Ion-tagged synthesis of an oligoribonucleotide pentamer — The continuing versatility of TBDMS chemistry[1]

Robert A. Donga, Tak-Hang Chan, and Masad J. Damha

Abstract: An oligoribonucleotide has been synthesized in solution, using an ionic-liquid-based soluble tag at a scale several hundred times that of a standard solid-phase synthesis approach. Ogilvie's 2′-TBDMS strategy was adopted, and because of the resultant increase in lipophilicity, it allowed an easier purification of the growing oligomer compared with the previously observed for DNA, which does not require 2′ protection. The procedure is illustrated by the synthesis of the pentaribonucleotide sequence AGAUC, corresponding to a segment of the $tRNA^{fMet}$ from *E. coli.*

Key words: solution-phase RNA synthesis, ionic-liquid tag.

Résumé : Nous avons réalisé la synthèse d'un oligoribonucléotide en solution en utilisant un support soluble à base d'un liquide ionique dont la quantité correspond à plusieurs centaines de fois celle qui serait utilisée dans une approche basée sur une synthèse en phase solide normale. On a adopté la stratégie 2′-TBDMS d'Ogilvie et on a trouvé que, en raison de son caractère lipophile, la purification de l'oligomère croissant se fait beaucoup plus facilement que ce qui a été observé avec l'ADN qui ne requiert pas de protection en 2′. La méthode est illustrée par la synthèse de la séquence du pentaribonulcléotide AGAUC qui correspond à un segment de la $t\text{-}ARN^{fMet}$ du *E. coli*.

Mots-clés : synthèse d'ARN en solution, traceur ionique liquide.

[Traduit par la Rédaction]

Introduction

Since it was first introduced by Ogilvie and co-workers in 1974 (1), the *tert*-butyl dimethylsilyl (TBDMS) group has become the standard 2′-protecting group in both solid- and solution-phase oligomerizations of ribonucleotides (2). Along with the phosphoramidite approach (3) to ribonucleoside coupling, the TBDMS group remains one of the most widely used 2′-protecting groups for commercially available ribo-phosphoramidites. With the advent of RNAi and an increasing number of RNA-based therapeutics progressing toward clinical development (4), the TBDMS approach will continue to be an important resource as more commercially viable processes are developed for the large-scale synthesis of oligoribonucleotides.

However, the limitation in RNA chemistry, thus far, has been the cost-efficient preparation of oligomers at a sufficiently large scale. A solid-phase approach to large-scale DNA and RNA synthesis is expensive because of the cost of the solid supports and the need for a large excess of phosphoramidite compared with what is usually required in a solution-phase approach (5). A conventional solution-phase approach, where the 3′-terminal nucleoside is not linked to a support but simply protected at the 3′ position (and 2′ for RNA), is intrinsically arduous because of the necessity of chromatographic purification after each coupling cycle. An alternative approach, developed by Bonara et al. (6), uses a soluble polyethylene glycol tag for DNA solution-phase synthesis. In an effort to improve this strategy, which requires an energy-intensive cooling step during purification, and to adapt it to use in RNA, we have been exploring the utility of ionic liquids as soluble supports in oligonucleotide synthesis (7). Herein, we report the preparation of a pentaribonucleotide in solution, requiring only simple precipitations at ambient temperature for purification during the chain-extension cycles. The process retains the versatile TBDMS group as the 2′-protecting group. The pentaribonucleotide was prepared on a 1 g scale and was assembled in 32 h (an average of 8 h per coupling cycle).

Received 10 January 2007. Accepted 28 February 2007. Published on the NRC Research Press Web site at canjchem.nrc.ca on 18 April 2007.

R.A. Donga, T.-H. Chan, and M.J. Damha.[2] Department of Chemistry, McGill University, Montreal, QC H3A 2K6 Canada.

[1]This article is part of a Special Issue dedicated to Professor Kelvin K. Ogilvie.

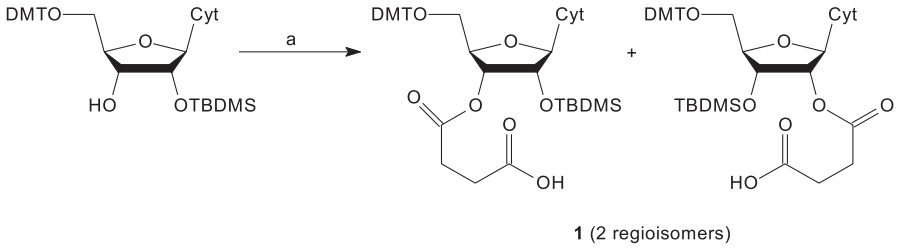
[2]Corresponding author (e-mail: masad.damha@mcgill.ca).

Discussion

The sequence of the selected pentaribonucleotide represents base positions 45 to 49 of an unpaired coil region of *N*-formyl methionine tRNA from *E. coli*. It was selected primarily because it contains all four common ribonucleosides. It is also a sequence that has been previously synthesized in solution by Damha using the phosphite triester approach (8), which required several weeks to achieve. The latter compound serves as a good reference standard for the pentaribonucleotide made by the process described here.

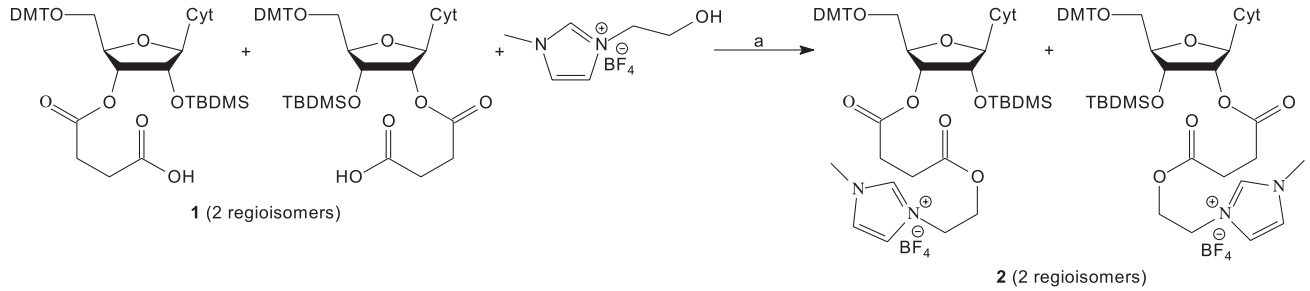
 doi:10.1139/V07-022

Scheme 1. Nucleoside succinylation.



a: Succinic anhydride, 4-dimethylaminopyridine, and anhydrous acetonitrile

Scheme 2. Coupling of ionic tag.

