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Acetal Levulinyl Ester (ALE) Groups for 2′-Hydroxyl Protection of Ribonucleosides in the Synthesis of Oligoribonucleotides on Glass and Microarrays

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Abstract: We describe a synthetic strategy that permits both the growth and deprotection of RNA chains that remain attached to a solid polymer support or chip surface. The key synthons for RNA synthesis are novel 5′-*O*-DMTr 2′-acetal levulinyl ester (2′-*O*-ALE) ribonucleoside 3′-phosphoramidite derivatives. In the presence of 4,5-dicyanoimidazole (DCI) as the activator, these monomers coupled to Q-CPG solid support with excellent coupling efficiency (∼98.7%). The method was extended to the light directed synthesis of poly rU and poly rA on a microarray through the use of a 5′-*O*-(2-(2-nitrophenyl)propoxycarbonyl)-2′-*O*-ALE-3′-phosphoramidite derivative. A two-stage deprotection strategy was employed to fully deblock the RNA directly on the Q-CPG or microarray support without releasing it from the support's surface: phosphate group deblocking with NEt$_3$ in acetonitrile (ACN) (2:3 v/v; 1 h, r.t.) followed by removal of the 2′-*O*-ALE groups under mild hydrazinolysis conditions (0.5−4 h, r.t.). This last treatment also removed the levulinyl (Lv) group on adenine (N^6) and cytosine (N^4) and the dimethylformamidine (dmf) group on guanine (N^2). The chemistry and methods described here pave the way to the fabrication of microarrays of immobilized RNA probes for analyzing molecular interactions of biological interest.

Introduction

The recent discovery of double-stranded RNAs (dsRNAs) as gene knockdown agents[1,2] and their potential therapeutics applications[3,4] has led to a resurgence in RNA synthesis[5,6] over the past several years. While classical strategies[7−9] (and others that have recently emerged[10−17]) will satisfy the need for RNA synthesis in the years to come, there is a demand for new methods tailored to the fabrication of RNA microarrays (RNA "chips"). RNA microarrays offer the potential to accelerate high-throughput screenings of RNA aptamers as well as to probe RNA−protein and RNA−RNA interactions of biological interest. Current strategies in the synthesis of RNA microarrays involve immobilization of a presynthesized RNA strand through enzymatic and chemical ligation steps,[18−23] which can limit the

complexity and versatility of the microarray. In addition, such methods leave RNA vulnerable to degradation as they are handled in the deprotected form.

Clearly, an ideal method should grow and deprotect the RNA directly on the chip's surface and be amenable to well-established DNA microarray technology platforms. In addition, selective deprotection of the 5′-position must be attainable in order to allow for chain growth and chip diversity. For example, protecting groups such as acid labile 5′-dimethoxytrityl (DMTr)

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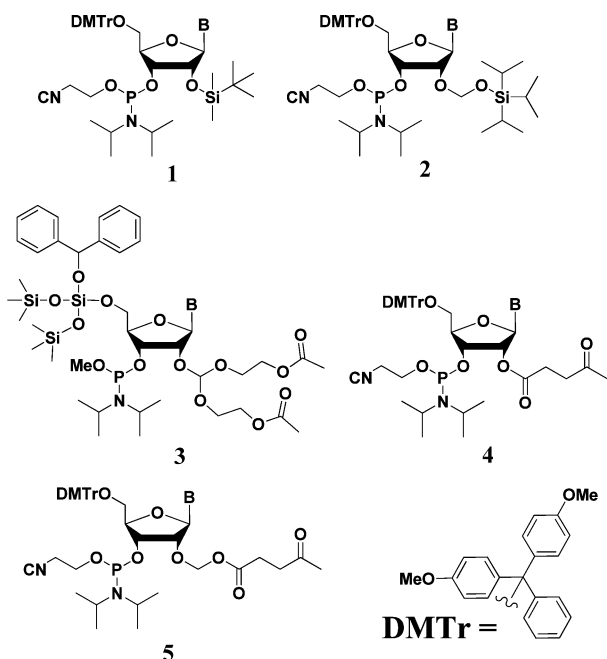
Figure 1. Ribonucleoside phosphoramidite synthons for solid phase synthesis of RNA: (**1**) 2′-*O*-TBDMS, *B* = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu}, Ura; (**2**) 2′-*O*-TOM, *B* = Ade^{Ac}, Cyt^{Ac}, Gua^{Ac}, Ura; (**3**) 2′-ACE, Ade^{Bz}, Cyt^{Ac}, Gua^{ibu}, Ura; (**4**) 2′-Lv, *B* = Ade^{Lv}, Cyt^{Lv}, Gua^{dmf}, Ura; (**5**) 2′-*O*-ALE, *B* = Ade^{Lv}, Cyt^{Lv}, Gua^{dmf}, Ura.

groups have been utilized in DNA microarray fabrication[24,25] as well as the photolabile 5′-*O*-(2-(2-nitrophenyl)propoxycarbonyl) group (NPPOC).[26] The latter is the protecting group of choice in maskless photolithographic DNA microarray technology yielding high complexity chips of up to 786 000 features.[27]

