11 mmol) is mixed with a solution of **18** (16 mmol) in DMF (20 mL) in the absence of tetra-*n*-butylammonium bromide.<sup>10b</sup> The reaction mixture is stirred at 60 °C and processed under conditions identical to those used in the preparation of **14c'**. Crude **14d** was also obtained as a mixture of regioisomeric acetals. Purification of **14d** was achieved by chromatography on silica gel (150 g in a 6.5 cm I.D. column) using a gradient of MeOH (2% → 8%) in CH<sub>2</sub>Cl<sub>2</sub> as the eluent.<sup>29</sup> Fractions containing pure **14d** were collected and rotoevaporated to dryness under reduced pressure affording a white solid (2 g, 3.3 mmol, 30%). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 12.07 (s, 1H), 11.66 (s, 1H), 8.27 (s, 1H), 7.49 (d, *J* = 7.9 Hz, 2H), 7.39 (d, *J* = 7.9 Hz, 2H), 6.20 (s, 1H), 5.85 (d, *J* = 6.6 Hz,



1H), 5.62 (d,  $J = 5.8$  Hz, 1H), 5.14 (t,  $J = 5.3$  Hz, 1H), 4.92 (d,  $^2J = 12.9$  Hz, 1H), 4.91 (d,  $^2J = 12.9$  Hz, 1H), 4.68 (s, 2H), 4.62 (m, 1H), 4.28 (2d,  $J = 4.7$  Hz, 1H), 4.04 (m, 1H), 3.65 (dd,  $J = 12.0$ , 4.8 Hz, 1H), 3.57 (dd,  $J = 12.0$ , 4.8 Hz, 1H), 3.23 (s, 3H), 2.77 (h,  $J = 6.9$  Hz, 1H), 1.12 (d,  $J = 6.9$  Hz, 6H).  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta$  180.0, 154.7, 148.9, 148.1, 140.4, 138.7, 137.2, 129.2, 127.0, 120.0, 93.8, 86.0, 83.6, 75.2, 73.2, 68.2, 64.6, 60.9, 54.8, 38.2, 34.6, 18.73, 18.7. APESI-HRMS: calcd for  $\text{C}_{25}\text{H}_{30}\text{Cl}_2\text{N}_6\text{O}_8$  (M) $^+$  612.1502, found 612.1493.

**General Procedure for the Preparation of 5'-O-(4,4'-Dimethoxytrityl)-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyloxy]-methyl Ribonucleosides 15a–d.** To a solution of a dry ribonucleoside (**14a–d**, 3.5 mmol) in anhydrous pyridine (15 mL) was added 4,4'-dimethoxytrityl chloride (1.4 g, 4.2 mmol). The solution was stirred at  $\sim 25$  °C and progress of the reaction was monitored by TLC ( $\text{CHCl}_3/\text{MeOH}$  (95:5 v/v)) until complete disappearance of **14a–d** ( $\sim 2$  h). The reaction mixture was then poured into satd  $\text{NaHCO}_3$  (200 mL) and the aqueous solution was extracted with  $\text{CH}_2\text{Cl}_2$  (3  $\times$  150 mL). The combined organic layers were dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Following filtration, the filtrate was evaporated under reduced pressure and the material left was coevaporated with toluene (3  $\times$  100 mL). The crude product was purified by chromatography on silica gel (20 g in a 2.5 cm i.d. column) using a gradient of  $\text{CH}_3\text{OH}$  (0  $\rightarrow$  2%) in  $\text{CH}_2\text{Cl}_2$  containing 0.2% (v/v) triethylamine as the eluent. Fractions containing the pure 5'-O-DMTr ribonucleoside were collected and rotoevaporated to a foam under low pressure. Ribonucleosides **15a**, **15b**, **15c**, and **15d** were then dissolved each in dry  $\text{C}_6\text{H}_6$  (10 mL); the resulting solutions were frozen and then lyophilized under high vacuum to produce yellowish powders in the following overall yields, relative to the amounts of the respective ribonucleosides **10a**, **10b**, **10c'**, and **10d** used as starting materials: **15a**, 55%; **15b**, 42%; **15c**, 26%; **15d**, 28%.

