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A convenient synthesis of the cytidyl 3′-terminal monomer for solid-phase synthesis of RNG oligonucleotides

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ABSTRACT

Replacing the phosphodiester backbone of RNA with positively charged guanidinium linkages has been shown to enable RNA oligomers to overcome electrostatic repulsion and bind double-stranded DNA in a triplex with high affinity. Ribonucleotide monomers with the ability to form guanidinium linkages have been synthesized for the generation of ribooligonucleotides with guanidinium linkages (RNGs) through solid-phase synthesis. We report herein an efficient method for the synthesis of N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*N*-(4-monomethoxytritylamino)-3′-*O*-succinyl-5′-deoxycytidine, a new monomer required for the solid-phase synthesis of cytidyl RNG oligonucleotides.

Synthetic oligonucleotides have been proposed as agents for sequence specific gene regulation by either antisense or RNAi technologies.[1] The efficacy of these oligonucleotides in vitro has been evaluated based upon their ability to exhibit a high affinity for DNA/RNA, maintain specific base-pairing, intracellular delivery capability, and resist degradation by nucleases.[2] In addition, triplex forming oligonucleotides bound to duplex DNA have been shown to display normal Watson–Crick base-pairing.[3] Chemical modifications of the pentose sugar as well as those of the phosphate linkages between nucleosides have tended to impact the affinity of oligonucleotides to their target nucleic acids, but have not yet been shown to affect the specificity of the hydrogen bonding that occurs between bases.[4] In previous experiments, oligonucleotides with negatively charged phosphate backbones have bound to duplex DNA poorly due to electrostatic repulsion, and were only effective in chains rich in adenine and guanosine, limiting the genes that could be targeted.[5]

The chemical modification of nucleotides to incorporate positively charged guanidinium linkages (Fig. 1A) has been observed to increase the binding affinity between duplex DNA and deoxynucleic guanidine (DNG) oligomers containing the bases adenosine, cytidine, and thymidine.[6] In addition, oligomers of uridyl and adenyl ribonucleic guanidine (RNG U and A, respectively) have been reported.[7] The discovery of antigene RNA demonstrated that ribonucleic acids are able to bind and silence genes at the transcriptional level without requiring methylation of the DNA.[8] Ribooligonucleotides have been proposed as therapeutic agents for transcriptional control, yet deoxyoligonucleotides have been focused on, partly due to their stability. Ribooligonucleotides are more sensitive to degradation by nucleases[9] and the propensity of the 2′-hydroxyl to perform a nucleophilic attack on the phosphate backbone of RNA limits their use without chemical modifications. However, RNG oligonucleotides possess stabilizing characteristics not found in RNA. Guanidinium linkages are resistant to nucleases.[10] In addition, guanidine linkages between

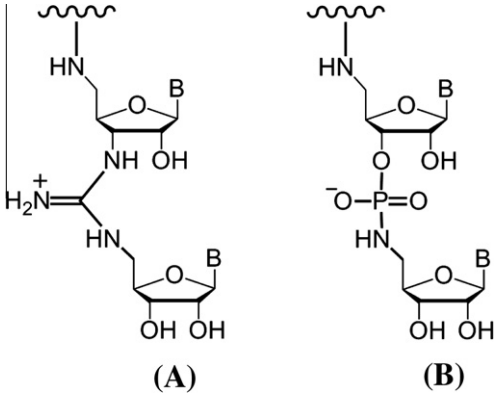

Figure 1. (A) Guanidinium linkage in RNG (B) 5′-amino-RNA.