Furthermore, the 2′-hydroxyl group of RNA requires protection and carefully controlled deprotection conditions during oligonucleotide synthesis. Fluoride labile 2′-*O*-protecting groups are unsuitable as they are incompatible with glass substrates used in RNA chip fabrication. A photolabile protecting group at the 2′-position is also undesirable, as this would interfere with photodeprotection of the 5′-*O*-NPPOC group. 2′-Acetal- and 2′-orthoester-based protecting groups[9,28,29] (e.g., synthon **3**, Figure 1) could in principle work on a chip if both the 5′- and N-protecting groups were converted to chip compatible groups and if the conditions required to deblock these acid (or base) labile protecting groups were adjusted as to minimize the detachment of the RNA from its surface.[30]

To the best of our knowledge, there are no examples in the literature of the in situ synthesis of RNA microarrays. Toward

this goal, we recently reported on 2′-*O*-levulinyl ribonucleoside phosphoramidites (synthon **4**, Figure 1) for use in RNA synthesis on a hydroquinone-*O*,*O*′-diacetic acid ("Q-linker") CPG support.[31] The average coupling yield of 2′-*O*-Lv phosphoramidite monomers was 98.5%, which was comparable to the average coupling yield of TBDMS (synthon **1**, Figure 1) monomers[7,32,33] under similar conditions. A two-stage deprotection strategy was employed to fully deblock the RNA while bound to the Q-CPG support: the β-cyanoethyl phosphate protecting groups were removed using a solution of NEt$_3$ in acetonitrile (ACN) (2:3 v/v; 1 h, r.t.) followed by removal of the 2′-*O*-Lv groups by cleavage under hydrazinolysis conditions (30 min, r.t.). This last treatment also removed the Lv group on adenine (N^6) and cytosine (N^4) and the dimethylformamide (dmf) group on guanine (N^2). When desired, the fully deprotected RNA chain was released from the Q-CPG support with fluoride ions under conditions that do not lead to internucleotide cleavage (1 M TBAF in THF; r.t., 15 min; 92% recovery).[34] While the 2′-*O*-Lv 3′-phosphoramites are satisfactory in the solid-phase synthesis of oligoribonucleotides, great care has to be exercised in the purification of these building blocks in order to avoid contamination with the isomeric 3′-*O*-Lv 2′-phosphoramidites, the presence of which will inevitably lead to 2′,5′-internucleotide linkages in the final RNA sequence.

To overcome this limitation, we describe herein a novel 2′-acetal levulinyl ester (ALE) 2′-hydroxyl protection strategy for the synthesis of RNA (synthon **5**, Figure 1). The ALE group may be regarded as an alternative to the levulinyl (Lv) group in that it is also removed on-column by treatment with buffered hydrazine solution; however, it has two advantages over the Lv group in that (i) it cannot migrate by virtue of its acetal function[35] and (ii) the 5′-*O*-DMTr 2′-*O*-ALE monomers can be prepared in higher yields. We show here that 2′-*O*-ALE chemistry, in conjunction with *N*-Lv (Ade/Cyt) and *N*-dmf (Gua) protection, provides a unique method that is well-suited for the synthesis of RNA on microarrays.

Results and Discussion

Monomer Synthesis. The synthesis of 2′-*O*-ALE 3′-phosphoramidite monomers is summarized in Scheme 1. Uridine (**6a**), *N*^{4}-Lv cytidine (**6b**), *N*^{6}-FMOC adenosine (**6c**), and *N*^{2}-FMOC guanosine (**6d**) were treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine to give **7a−d** in near quantitative yield.[36] These materials were then reacted with DMSO, AcOH, and Ac$_2$O giving the 2′-*O*-thiomethyl ethers **8a−d** in 63−88% yield.[16,13] Compounds **8a−c** were treated with sulfuryl chloride for 1 h, and the resulting 2′-*O*-CH$_2$Cl intermediates were combined with sodium levulinate (NaOLv) and 15-crown-5 ether to afford **9a−c** in 78−94% yield. These conditions did not work well for **8d**. Instead, this compound was reacted with sulfuryl chloride in the presence of 4-chloro-styrene to avoid side reactions on the guanine moiety. Without product isolation, this mixture was added to cesium carbonate and levulinic acid to provide **9d** in 85% yield.