**General Procedure for the Preparation of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-[(N,N-diisopropylamino)(2-cyanoethoxy)]phosphinyl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyloxy]-methyl Ribonucleosides (9a–d).** To a solution of a suitably protected ribonucleoside (**15a–d**, 3.0 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (15 mL) containing  $\text{Et}_3\text{N}$  (2 mL, 15 mmol) was added 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.15 mL, 5.10 mmol). The reaction mixture was stirred at  $\sim 25$  °C under argon until complete disappearance of **15a–d** ( $\sim 2$  h) was observed by TLC ( $\text{C}_6\text{H}_6/\text{Et}_3\text{N}$  (9:1 v/v)). The reaction mixture was then poured into water (50 mL) and was extracted with  $\text{CH}_2\text{Cl}_2$  (100 mL). The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and then filtered. The filtrate was rotoevaporated to dryness under reduced pressure. The crude phosphoramidite product was purified by chromatography on silica gel (20 g in a 2.5 cm i.d. column) using  $\text{C}_6\text{H}_6/\text{Et}_3\text{N}$  (9:1 v/v) as the eluent (**9d** required  $\text{C}_6\text{H}_6/\text{CH}_2\text{Cl}_2/\text{Et}_3\text{N}$  (45:45:10 v/v/v) to elute off the column). Fractions containing the pure product (TLC) were pooled together and rotoevaporated to dryness under vacuum. The material was dissolved in dry  $\text{C}_6\text{H}_6$  (3 mL), and the resulting solution was added to cold ( $-78$  °C) stirred hexane (100 mL). The pure ribonucleoside phosphoramidite precipitated immediately as a white solid. After careful decantation of hexane, the solid was dissolved in dry  $\text{C}_6\text{H}_6$  (10 mL); the solution was frozen and then lyophilized under high vacuum.  $\text{Et}_3\text{N}$ -free **9a–d** were isolated each as a powder in yields varying from 62 to 85%.

**5'-O-(4,4'-Dimethoxytrityl)-3'-O-[(N,N-diisopropylamino)(2-cyanoethoxy)]phosphinyl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyloxy]methyluridine (9a).**  $^{31}\text{P}$  NMR (121 MHz,  $\text{C}_6\text{D}_6$ ):  $\delta$  151.9, 150.2. APESI-HRMS: calcd for  $\text{C}_{50}\text{H}_{58}\text{Cl}_2\text{N}_5\text{O}_{11}\text{P}$  (M) $^+$  1005.3248, found 1005.3248.

***N*<sup>4</sup>-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-3'-O-[(N,N-diisopropylamino)(2-cyanoethoxy)]phosphinyl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyloxy]methylcytidine (9b).**  $^{31}\text{P}$  NMR (121 MHz,  $\text{C}_6\text{D}_6$ ):  $\delta$  152.7, 150.6. APESI-HRMS: calcd for  $\text{C}_{57}\text{H}_{63}\text{Cl}_2\text{N}_6\text{O}_{11}\text{P}$  (M) $^+$  1108.3670, found 1108.3668.

***N*<sup>6</sup>-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-3'-O-[(N,N-diisopropylamino)(2-cyanoethoxy)]phosphinyl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyloxy]methyladenosine (9c).**  $^{31}\text{P}$  NMR (121 MHz,  $\text{C}_6\text{D}_6$ ):  $\delta$  152.1, 150.7. APESI-HRMS: calcd for  $\text{C}_{58}\text{H}_{63}\text{Cl}_2\text{N}_6\text{O}_{10}\text{P}$  (M) $^+$  1132.3782, found 1132.3770.

***N*<sup>2</sup>-Isobutyryl-5'-O-(4,4'-dimethoxytrityl)-3'-O-[(N,N-diisopropylamino)(2-cyanoethoxy)]phosphinyl-2'-O-[4-(N-dichloroacetyl-N-methylamino)benzyloxy]methylguanosine (9d).**  $^{31}\text{P}$  NMR (121 MHz,  $\text{C}_6\text{D}_6$ ):  $\delta$  151.4, 151.3. APESI-HRMS: calcd for  $\text{C}_{55}\text{H}_{65}\text{Cl}_2\text{N}_8\text{O}_{11}\text{P}$  (M) $^+$  1114.3887, found 1114.3880.

**Solid-Phase Oligonucleotide Synthesis.** Solid-phase synthesis of AUCCGUAGCUAACGUCAUGG was conducted on a scale of 0.2  $\mu\text{mole}$  in the "trityl-off" mode using a succinyl long chain alkylamine controlled-pore glass (Succ-LCAA-CPG) support functionalized with *N*<sup>2</sup>-dimethylaminomethylene-5'-O-(4,4'-dimethoxytrityl)-2'-/3'-O-acetylguanosine as the leader nucleoside. The solid support was mixed with 0.5 mL of each Cap A solution ( $\text{Ac}_2\text{O}$ /pyridine/THF) and Cap B solution (10% 1-methylimidazole in THF) for a period of 60 s to acetylate any unprotected hydroxyls of the leader nucleoside. RNA oligonucleotide synthesis was carried out using a DNA/RNA synthesizer and phosphoramidites **9a–d**, which were dissolved in dry MeCN to give 0.2 M solutions. 5-Benzylthio-1*H*-tetrazole was employed for phosphoramidite activation and used as a 0.25 M solution in dry MeCN. All other ancillary reagents necessary for the preparation of oligonucleotides, with the exception of trichloroacetic acid (TCA), were purchased and utilized as recommended by the manufacturer. The reaction time for each phosphoramidite coupling step was set to 180 s. The dedimethoxytritylation step was effected using 4% TCA in  $\text{CH}_2\text{Cl}_2$ <sup>17b</sup> during a period of 60 s. The capping step of each synthesis cycle was also programmed for a duration of 60 s.