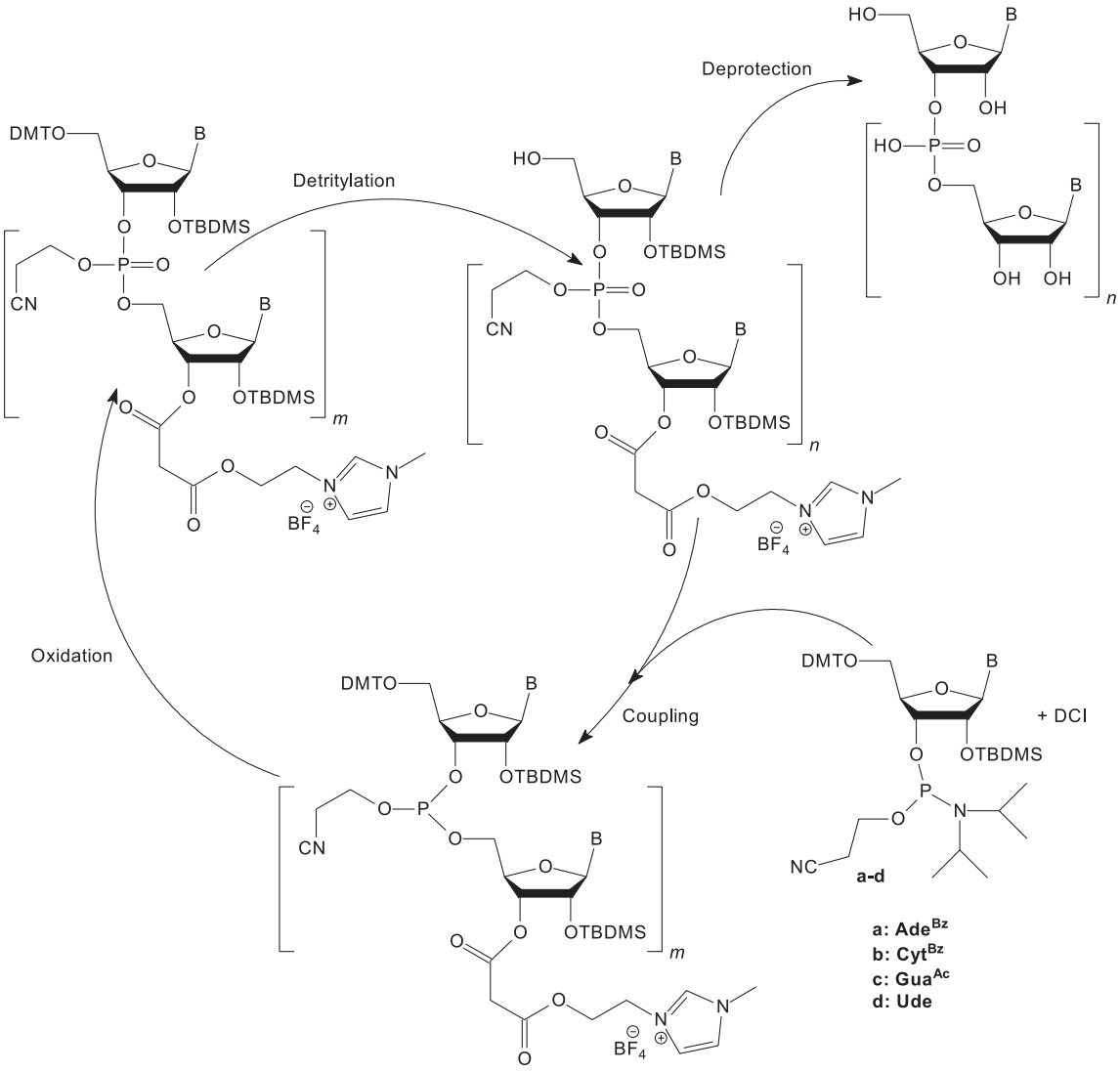


a: *O*-(benzotriazol-l-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate, triethylamine, and anhydrous acetonitrile

Synthesis began with the attachment of the 3′-cytidine to the ionic ethyl-methyl-imidazolium tag through a succinyl linker. Thus, *N*-benzoyl-2′-*tert*-butyldimethylsilyl-5′-dimethoxytrityl-cytidine was reacted with succinic anhydride in the presence of 4-dimethylaminopyridine (DMAP). The presence of the base, DMAP, caused the 2′-TBDMS protecting group to isomerize, yielding a statistical mixture of 2′-TBDMS- and 3′-TBDMS-protected cytidine with no alteration of the other protecting groups (Scheme 1). However, this isomerization is inconsequential, since the 2′/3′-silyl group and the 3′/2′-succinyl linker of this terminal nucleoside will be removed to release the required 2′- and 3′-OH groups during the final deprotection step of the desired full-length pentamer. In addition, it would be expected that the solubility properties of the two regioisomers, an important consideration for the purification steps, would be very similar. Succinylation at the free 3′- or 2′-hydroxyl group provided the 2′/3′-succinylated nucleoside along with some unreacted nucleoside. This crude mixture was then esterified with the free hydroxyl group of the ionic tag (Scheme 2). In our previous work (7), dicyclohexylcarbodiimide (DCC) was used as the condensing reagent, but long reaction times were required (3–7 d), and the yield of the desired succinate ester was only 65%–85%. An alternative coupling reagent, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), proved to be more suitable for this reaction. In addition, its counterion, tetrafluoroborate, matched that of the ionic liquid employed as the tag. Thus, the succinylated nucleoside **1** and TBTU were first mixed in the solid state, followed by the addition of the ionic liquid [3-(2′-hydroxyethyl)-1-methylimidazolium tetrafluoroborate], triethylamine, and finally the solvent. The reaction was complete in less than 90 min, yielding the desired product **2** and a small amount of nucleoside dimer; the latter formed by the reaction of **2** with a small amount of 5′-DMT rC^{NBz} TBDMS that was carried forward from the succinylation step. At this point, a water–chloroform extraction was performed to remove any excess ionic liquid, the excess coupling reagent, and the resulting tetramethylurea by-product of the reaction, leaving a mixture of nucleoside dimer and the desired product **2**. The ion-tagged nucleoside **2** was easily separated from the dimer and any other remaining impurities by two simple precipitations. In our previous work on DNA oligomers (7), the preferred precipitation medium consisted of a mixture of ethyl acetate and diethyl ether. This yielded purer precipitates than diethyl ether alone by effectively solubilizing the undesired by-products while generating the desired tagged oligomer as a finely divided precipitate. However, when this media was used here, it was found that the nucleoside **2** was slightly soluble, resulting in a loss of the desired product and collapse of the precipitate into a slightly impure waxy solid. Therefore, the system was changed to neat diethyl ether as this was found to restore the desired properties of the precipitation medium with no appreciable loss of the tagged materials.