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Scheme 1. Synthesis of 2′-*O*-ALE Monomers[a]
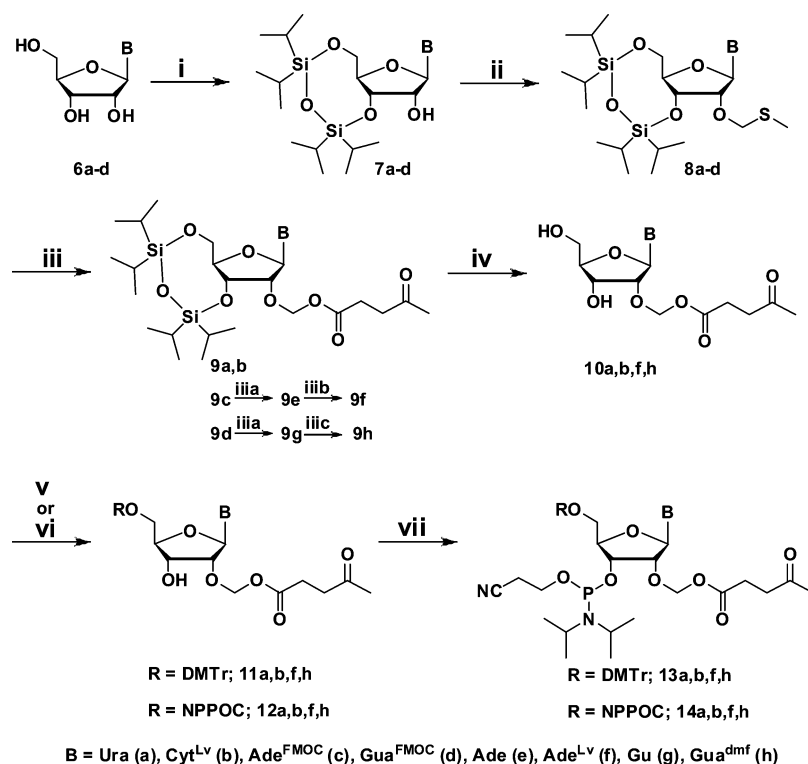
^{a} Reagents and conditions: (i) TIPDSCl, py; (ii) DMSO, AcOH, Ac$_2$O; (iii) 1 M SO$_2$Cl$_2$, CH$_2$Cl$_2$, NaOLv, 15-C-5, CH$_2$Cl$_2$ (2 steps); for **8d**, 1 M SO$_2$Cl$_2$, CH$_2$Cl$_2$, 4-Cl-styrene, Cs$_2$CO$_3$, levulinic acid; (iiia) 2:3 NEt$_3$/py; (iiib) levulinic acid, EEDQ, THF; (iiic) *N,N*-dimethylformamide dimethyl acetal, THF; (iv) NEt$_3$-3HF, THF; (v) DMTrCl, py; (vi) NPPOCCl/py; (vii) CEtOP(Cl)N*i*Pr$_2$, *i*Pr$_2$Net, CH$_2$Cl$_2$.

At this point, the *N*-FMOC protected purines **9c** and **9d** were converted into the desired *N*-Lv (**9f**) and *N*-dimethylformamidine (dmf) (**9 h**) derivatives. This "transient" FMOC protection was necessary as *N*-Lv and *N*-dmf groups on Ade and Gua, respectively, do not survive the conditions used to install the 2′-*O*-thiomethyl ether or 2′-*O*-ALE moieties (e.g., **7→8** and **8→9**). While FMOC protection remains an attractive option, it is incompatible with the 5′-*O*-NPPOC protection used in microarray fabrication (data not shown). Thus, compounds **9c** and **9d** were treated with 2:3 triethylamine/pyridine to remove the FMOC group in quantitative yield. Next, the resulting Ade **9e** was reacted with EEDQ and levulinic acid to give the *N*^{6}-Lv Ade[37] **9f** (86%), whereas Gua **9g** was treated with *N,N*-dimethylformamide dimethyl acetal to give *N*^{2}-dmf Gua[38] **9h** in quantitative yield. Compounds **9a,b,f,** and **h** were then treated with NEt$_3$-3HF to afford **10a,b,f,** and **h** in nearly quantitative yields. To obtain monomers suitable for standard synthesis on CPG solid supports, these nucleosides were treated with DMTrCl/pyr to afford **11a,b,f,** and **h** (78−90%), which were then 3′-phosphitylated under standard conditions to give **13a,b,f,** and **h** (70−90%). The corresponding 5′-*O*-NPPOC monomers were prepared from **10a,b,f,** and **h** by reaction with NPPOCCl/py and then CEtOP(Cl)N*i*Pr$_2$/DIPEA to afford **12a,b,f,** and **h** (30−65%) and **14a,b,f,** and **h** (85−88%), respectively.

Oligonucleotide Synthesis. As an initial test to demonstrate the suitability of 2′-*O*-ALE monomers for RNA synthesis, we synthesized four oligomers (i.e., dT$_9$-rN-dT$_5$ (rN = U, C, A, and G)) on a Q-CPG solid support (1 μmol scale). The Q-CPG

support consisted of 5′-*O*-DMTr-dT (loading: 45 μmol/g) appended through a hydroquinone-*O,O′*-diacetic acid ("Q-linker"),[31] making it possible to release an oligonucleotide chain by a brief fluoride treatment.[34] The phosphoramidites **13a,b,f,** and **h** (0.1 M in ACN) were activated with 4,5-dicyanoimidazole (DCI; 0.25 M in ACN) and allowed to couple to the support for 1 min. Standard capping, oxidation, and detritylation steps ensue the coupling step. After the completion of each synthesis, the Q-CPG was treated with 2:3 NEt$_3$/ACN for 1 h to effect removal of the β-cyanoethyl phosphate protecting groups. Half of the solid support was treated with 0.5 M NH$_2$NH$_2$·H$_2$O in 3:2 v/v pyr/AcOH for 1 h (*N*-Lv/dmf and 2′-*O*-ALE removal) followed by 1 M TBAF in THF for 16 h to release the oligonucleotide from the Q-CPG. The remaining solid support was treated directly with 1 M TBAF (16 h) to release the protected oligonucleotide from the support. This material was purified on a reverse phase-high performance liquid chromatography (RP-HPLC) column, and then treated under hydrazinolysis conditions for time-varying periods (1−24 h). Both methods of deprotection yielded the same results with no indication of base modification or internucleotide strand cleavage occurring even after extended periods of time (Figure 2, Table 1, Tables 1−4 of the Supporting Information).