**Characterization and Deprotection of AUCCGUAGCUAACGUCAUG[2'-O-(4-MABOM)]<sub>19</sub>G.** The solid-phase-linked 5'-dedimethoxytritylated oligonucleotide was placed into a 4 mL screw-capped glass vial to which was added concd  $\text{NH}_4\text{OH}$  (1 mL). The suspension was shaken occasionally over a period of 30 min. The ammoniacal solution was then transferred to another 4 mL glass screw-capped and was heated at 55 °C for 10 h. A sample of the ammoniacal solution (0.9 OD<sub>260</sub>) was evaporated to dryness using a stream of air. The oligonucleotide was then dissolved in 0.1 M triethylammonium acetate, (pH 7.0, 100  $\mu\text{L}$ ) and was analyzed by RP-HPLC. The analysis was performed using a 5  $\mu\text{m}$  Supelcosil LC-18S column (25 cm  $\times$  4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min and was then held, isocratically, for 20 min. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit. The RP-HPLC chromatogram of unpurified AUCCGUAGCUAACGUCAUG[2'-O-(4-MABOM)]<sub>19</sub>G ( $t_R = 33.7$  min) is shown in Figure 1A. The remaining portion of the ammoniacal solution of AUCCGUAGCUAACGUCAUG[2'-O-(4-MABOM)]<sub>19</sub>G (15 OD<sub>260</sub>) was evaporated to dryness employing a stream of air. The oligonucleotide was dissolved in 0.1 M AcOH buffered to pH 3.8 with *N,N,N',N'*-tetramethylethylenediamine (TEMED).<sup>21b</sup> The solution was kept at 90 °C for 30 min. A sample (0.8 OD<sub>260</sub>) of the fully deprotected AUCCGUAGCUAACGUCAUGG was analyzed by RP-HPLC under chromatographic conditions identical to those used for the analysis of AUCCGUAGCUAACGUCAUG[2'-O-(4-MABOM)]<sub>19</sub>G. The RP-HPLC chromatogram of unpurified and fully deprotected AUCCGUAGCUAACGUCAUGG ( $t_R = 17.5$  min) is shown in Figure 1B. The solution of the fully deprotected oligoribonucleotide was applied on the top of a 10-mL PD-10 (Sephadex G-25M) column. The oligonucleotide was eluted from the column using DEPC-treated  $\text{H}_2\text{O}$  as the eluent. Fractions of 1 mL were collected and those containing the RNA oligomer ( $A_{260}$ ) were pooled together and evaporated to dryness using a stream of air. The unpurified oligoribonucleotide was dissolved in DEPC-treated  $\text{H}_2\text{O}$  and its

concentration was determined by UV spectroscopy at 260 nm. RP-HPLC analysis of the desalted unpurified oligomer was performed and the chromatogram resulting from this analysis is shown in Figure 1C. MALDI-TOF MS: calcd for  $C_{190}H_{217}N_{75}O_{138}P_{19}$   $[M - H]^-$  6346, found 6346.

**Supporting Information Available:** Materials and methods; procedure for polyacrylamide gel electrophoresis analysis of unpurified and desalted AUCCGUAGCUAACGUCAUGG; procedures for hydrolysis of AUCCGUAGCUAACGUCAUGG catalyzed by both snake venom phosphodiesterase/bacterial alkaline phosphatase and bovine spleen phosphodiesterase/bacterial alkaline phosphatase;  $^1H$  NMR spectra of **14a-d** and **17**;  $^{13}C$  NMR spectra

of **14a-d** and **17**;  $^{31}P$  NMR spectra of **9a-d**; mass spectra of **14a-d** and **9a-d**; expanded Figures 1 and 4A,B; RP-HPLC chromatograms comparing AUCCGUAGCUAACGUCAUGG obtained commercially and through solid-phase synthesis employing phosphoramidites **9a-d**; RP-HPLC analysis of synthetic (3'→5')-UpU and (2'→5')UpU; RP-HPLC chromatograms of the enzymatic digestion of synthetic (3'→5')UpU and (2'→5')UpU catalyzed by either snake venom phosphodiesterase or bovine spleen phosphodiesterase; RP-HPLC chromatogram of the conversion of adenosine to inosine by bovine spleen phosphodiesterase. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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