The synthesis cycle (Scheme 3) requires the removal of the DMT group at the 5′-terminus of the growing oligonucleotide chain. Generally, this is achieved by treatment with trifluoroacetic acid in dichloromethane. In our earlier work on DNA, this step was often problematic, especially for removing the DMT group from the nucleoside unit directly linked to the ionic tag. We ascribe the slower rate of DMT cleavage to the proximal imidazolium ion, which makes protonation of the O5′ rate limiting. A similar phenomenon is generally invoked to explain the slower rate of esterification of glycine (NH_3^+–CH_2–COOH), relative to propanoic acid under acidic conditions (9). Consistent with this notion, the rate of detritylation increases as the site of

Scheme 3. Oligoribonucleotide coupling cycle (illustrated as one regioisomer).



protonation is spatially removed from the positively charged ion tag (i.e., rate of detritylation of trimer > monomer; see later). Alternate detritylation methods employing Lewis acids, such as zinc bromide, were tested and found to be somewhat successful, but Lewis acids were more difficult to remove via precipitation. Thymine and cytosine, and to a greater extent adenine and guanine, are sensitive to acid-mediated depyrimidation/depurination (10), rendering the application of stronger acids troublesome in DNA synthesis. Because of the inductive effects of 2′-OH groups, RNA is not as prone to acid-mediated depurination; therefore, a stronger acid may be employed in the detritylation step. Thus, *p*-toluenesulphonic acid was selected as the detritylation reagent, as it was found that a single treatment was sufficient to achieve complete detritylation of the first nucleoside. Weaker acids, such as trifluoroacetic acid, required multiple treatments and longer reaction times to achieve the same result.

With the 5′ position of the 3′-nucleoside deblocked, the oligomerization cycle could begin (Scheme 3). The nucleoside **3** was mixed with 1.66 equiv. of ribouridine (2′-TBDMS-protected) 3′-cyanoethylphosphoramidite, using 4,5-dicyanoimidazole (DCI) as the activator (0.06 mol/L in anhyd. acetonitrile). After 2 h of stirring, excess *tert*-butanol (approximately 6 equiv.) was added to quench the excess phosphoramidite and to enhance the solubility of the resulting nucleoside 3′-(*tert*-butyl-2-cyanoethyl) phosphite triester by-product in the precipitation medium. The intermediate UpC dimer was isolated and purified by adding the reaction mixture dropwise to stirred diethyl ether. The resultant fine precipitate was filtered over celite and recovered from the filter by dissolving in acetone.

Once purified, the intermediate UpC phosphite triester was redissolved in anhydr. acetonitrile, and a capping step was performed using acetic anhydride and DMAP. In our DNA synthesis (7), oxidation of the intermediate phosphite triester to the phosphate was achieved using iodine as the oxidant. This procedure necessitated a difficult extraction to remove the generated salts but precluded the need for a capping step, since any unreacted oligomer would be removed with the aq. phase. The difficulty of the extraction (because of the formation of strong emulsions) outweighed the advan-

Table 1. Physical data.

Compound	Recovery (%)	Formula[a]	^{31}P NMR (ppm)	*m/z* (Calculated)	*m/z* (Experimental)
5-DMT rC Succ-IL (**2**)	73	$C_{53}H_{62}N_5O_{11}Si^+$	—	972.4	972.3
5-HO rC Succ-IL (**3**)	93	$C_{32}H_{44}N_5O_9Si^+$	—	670.3	670.2
5-DMT rUC Succ-IL (**4**)	93	$C_{71}H_{90}N_8O_{19}PSi_2^+$	—	1445.6	1446.0
5-HO rUC Succ-IL (**5**)	>100	$C_{50}H_{72}N_8O_{17}PSi_2^+$	0.082 to –0.513	1143.4	1143.5
5-DMT rAUC Succ-IL (**6**)	91	$C_{97}H_{123}N_{14}O_{26}P_2Si_3Na^{2+}$	—	1034.4	1034.5
5-HO rAUC Succ-IL (**7**)	>100	$C_{76}H_{105}N_{14}O_{24}P_2Si_3^+$	0.510 to –0.508	1743.6	1744.4
5-DMT rGAUC Succ-IL (**8**)	99	$C_{118}H_{154}N_{20}O_{34}P_3Si_4Na^{2+}$	—	1311.5	1311.7
5-HO rGAUC Succ-IL (**9**)	91	$C_{97}H_{136}N_{20}O_{32}P_3Si_4Na^{2+}$	0.637 to –0.466	1160.4	1161.0
5-DMT rAGAUC Succ-IL (**10**)	95	$C_{144}H_{187}N_{26}O_{41}P_4Si_5Na^{2+}$	—	1611.6	1611.8
5-HO rAGAUC Succ-IL (**11**)	96	$C_{123}H_{169}N_{26}O_{39}P_4Si_5Na^{2+}$	0.599 to –1.318	1460.5	1460.8