As a further check for the integrity of the oligonucleotide chains, each strand obtained by on-column deprotection of 2′-*O*-ALE, *N*-Lv/dmf groups was hybridized to its complementary (dA$_5$-rN-dA$_9$) strand. The thermal stability of the resulting duplexes, as assessed by their T_m value, was the same as the hybrids synthesized from 2′-*O*-TBDMS monomers (Table 1). Furthermore, appropriate molecular weights were observed for the various oligomers that were synthesized.

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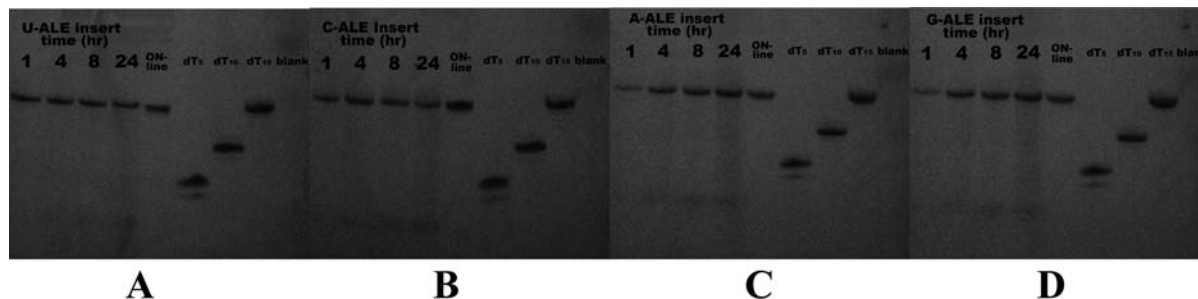
Figure 2. Assessment of base modification or internucleotide strand cleavage during deprotection: 24% denaturing gel (8.3 M urea) visualized by UV shadowing. dT$_9$-rN-dT$_5$ prepared using rN = (A) **8a**, (B) **8b**,(C) **8f**, and (D) **8g**. Half of the material was first treated with 1 M TBAF (16 h) and then purified by reverse phase HPLC. The material obtained is then treated for time-varying periods (1−24 h lanes) with 0.5 M hydrazine hydrate in 3:2 pyr/HOAc. Comparison is made to the same oligonucleotide sequences obtained through online deprotection ("on-line" lanes) that consists of sequential treatments of the solid support with 0.5 M NH$_2$NH$_2$·H$_2$O in 3:2 v/v pyr/AcOH (1 h) followed by 1 M TBAF in THF (16 h) to release the oligonucleotide from the Q-CPG support.

Table 1. Sequence and Properties of Oligonucleotides[a]

chemistry	sequence	T_m (°C)	found MW	calcd MW
TBDMS	ttt ttt ttt **A**tt ttt	29.9	4525.8	4525.9
ALE	ttt ttt ttt **A**tt ttt	30.0	4525.9	4525.9
TBDMS	ttt ttt ttt **G**tt ttt	35.2	4541.9	4542.0
ALE	ttt ttt ttt **G**tt ttt	35.3	4541.7	4542.0
TBDMS	ttt ttt ttt **C**tt ttt	34.7	4502.1	4501.9
ALE	ttt ttt ttt **C**tt ttt	35.8	4501.9	4501.9
TBDMS	ttt ttt ttt **U**tt ttt	33.0	4502.7	4502.9
ALE	ttt ttt ttt **U**tt ttt	32.6	4503.1	4502.9

[a] dT$_9$-**rN**-dT$_5$ strands were prepared from TBDMS and ALE monomers. Oligonucleotides were dissolved to give a concentration of 1 μM [dA$_5$-rN-dA$_9$]/[dT$_9$-rN-dT$_5$] hybrid in 140 mM KCl, 5 mM MgCl$_2$, and 3 mM Na$_2$HPO$_4$ buffer (pH 7.2).

Comparative Studies. Although not the thrust of this study, we compared the 2′-acetal levulinyl ester (ALE) 2′-hydroxyl protection strategy to the classical methods for RNA synthesis on CPG supports. Thus, we synthesized and compared luciferase gene knockdown of four 21-nt siRNA duplexes derived from ALE and the well-established RNA methods; namely, TBDMS,[7,32,33] TOM,[8] and ACE[9] chemistries (synthons **1**, **2**, and **3**, Figure 1, respectively). Antisense strands of these duplexes were prepared in house from 2′-O-TBDMS, 2′-O-TOM, or 2′-O-ALE monomers. The sense strand was assembled by 2′-TBDMS chemistry. Syntheses of mixed-nucleobase siRNAs via ALE monomers (0.1 M) was carried out as above, whereas those derived from TBDMS (0.15 M) and TOM (0.1 M) monomers followed literature procedures.[39] As our synthesizer was not compatible with the ACE phosphoramidite protocols, a crude sample of the same RNA sequence was obtained from Dharmacon. Detailed protocols for synthesizing RNA via ACE chemistry have been reported by Scaringe and coworkers,[9,39] and we assume that similar procedures were followed during the preparation and isolation of our commercial sample. Normally, once the 2′-O-ACE oligomer is synthesized, deprotection of the methyl phosphate group is effected by S$_2$Na$_2$ (10 min); followed by treatment with aqueous 40% methylamine at 60 °C for 15 min, that produces an intermediate 2′-O-orthoester, which is heated to 60 °C for 30 min in a pH 3.8 buffer. This final step cleaves any remaining 2′-O-formyl groups that result from the orthoester deprotection. Coupling efficiencies of 99% and higher have been reported with coupling times of <1 min. Typically this method produces a crude product of high purity.

Table 2. Comparative Study of 21-nt RNAs Synthesized from Various Chemistries[a]

2′-O-PG	found MW[b]	T_m (°C)	10 min coupling % purity[c]	avg coupling yield[d]	1 min coupling % purity[c]	avg coupling yield[e]
TBDMS	6616.4	59.8	70.6	98.4	45.4	96.3
TOM	6616.5	60.1	67.2	98.1	32.0	94.7
ACE	6616.5	59.5	81.8[f]	99.0	n.d.	n.d.
ALE	6616.2	59.4	76.2	98.7	61.8	97.7

[a] Base sequence: r(GCUUGAAGUCUUUAAUUAA)-d(TT). [b] Calcd molecular weight: 6617 g/mol. [c] % yield calculated by HPLC (% area of major peak). [d] Calculated from 10 min coupling time. [e] Calculated from 1 min coupling time. [f] Coupling time unknown.

To assess coupling and kinetic efficiencies, monomer coupling times were set at 1 and 10 min. Deprotection of the 2′-O-TOM and 2′-O-TBDMS RNA oligomers from the CPG support was achieved with 29% aq. NH$_3$/ethanol, 3:1, 55 °C, 30 min followed by 1 M TBAF in THF (16 h; r.t.). Deprotection of the ALE oligomer was carried out on-column as described above, except that the hydrazine treatment was extended to 4 h to achieve complete deprotection of the mixed sequence. As expected, extensive degradation of the RNA occurred if the hydrazine deprotection step was replaced by 29% aq. NH$_3$/ethanol, 3:1, r.t., 3 h (data not shown).

Coupling data and HPLC profiles obtained for the crude oligomers deprotected under optimum conditions are given in Table 2, Figure 3, and Figure SI2 of the **Supporting Information**.

The quality of the HPLC trace of the ACE oligomer is excellent (purity 81.8%; unknown coupling time), from which an average coupling efficiency of 99% was calculated. At 1 min coupling, average stepwise coupling yield for the 2′-O-ALE monomers, (**13a, b, f, h**) were higher (97.7%) than those obtained with 2′-O-TOM (96.3%) and 2′-O-TBDMS (94.7%) monomers (Table 2). At longer coupling times (10 min), the values obtained were 98.7, 98.1, and 98.4, respectively. Careful analysis of the deprotected oligomers showed in each case that there was no base modification (Table 2, Figures SI1−SI7 of the Supporting Information).

As a final check, we evaluated the activity of all RNAs synthesized in an RNAi assay that targets luciferase mRNA.[40] Each of the antisense strands prepared by the various chemistries were allowed to anneal to a common sense strand. As shown in Figure 4, the siRNA duplex prepared by 2′-O-ALE chemistry

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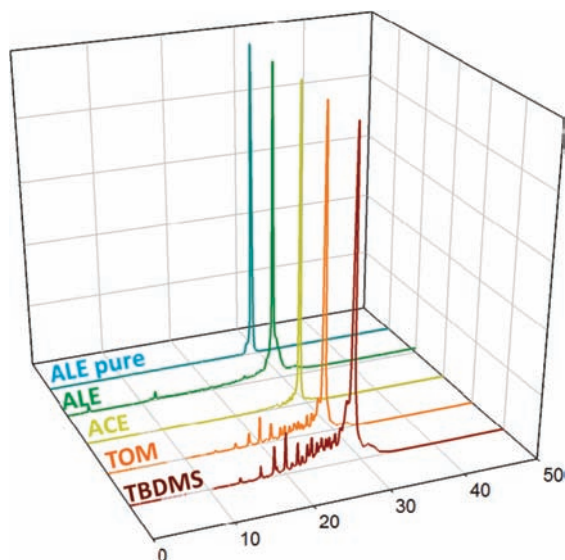
Figure 3. Anion exchange HPLC traces of crude antisense siRNA strands synthesized from 2′-*O*-TBDMS (red), 2′-*O*-TOM (orange), 2′-*O*-ACE (yellow), and 2′-*O*-ALE (green) chemistries. Purified oligomer from 2′-*O*-ALE chemistry is shown in blue.
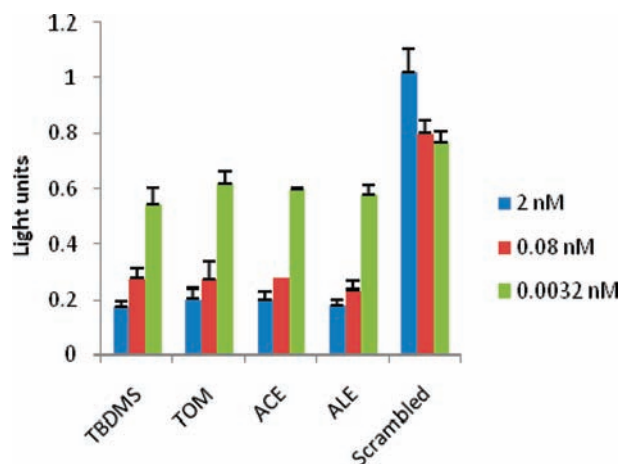
Figure 4. Luciferase gene knockdown by siRNA duplexes (light units are relative to Renilla control). The fully deprotected antisense strands were synthesized by TBDMS, TOM, ACE, and ALE chemistries whereas the complementary sense strand was synthesized by TBDMS chemistry.