tage of the removal of the capping step, especially since the capping step overcomes the undesirable O6 extension sometimes observed for guanosine (11). Thus, in the present protocol for RNA, the capping step was reinstated, and the oxidation procedure was changed to employ a reagent that would not require extractions. *tert*-Butylhydroperoxide (6 mol/L) in decane was chosen and found to completely oxidize the phosphite triester in less than 15 min. A further advantage is that it could easily be removed, together with its by-product *tert*-butanol, since it was completely soluble in the precipitation medium. At this point, it was observed by mass spectroscopy that the original tetrafluoroborate counteranion of the tag had been exchanged for dicyanoimidazolide, an effect that was not observed in our previous work on DNA synthesis. Indeed, anion exchange occurred several times during the synthesis cycles, alternating between dicyanoimidazolide and *p*-toluenesulphonate, but this did not appear to adversely affect the solubility properties of the intermediate di (UpC), tri (ApUpC), tetra (GpApUpC), and pentanucleotides (ApGpApUpC), isolated during and after each coupling step (see later). It had been previously observed in other experiments (data not shown) that the solubility properties of these types of molecules were adversely affected when exchanges occurred for halides; thus, the identity of the counterion is of importance, since a change in solubility can have a dramatic impact on the quality and yield of the synthesis cycle. Therefore, this must be taken into account during the design of the oligomerization cycle such that if anion exchanges occur, the resulting ion-paired molecule maintains an acceptable level of solubility in the solvents used for synthesis and recovery, while also maintaining its insolubility in the precipitation medium.

Chain extension proceeded as previously outlined, but it was found that the yields exceeded 100% after the detritylation steps, as determined by the weight of the product (Table 1). This was due to the incomplete removal of *p*-toluenesulphonic acid, which subsequently interfered with the next coupling steps (e.g., premature detritylation following the next coupling step). Once this effect was realized, the detritylating reagent was substituted with the volatile trifluoroacetic acid, which could be more readily removed during the precipitation step or afterwards, by evaporation if necessary. Again, this approach necessitated two acid treatments per detritylation step, but no further premature detritylation was observed at the coupling stage, and precipitations alone were sufficient to remove the acid. Thus, while it has been found that weaker acids may be used for the detritylation step of the oligomerization cycle shortly after the initial nucleoside is deprotected, multiple treatments are still required so it would be advantageous to use stronger acids if their complete removal could be assured.

One of the advantages of solution-phase over solid-phase synthesis of oligomers is the capacity to directly monitor chain elongation by various means, including mass spectrometry (MS). Indeed, ion-tagged oligomers are especially amenable to this technique, since the molecules contain permanent positive charges. This means that ion-tagged molecules only need to have their solutions nebulized in an electrospray ionization (ESI) source to give very strong MS signals. Indeed, when using an octopole analyzer, short oligomers provided excellent results. Long oligomers quickly exceed the upper limits of the mass detector; however, in these cases multiple charging occurred more readily, pulling the analyte back into an observable window (Fig. 1). This is noticed by comparing the mass spectrum for longer oligomers to that of UpC (Fig. 2). For the dimer, the ion tag creates a high-energy barrier to protonation and likewise to formation of these adducts. As the chain length increases, the site of protonation is spatially removed from the positively charged ion tag, and adducts are readily observed. Using this technique, the identities of all post-detritylation compounds were confirmed by HR-ESIMS (Table 2).

Finally, the pentamer **11** was deprotected to the free nucleotide in parallel with a sample of the original sequence **8**, made by the original solution-phase approach (8). This consisted of treatment of 1–2 mg of each detritylated (but otherwise fully protected oligomer) with 1 mL of 40% (*v*/*v*) aq. methylamine and incubating at 60 °C for 20 min. The resultant solution was cooled to –78 °C and dried under vacuum. The resultant solid was treated with 300 µL of 1.5:0.75:1 (*v*/*v*/*v*) triethylamine trihydrofluoride – 1-methyl-2-pyrrolidinone – triethylamine solution and incubated for 90 min at 60 °C (12). The reaction mixture was then cooled to ambient temperature and 1 mL of anhyd. ethanol was added. The samples were mixed and cooled at –78 °C for 30 min, resulting in precipitates. The samples were then centrifuged at 14000 r/min at 4 °C for 30 min, and the supernatant was removed. The precipitates were washed

Fig. 1. Low resolution ESIMS of **9**.

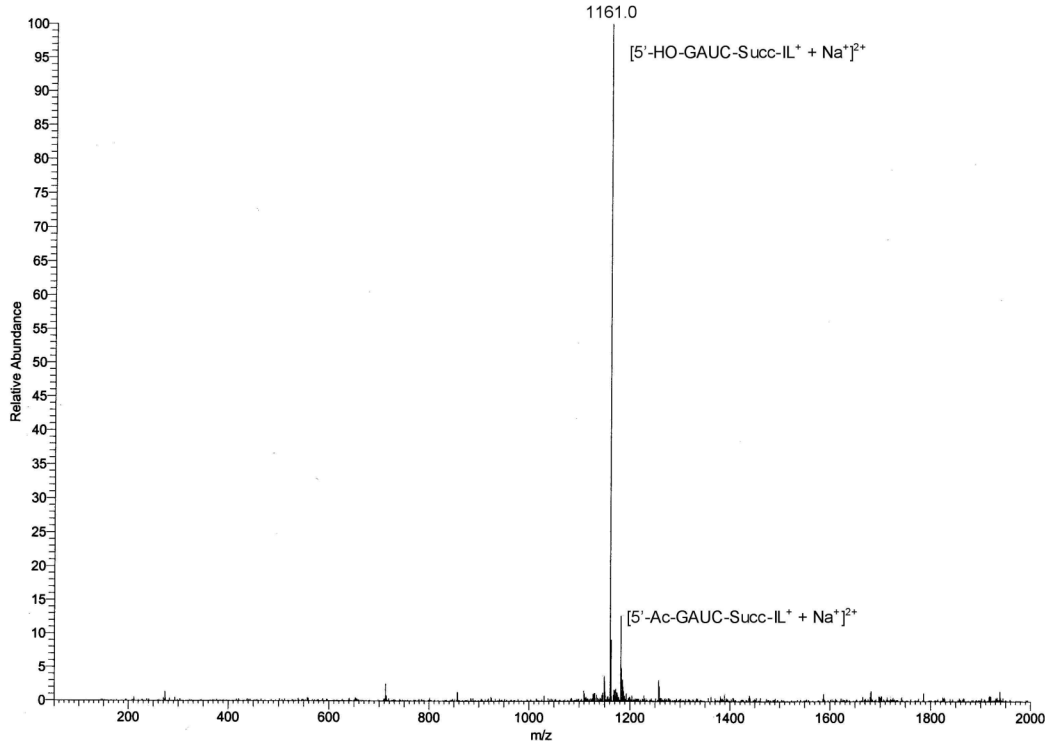
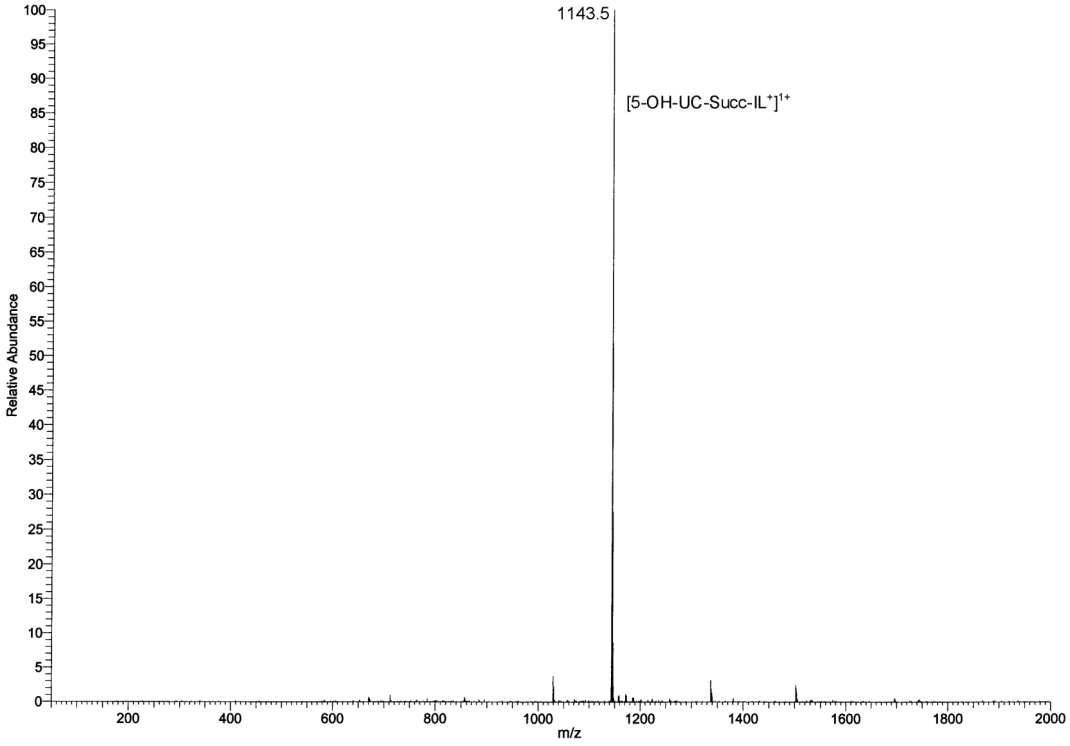


Fig. 2 Low resolution ESIMS of **3**.



with 200 μL of –78 °C ethanol, and then the residual solvent was removed under vacuum. The precipitates were dissolved in 1 mL of autoclaved, deionized water and loaded onto Waters® Sep-Pak™ C_{18} cartridges for desalting. The cartridges were rinsed with water, and then the desired products were eluted with 1:1:1 (*v*/*v*/*v*) water–methanol–acetonitrile. The solvent was removed under vacuum to yield the crude, deprotected AGAUC pentamers.

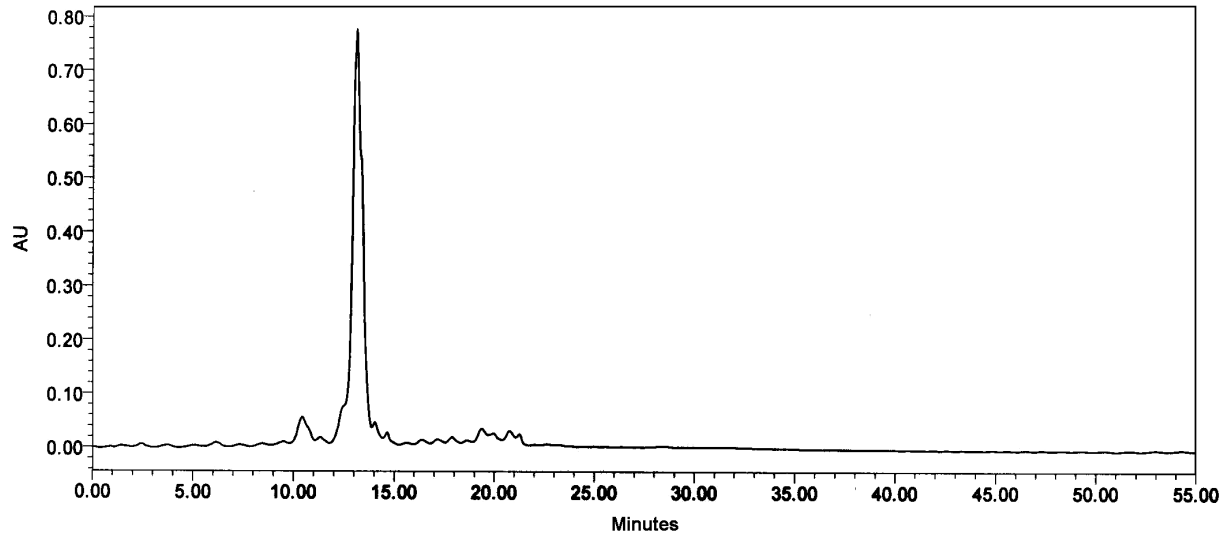
The identity and purity of the pentamers were assessed using a triethylammonium acetate ion-pairing reverse-phase

Table 2. HR-ESIMS data.