had the same gene silencing activity as the siRNA duplexes derived from TBDMS, TOM, and ACE protocols, further confirming the integrity of the synthesized RNA strands.

Synthesis of RNA on Chip. Microarray technology is readily used in biological research as it provides unprecedented information on nucleic acids in a wide range of applications such as gene expression and genotyping.[41] Like DNA, RNA microarrays will likely emerge as combinatorial tools as a result of the increasing interest in the use of siRNA, RNA aptamers, and protein−RNA interactions. However, unlike DNA, the construction of RNA microarrays is limited to spotting[18−23] as they are far more challenging to synthesize in situ.

Following demonstration of efficient synthesis of RNA from ALE chemistry on CPG, the 5′-NPPOC-modified phosphoramidites (**14a, b, f, h**) were employed in the synthesis of RNA microarrays. Synthesis was carried out on a maskless array

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Table 3. Microarray Synthesis Coupling Parameters and Efficiencies

| monomer	concentration (mM)	coupling time (min)	coupling efficiency (%)
rA, **14f**	50	10	86[a]
rC, **14b**	50	10	95
rG, **14h**	60	15	96
rU, **14a**	50	10	97

[a] Unoptimized.

synthesizer (MAS) with glass substrates ("chips") mounted on a flow cell connected to a DNA synthesizer. The MAS system uses virtual masks generated by computer and imaged by a digital light processor (DLP) with dimensions of a 768×1024 array of 13 μm wide micromirrors.[27] The MAS instrument may generate chips of high complexity of up to 786 000 features. To determine the coupling efficiency of the RNA monomers, sequences of 1−12 nucleotides in length were synthesized onto chips and terminally labeled with a Cy3 phosphoramidite. dT_5 linker strands made with 5′-*O*-NPPOC-modified thymine phosphoramidites were used to distance the RNA strand from the chip surface. All the monomers (0.05−0.06 M in ACN) were activated with 4,5-dicyanoimidazole (DCI; 0.25 M in ACN) and allowed to couple to the support for 10−15 min. Standard capping (Ac_2O) was performed followed by oxidation (0.02 M I_2/water/pyridine). UV light energy dose of 6.5 J/cm^2 at 365 nm was required for a complete exposure of the photolabile 5′-*O*-NPPOC group (Figure SI8 of the Supporting Information). Fluorescence intensities from the coupling steps were fit with a single exponential decay to determine average coupling efficiency. Coupling parameters and efficiencies for the four monomers are given in Table 3 (Figure SI9 of the Supporting Information).

Following the determination of coupling efficiencies, two microarrays (rU_{12} and rA_{12}) were deprotected. Decyanoethylation was first conducted by immersing the synthesized microarrays in 2:3 NEt$_3$/ACN, 80 min with agitation at room temperature. The slides were rinsed five times in anhydrous ACN and dried under Ar. The 2′-*O*-ALE protecting groups are removed by treatment with 0.5 M NH$_2$NH$_2$·H$_2$O (3:2 v/v pyr/AcOH), shaken for 1 h at r.t. The slides were washed with 1:1 pyr/AcOH (pH > 5) to remove any salts formed on the glass substrate. DNA control experiments (dA_{10}/dT_{10} microarray hybridizations) were conducted prior to RNA microarray synthesis to ensure compatibility with deprotection conditions required for RNA (data not shown). Hybridization results indicated no loss in oligonucleotide from the glass substrate when DNA microarrays were exposed to the reagents that remove phosphate and 2′-hydroxyl protecting groups. Following deprotection, the oligonucleotides on the chip were hybridized with either Cy5-labeled dA_{20} or Cy5-labeled dT_{20} (Figure 5).