Compound	Formula[a]	*m/z* (Calculated)	*m/z* (Experimental)
5-HO rC Succ-IL (**3**)	$C_{32}H_{44}N_5O_9Si^{+1}$	670.29069	670.29028
5-HO rUC Succ-IL (**5**)	$C_{50}H_{73}N_8O_{17}Si_2P^{+2}$	572.21727	572.21795
5-HO rAUC Succ-IL (**7**)	$C_{76}H_{106}N_{14}O_{24}Si_3P_2^{+2}$	872.31268	872.31383
5-HO rGAUC Succ-IL (**9**)	$C_{97}H_{137}N_{20}O_{32}Si_4P_3^{+2}$	1149.39748	1149.39934
5-HO rAGAUC Succ-IL (**11**)	$C_{123}H_{171}N_{26}O_{39}Si_5P_4^{+3}$	966.66590	966.66546

[a]Excluding counterion.

Fig. 3. HPLC chromatogram of the co-injection of **11** and solution-phase synthesized standard.



HPLC analysis on a Phenomenex® Clarity™ semipreparatory-scale column. The samples were injected individually, and the major peaks in the chromatograms were collected and analyzed by low resolution ESIMS in the negative mode. The major ion found in both cases corresponded exactly to the expected mass of the fully deprotected AGAUC pentamers missing a single proton (1551 daltons). The two samples were also co-injected at a 1:1 ratio in the HPLC column and found to overlap perfectly (Fig. 3). As a final test to show that the deprotected compounds were identical, they were analyzed side by side, co-loaded on a 24% polyacrylamide gel, and electrophoresed (data not shown). In all cases, single bands with the same electrophoretic mobility were found, showing that indeed the synthesis using the ionic tag yielded, after deprotection, an identical compound to that of the standard solution-phase-derived molecule.

Conclusion

In summary, a functional method has been developed to rapidly synthesize oligoribonucleotides in solution and in excellent yields. The method utilizes simple precipitations as the sole purification method during chain elongation and is amenable to scales several hundred times that of a standard solid-phase synthesis. Although 5′-detritylation continues to pose a challenge, the presence of the lypophilic TBDMS group greatly enhances the solubility of the phosphoramidite monomers in the medium used for the precipitation-based purifications. Thus, the purification of RNA using this approach is superior to that of DNA, as observed in our previous work (7). We believe that the soluble ionic tag we have developed, in conjunction with Ogilvie's RNA method, enable the synthesis of short RNA oligomers as rapidly as DNA. We will further explore the limits of this method with target sequences corresponding to those of short interfering RNA (19–23 nucleotides).

Experimental

N-benzoyl-2′/3′-*tert*-butyldimethylsilyl-3′/2′-succinyl-5′-dimethoxytrityl-cytidine (1)

N-benzoyl-2′-*tert*-butyldimethylsilyl-5′-dimethoxytrityl-cytidine (1 g, 1.31 mmol) was mixed with succinic anhydride (0.39 g, 3.93 mmol) and 4-dimethylaminopyridine (0.16 g, 1.34 mmol), and the mixture was dissolved in 30 mL of dry acetonitrile. The reaction was stirred for 4 d at room temperature, and the progress was determined by TLC (9:1 dichloromethane–methanol), which showed the disappearance of the starting material and the appearance of two products (2′-3′ isomerization of the TBDMS-protecting group). The solvent was removed under vacuum, and the resulting solid was taken up in chloroform and extracted with water. The organic layer was dried with anhyd. sodium sulphate, filtered, and the solvent was removed under vacuum. The resulting crude product (1.1 g,

97%) was used without further purification. $C_{47}H_{54}N_3O_{11}Si^+$ (low resolution ESIMS) calculated: 864.3; found: 863.9.

N-benzoyl-2′/3′-*tert*-butyldimethylsilyl-3′/2′-(1-succinyl-4-[3-(2″-oxyethyl)-1″-methylimidazolium tetrafluoroborate])-5′-dimethoxytrityl-cytidine (2)

Compound **1** (1.1 g, 1.31 mmol) was mixed with TBTU (0.5 g, 1.58 mmol), 3-(2′-hydroxyethyl)-1-methylimidazolium tetrafluoroborate (0.34 g, 1.58 mmol) and triethylamine (0.4 mL, 2.62 mmol). The mixture was dissolved in 20 mL dry acetonitrile and stirred for 90 min. The solvent was then removed under vacuum, and the resulting oil was taken up in chloroform and extracted with water. The organic layer was dried over sodium sulphate. The solvent was removed under vacuum, and the resulting solid was dissolved in 5 mL of acetone. The solution was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, taken up in acetone, and precipitated again. The solid was filtered off, rinsed, and collected from the filter by dissolution in acetone (1.01 g, 73%) and was used without further purification. $C_{53}H_{62}N_5O_{11}Si^+$ (low resolution ESIMS) calculated: 972.4; found: 972.3, tetrafluoroborate counterion by ESIMS.

N-benzoyl-2′/3′-*tert*-butyldimethylsilyl-3′/2′-(1-succinyl-4-[3-(2″-oxyethyl)-1″-methylimidazolium *p*-toluenesulphonate])-cytidine (3)

Compound **2** (0.69 g, 0.65 mmol) was dissolved in 10 mL of 0.1 mol/L *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 min. Methanol (5 mL) was added to quench the trityl cation, and the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, taken up in acetone, and precipitated again. The solid was filtered off, rinsed, and collected from the filter by dissolution in acetone (0.51 g, 93%) and was used without further purification. $C_{32}H_{44}N_5O_9Si^{+1}$ (HR-ESIMS) required: 670.29069; found: 670.29028, *p*-toluenesulphonate counterion by ESIMS.

5′-DMT-rU-(2′-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as dicyanoimidazolide salt) (4)

Compound **3** (0.44 g, 0.53 mmol) was mixed with 2-*tert*-butyldimethylsilyl-5-dimethoxytrityl-uridine-3-cyanoethyl-phosphoramidite (0.75 g, 0.88 mmol) and dicyanoimidazole (0.14 g, 1.2 mmol), and the mixture was dissolved in 20 mL of dry acetonitrile. The reaction mixture was stirred for 2 h, and then 0.5 mL of *tert*-butanol was added. The mixture was stirred a further 15 min and was then precipitated from diethyl ether as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone and then removing the solvent under vacuum. The resulting solid was dissolved in 5 mL of dry acetonitrile and 250 μL each of a 17.5% (*v*/*v*) solution of acetic anhydride in anhyd. acetonitrile and saturated dimethylaminopyridine in 25% (*v*/*v*) anhyd. pyridine – acetonitrile. This reaction mixture was stirred for 10 min, and then 0.5 mL of 6 mol/L *tert*-butylhydroperoxide in decane was added. After a further 10 min of stirring, the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, and collected from the filter by dissolution in acetone (0.77 g, 93%) and was used without further purification. $C_{71}H_{90}N_8O_{19}PSi_2^+$ (low resolution ESIMS) calculated: 1445.6; found: 1446.0, dicyanoimidazolide counterion by ESIMS.