The microarray features shown in the fluorescence micrographs in Figure 5 are arranged in such a way that the length of the oligomers increases progressively ($n = 0-12$); that is, the sequences on the chip above or below a numbered label n are (surface)-dT_5-r_n, where n is between 0 and 12. Zero coupling means that the area was subject to a complete coupling cycle, but without monomer, and shows that capping on the chip is ∼90% efficient. The chip surface corresponding to each "n" labeled coupling step is subdivided into four sections: (1) single RNA coupling (dT_5-rN) is followed by (2) a very bright, single terminally labeled RNA coupling (dT_5-rN-Cy3), followed by (3) dT_5-rN$_n$, and (4) dT_5-rN$_n$-Cy3. The unlabeled regions are used for background subtraction of the fluorescence signal. The
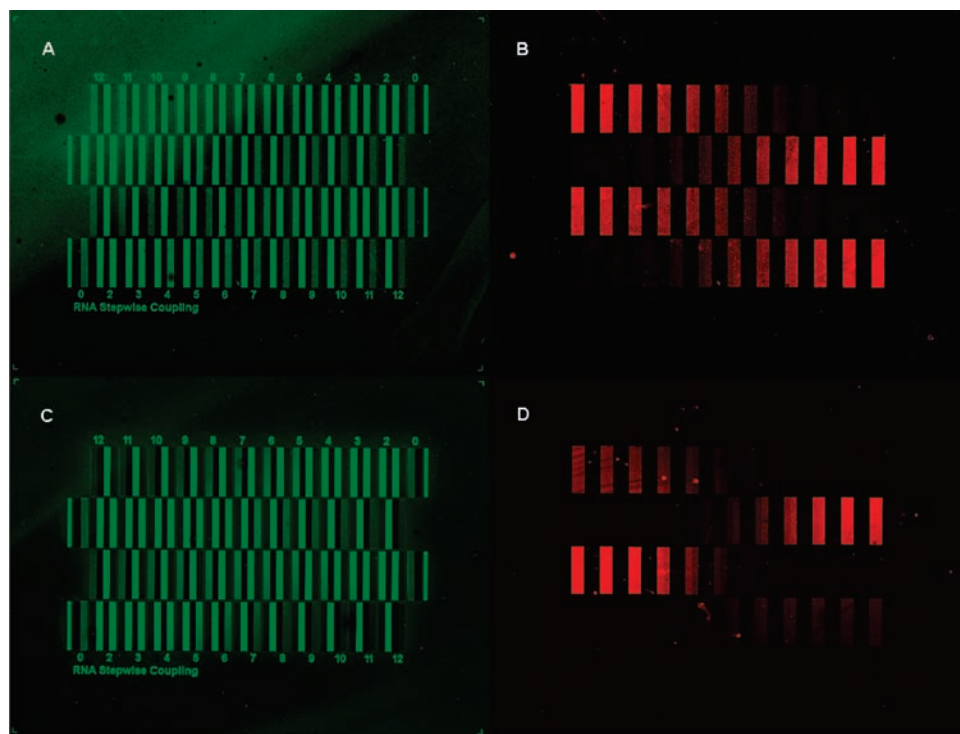
Figure 5. Coupling and hybridization of rU and rA microarrays. (A) Coupling efficiency microarray for rU with zero (blank) through 12 coupling steps and 5′-terminal Cy3 label. Each coupling step feature includes an adjacent area with the same number of couplings but no terminal label, as well as a one coupling reference. Intensity data was fit with a single exponential to obtain the average coupling efficiency for rU in Table 3. (B) The same microarray in A, hybridized with Cy5-labeled dA_{20}. (C) Equivalent microarray with rA couplings. (D) rA chip hybridized with Cy5-labeled dT_{20}.

numbers and label on the chip have the terminally labeled, single RNA coupling pattern (dT_5-rN-Cy3).

Both the terminally labeled and unlabeled *n*-mers are visible upon hybridization with the probes (Cy5-labeled dA_{20} or Cy5-labeled dT_{20}); the longer (and more stable) duplexes provide as expected the brightest signal, which gradually decreases as the length of probes decreases.

In summary, the 2′-*O*-ALE and *N*-Lv/dmf protecting group combination provides unique ribonucleoside 3′-phosphoramidite synthons for RNA synthesis that couple with excellent rates and efficiencies. In addition, this protecting group strategy provides two distinct advantages: (1) it prevents the common 2′ to 3′ isomerization that can occur with acyl protecting groups; (2) the removal of the protecting groups can be efficiently performed on the solid support, which simplifies postsynthesis deprotection of RNA chains and minimizes the potential for degradation of the oligomers by RNases. The chemistry and methods described also pave the way to the fabrication of microarrays of immobilized RNA probes for analyzing molecular interactions of biological interest.

Experimental Section

Solid-Phase Oligonucleotide Synthesis Using 2′-*O*-ALE Chemistry. The solid-phase synthesis of r(GCUUGAAGUCU-UUAAUUAA)-d(TT) was performed on an ABI-3400 DNA/RNA synthesizer. A 1 μmol scale was conducted in the trityl-off mode using 500 Å 5′-DMTr-dT-Q-linker long chain alkylamine controlled-pore glass (LCAA-CPG). The support was first subjected to a standard capping cycle, CAP A solution (Ac$_2$O/pyr/THF) and Cap B solution (10% 1-methylimidazole in THF) for 3 × 180 s to acetylate and dry the solid support. RNA synthesis was carried out using 0.1 M solutions of phosphoramidites **13a**, **b**, **f**, **g** in dry ACN with 0.25 M DCI as the activator. All other ancillary agents necessary for oligonucleotide synthesis were obtained commercially.