5′-HO-rU-(2′-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (5)

Compound **4** (0.77 g, 0.49 mmol) was dissolved in 10 mL of 0.1 mol/L *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 min. Methanol (5 mL) was added to quench the trityl cation, and the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, taken up in acetone, and precipitated again. The solid was filtered off, rinsed and collected from the filter by dissolution in acetone (0.68 g, 0.65 g theoretical yield, excess thought to be residual salts), dried under vacuum, and was used without further purification. ^{31}P NMR (ppm): 0.082 to –0.513. $C_{50}H_{73}N_8O_{17}Si_2P^{+2}$ (HR-ESIMS) required: 572.21727; found: 572.21795, *p*-toluenesulphonate counterion by ESIMS.

5′-DMT-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rU-(2′-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as dicyanoimidazolide salt) (6)

Compound **5** (0.60 g, 0.46 mmol) was mixed with *N*-benzoyl-2′-*tert*-butyldimethylsilyl-5′-dimethoxytrityl-adenosine (1.65 g, 1.67 mmol) and dicyanoimidazole (0.12 g, 1.0 mmol), and the mixture was dissolved in 10 mL of dry acetonitrile. Upon addition of solvent, the solution turned pale orange, indicating that the excess mass appreciated in the previous step was likely *p*-toluenesulphonic acid, causing some detritylation of the incoming phosphoramidite. The reaction was stirred for 1 h, and then 0.5 mL of *tert*-butanol was added. The mixture was stirred a further 15 min and was then precipitated from diethyl ether, as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone and then removing the solvent under vacuum. The resulting solid was dissolved in 5 mL of dry acetonitrile and 250 μL each of a 17.5% (*v*/*v*) solution of acetic anhydride in anhyd. acetonitrile and saturated dimethylaminopyridine in 25% (*v*/*v*) anhyd. pyridine – acetonitrile. This reaction mixture was stirred for 10 min, and then 0.5 mL of 6 mol/L *tert*-butylhydroperoxide in decane was added. After a further 10 min of stirring, the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, collected from the filter by dissolution in acetone (0.89 g, 91%), and was used without further purification. $C_{97}H_{123}N_{14}O_{26}P_2Si_3Na^{2+}$ (low resolution ESIMS) calculated: 1034.4; found: 1034.5, dicyanoimidazolide counterion by ESIMS.

5′-HO-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rU-(2-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (7)

Compound **6** (0.89 g, 0.41 mmol) was dissolved in 5 mL of 0.1 mol/L *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 min. Methanol (1 mL) was added to quench the trityl cation, and the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided

precipitate. The precipitate was filtered off, rinsed with diethyl ether, dissolved off the filter with acetone, and dried under vacuum. TLC analysis indicated that detritylation was incomplete, so the solid was dissolved in a further 5 mL of 0.1 mol/L *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 min. Methanol (1 mL) was again added to quench the trityl cation, and the reaction mixture was precipitated as before. The precipitate was filtered off, rinsed with diethyl ether, and collected off the filter by dissolution in acetone. The solution was concentrated under reduced pressure to an approximate volume of 5 mL and was again precipitated from diethyl ether. The solid was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.81 g, 0.79 g theoretical yield, excess thought to be residual salts), dried under vacuum, and was used without further purification. ^{31}P NMR (ppm): 0.510 to –0.508. $C_{76}H_{106}N_{14}O_{24}Si_3P_2^{+2}$ (HR-ESIMS) required: 872.31268; found: 872.31383, *p*-toluenesulphonate counterion by ESIMS.

5′-DMT-rG-(*N*-Ac, 2′-TBDMS)-3′-5′-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rU-(2′-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (8)

Compound **7** (0.71 g, 0.37 mmol) was mixed with *N*-acetyl-2′-*tert*-butyldimethylsilyl-5′-dimethoxytrityl-guanosine (0.72 g, 0.77 mmol) and dicyanoimidazole (0.10 g, 0.84 mmol), and the mixture was dissolved in 7 mL of dry acetonitrile. Upon addition of the solvent, the solution turned slightly orange, indicating that the excess mass appreciated in the previous step was likely *p*-toluenesulphonic acid, causing some detritylation of the incoming phosphoramidite. The reaction was stirred for 1 h, and then 0.5 mL of *tert*-butanol was added. The mixture was stirred a further 15 min and was then precipitated from diethyl ether, as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone and then removing the solvent under vacuum. The resulting solid was dissolved in 5 mL of dry acetonitrile and 250 µL each of a 17.5% (*v*/*v*) solution of acetic anhydride in anhyd. acetonitrile and saturated dimethylaminopyridine in 25% (*v*/*v*) anhyd. pyridine – acetonitrile. This reaction mixture was stirred for 10 min, and then 0.5 mL of 6 mol/L *tert*-butylhydroperoxide in decane was added. After a further 10 min of stirring, the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (1.02 g, 99%), and was used without further purification. Low resolution MS indicated that no anion exchange occurred in this cycle (i.e., counterion remained *p*-toluenesulphonate). $C_{118}H_{154}N_{20}O_{34}P_3Si_4Na^{2+}$ (low resolution ESIMS) calculated: 1311.5; found: 1311.7, *p*-toluenesulphonate counterion by ESIMS.