The detritylation step used 3% trichloroacetic acid (TCA) for 80 s. Each phosphoramidite coupling step was set for 1 or 10 min. The capping step (using CAP A and CAP B) was set for 20 s, and the oxidization step using 0.1 M iodine/pyridine/water/THF was 30 s. 2′-*O*-TBDMS phosphoramidite monomers were used at 0.15 M concentration in ACN.[42] The RNA synthesized using 2′-*O*-TOM phosphoramidite monomers were obtained commercially and treated as above except a 0.10 M phosphoramidite concentration in acetonitrile was used, as recommended by Glen Research. Crude RNA synthesized from 2′-*O*-ACE chemistry was purchased from Dharmacon. The synthetic conditions are unknown but are assumed to be similar to reported procedures.[39]

On-Column Deprotection of *N*-Lv/dmf-2′-*O*-ALE RNA. After completion of the synthetic cycle, the fully protected oligomer was treated with anhydrous 2:3 v/v NEt$_3$/ACN (1 h; r.t.) through the column to deblock the cyanoethyl phosphate groups. The column was then washed thoroughly with ACN and dried under high vacuum. Next, the *N*-Lv/dmf and 2′-*O*-ALE groups were removed simultaneously by passing a solution of 0.5 M NH$_2$NH$_2$·H$_2$O in 3:2 v/v pyr/HOAc, 4 h, r.t. through the column. This was followed by washing the solid support with CH$_2$Cl$_2$ and ACN and evacuating trace solvents on high vacuum. At this stage, the naked RNA strand bound to the Q-CPG was transferred to a 1 mL Eppendorf tube. The RNA was released from the Q-CPG support using fluoride treatment (1 mL of 1 M TBAF, 16 h, r.t.). The material was then centrifuged (14 000 rpm), and the supernatant was removed. The CPG was subsequently washed 4 × 250 μL with 1:1 water/ethanol. This material was evaporated to dryness and redissolved in water. It was then passed through a sephadex G-25 column to remove salts and purified further by denaturing polyacrylamide gel electrophoresis (24% acrylamide, 8.3 M urea).

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(42) Damha, M. J.; Ogilvie, K. K. *Protocols for Oligonucleotide Analogs*; Humana Press: Totowa, NJ, 1993.

MAS (Maskless Array Synthesizer) Light Directed Synthesis. Experiments were conducted according to methods described by Singh-Gasson and coworkers.[27] A MAS instrument and a Perspective Biosystems expedite 8909 DNA pump system were used in the light direct synthesis approach.[27] The MAS instrument was equipped with a Texas Instruments digital light processor (DLP) with dimensions of a 768 × 1024 array of 13 μm wide micromirrors. An exposure wavelength of 365 nm by a 1000 W Hg lamp was used for 5′-NPPOC deprotection during DNA and RNA synthesis (Roche NimbleGen exposure solvent). DNA microarrays were prepared according to standard protocol[24] using NPPOC-phosphoramidites (30 mM, 60 s coupling times) with photodeprotection at 6 J for 111 s at 54 mW/cm^2. Deprotection of DNA microarrays was effected using a solution of ethylenediamine in EtOH (1:1 v/v; r.t. 2 h) followed by EtOH washings. Slides were dried under argon prior to hybridization experiments. Both DNA and RNA microarrays were prepared with a 3′-dT$_5$ linker on the glass substrate.

RNA Microarrays. As demonstrated by fluorescence measurement of hybridized samples, sufficient 5′-NPPOC deprotection occurs at 6.5 J/cm^2 exposure. Standard acetic anhydride capping (CAP A and CAP B) was performed followed by oxidation in aqueous iodine solution (0.02 M). Prior to hybridization, the protecting groups were removed as follows. Decyanoethylation was effected by immersing the synthesized RNA microarray in a 2:3 (v/v) solution of anhydrous NEt$_3$/ACN for 80 min with agitation at room temperature. The slide was rinsed five times in anhydrous acetonitrile and dried with argon. The 2′-*O*-ALE protecting groups were removed under buffer conditions, 0.5 M NH$_2$NH$_2$·H$_2$O in (3:2 v/v pyr/AcOH) and shaken for 60 min at room temperature. The slide was washed in a 1:1 pyr/AcOH (pH > 5) to remove any salts formed on the glass substrate. The slide was then flushed repeatedly with ACN and dried under argon prior to hybridization.

Hybridization. Water was autoclaved with diethylpyrocarbonate to inactivate RNase and thus prevent enzymatic degradation of the RNA microarrays. Hybridization experiments were carried out in a buffer consisting of 40 mM TRIS−HCl, 10 mM MgCl$_2$, pH 7.2. A 300 μL solution of 500 nM DNA probes (dA$_{10}$-5′-Cy5 and dA$_{20}$-5′-Cy5) were hybridized to the respective rU complements. Hybridizations were conducted for 1 h at 4 °C for rU$_{10}$/dA$_{10}$ and ambient temperature for rU$_{20}$/dA$_{20}$. The slides were washed with 300 μL of buffer (0.5 M NaCl, 0.03 M phosphate, 0.3 mM EDTA, 0.01% Tween-20) prior to fluorescence scanning. The hybed chips were scanned and analyzed on an Applied Precision ArrayWorx biochip reader.

Supporting Information Available: Experimental details and NMR spectra of nucleoside intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

JA9002074