5′-HO-rG-(*N*-Ac, 2′-TBDMS)-3′-5′-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rU-(2′-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (9)[3]

Compound 8 (1.02 g, 0.37 mmol) was dissolved in 5 mL of 3% (*v*/*v*) trifluoroacetic acid in acetonitrile (~0.4 mol/L) and stirred for 10 min. Acetone (1 mL) was added to facilitate precipitation, and the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, dissolved off the filter with acetone, and dried under vacuum. The solid was redissolved in trifluoroacetic acid solution and stirred for 10 min. Acetone (1 mL) was again added, and the reaction mixture was precipitated as before. The precipitate was filtered off, rinsed with diethyl ether, and collected off the filter by dissolution in acetone. The solution was concentrated under reduced pressure to an approximate volume of 10 mL and was again precipitated from diethyl ether. The solid was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.82 g, 91%), dried under vacuum, and was used without further purification. ^{31}P NMR (ppm): 0.637 to –0.466. $C_{97}H_{137}N_{20}O_{32}Si_4P_3^{+2}$ (HR-ESIMS) required: 1149.39748; found: 1149.39934, *p*-toluenesulphonate counterion by ESIMS.

5′-DMT-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rG-(*N*-Ac, 2′-TBDMS)-3′-5′-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rU-(2-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (10)

Compound **9** (0.71 g, 0.29 mmol) was mixed with *N*-benzoyl-2-*tert*-butyldimethylsilyl-5′-dimethoxytrityl-adenosine (0.72 g, 0.77 mmol) and dicyanoimidazole (0.08 g, 0.65 mmol), and the mixture was dissolved in 7 mL of dry acetonitrile. No colour change was observed upon solvent addition this time. The reaction mixture was stirred for 2 h, and then 0.5 mL of *tert*-butanol was added. The mixture was stirred a further 15 min and was then precipitated from diethyl ether, as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone and then removing the solvent under vacuum. The resulting solid was dissolved in 5 mL of dry acetonitrile and 250 µL each of a 17.5% (*v*/*v*) solution of acetic anhydride in anhyd. acetonitrile and saturated dimethylaminopyridine in 25% (*v*/*v*) anhyd. pyridine – acetonitrile. This reaction mixture was stirred for 10 min, and then 0.5 mL of 6 mol/L *tert*-butylhydroperoxide in decane was added. After a further 10 min of stirring, 1 mL of acetone was added, and the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.92 g, 95%), and was used without further purification. $C_{144}H_{187}N_{26}O_{41}P_4Si_5Na^{2+}$ (low resolution ESIMS) calculated: 1611.6; found: 1611.8, *p*-toluenesulphonate counterion by ESIMS.

5′-HO-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rG-(*N*-Ac, 2′-TBDMS)-3′-5′-rA-(*N*-Bz, 2′-TBDMS)-3′-5′-rU-(2′-TBDMS)-3′-5′-rC-(*N*-Bz)-2′/3′-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (11)

Compound **10** (0.92 g, 0.27 mmol) was dissolved in 5 mL of 3% (*v*/*v*) trifluoroacetic acid in acetonitrile (~0.4 mol/L) and stirred for 10 min. Acetone (1 mL) was added to facilitate precipitation, and the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with di-

[3] Because of the residual *p*-toluenesulphonic acid observed carrying over in the previous two coupling cycles, it was decided that a volatile acid would be used for subsequent detritylations.

ethyl ether, dissolved off the filter with acetone, and dried under vacuum. The solid was redissolved in trifluoroacetic acid solution and stirred for 10 min. Acetone (1 mL) was again added, and the reaction mixture was precipitated as before. The precipitate was filtered off, rinsed with diethyl ether, and collected off the filter by dissolution in acetone. The solvent was removed under reduced pressure. TLC analysis indicated that detritylation was incomplete, so a third acid treatment was performed following exactly the same procedure as before. After precipitation and redissolution off the filter with acetone, the solution was concentrated under reduced pressure to an approximate volume of 10 mL and was again precipitated from diethyl ether. The solid was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.81 g, 96%), and dried under vacuum. No further purification was performed. ^{31}P NMR (ppm): 0.599 to –1.318. $C_{123}H_{171}O_{39}N_{26}P_4Si_5^{+3}$ (HR-ESIMS) required: 966.66590; found: 966.66546, *p*-toluenesulphonate counterion by low resolution ESIMS.[4]

Acknowledgements

We acknowledge the financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC) in the form of grants to M.J.D. and T.-H.C. This work was also supported by a scholarship award from the Chemical Biology Strategic Training Initiative of the Canadian Institutes of Health Research to R.A.D. We would like to acknowledge Alain Lesimple and Orval Mamer for the HR-ESIMS measurements.

References

1. K.K. Ogilvie, K.L. Sadana, E.A. Thompson, M.A. Quilliam, and J.B. Westmore. Tetrahedron Lett. 2861 (1974).
2. K.K. Ogilvie, N. Usman, K. Nicoghosian, and R.J. Cedergren. Proc. Natl. Acad. Sci. USA. **85**, 5764 (1988).
3. S.L. Beaucage and M.H. Caruthers. Tetrahedron Lett. **22**, 1859 (1981).
4. K.K. Jain. Mol. BioSyst. **2**, 523 (2006).
5. R.T. Pon, S. Yu, Z. Guo, R. Deshmukh, and Y.S. Sanghvi. J. Chem. Soc., Perkin Trans. 1, 2638 (2001).
6. G.M. Bonora, C.L. Scremin, F.P. Colonna, and A. Garbesi. Nucleic Acids Res. **18**, 3155 (1990).
7. R.A. Donga, M. Khaliq-Uz-Zaman Syed, T.-H. Chan, and M.J. Damha. J. Org. Chem. **71**, 7907 (2006).
8. M.J. Damha. Developments in the chemical synthesis of nucleic acids and their analogues. Ph.D. thesis, McGill University, Montreal. 1988.
9. A. Streiwieser, C.H. Heathcock, and E.M. Kosower. Introduction to organic chemistry. 4th ed. Macmillan Publishing Company, NY. 1992.
10. M. Roger and R.D. Hotchkiss. Proc. Natl. Acad. Sci. USA. **47**, 653 (1961).
11. R.T. Pon, M.J. Damha, and K.K. Ogilvie. Nucleic Acids Res. **13**, 6447 (1985).
12. L. Bellon and C. Workman. Deprotection of RNA. US Patent 6-054-576, 2000.

[4] Supplementary data for this article are available on the journal Web site (http://canjchem.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0S2, Canada. DUD 5148. For more information on obtaining material, refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.