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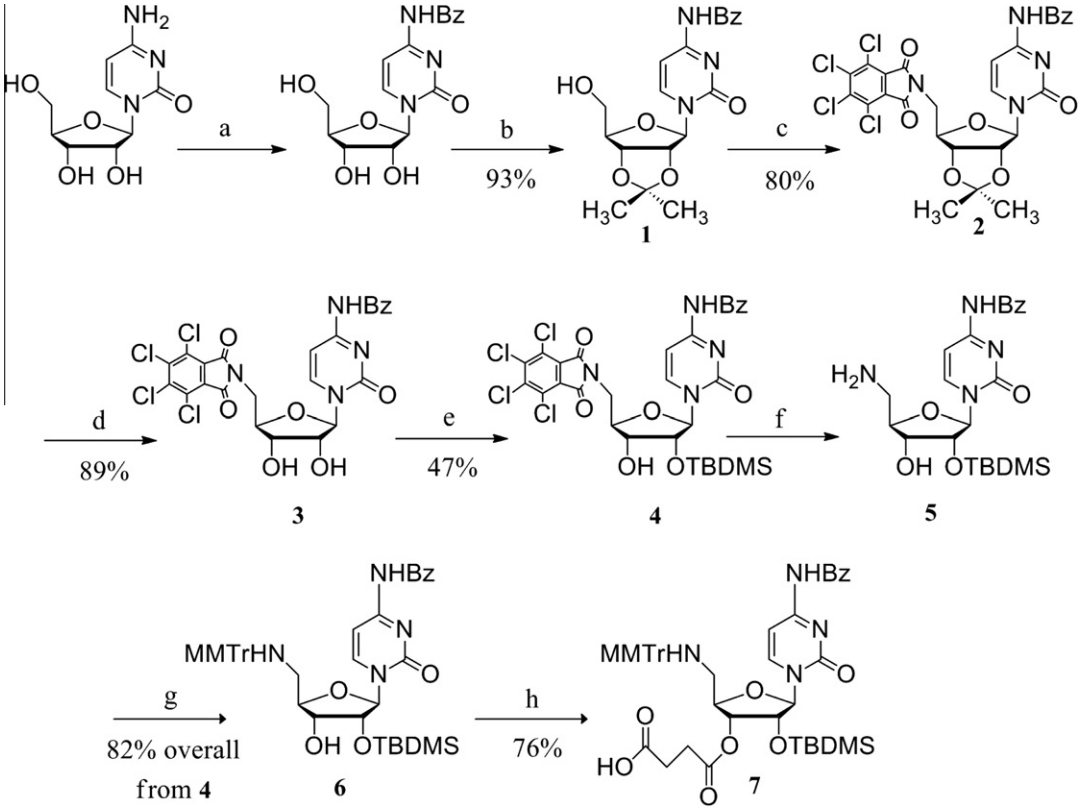

Scheme 1. Reagents and conditions: (a) (i) TMSCl, pyridine; (ii) BzCl; (iii) aq NH_4OH; (b) 2,2-dimethoxypropane, $TsOH{\cdot}H_2O$, acetone; (c) Mitsunobu reaction, tetrachloroph thalimide, PPh_3, THF, 15 h reflux; (d) 75% aq TFA; (e) TBDMSCl, $AgNO_3$, pyridine, DMF; (f) ethylenediamine, THF; (g) MMTrCl, Et_3N, CH_2Cl_2; (h) succinic anhydride, Et_3N, CH_2Cl_2.

ribonucleic monomers should stabilize oligonucleotide backbones against nucleophilic attack from the 2′-hydroxyl.[7]

Triplex-forming antigene agents with increased affinity for duplex DNA, enable the regulation of gene expression at the transcriptional level and require a miniscule concentration of oligonucleotides in comparison to that which would be required to inhibit the expression of multiple mRNA molecules. While previous experiments have demonstrated the selectivity of DNG, the interaction between template DNA and RNG oligonucleotides has not yet been observed, as RNG monomers for solid-phase synthesis exists only for adenyl RNG and uridyl RNG.[7] Our experiment provides a convenient, high-yield, synthesis of the 3′-terminal monomer N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*N*-(4-monomethoxytr itylamino)-3′-*O*-succinyl-5′-deoxycytidine **7** (Scheme 1), which may be incorporated into the solid-phase synthesis of cytidyl RNG oligonucleotides. In addition, this monomer can be also used for the solid-phase synthesis of P3′→ N5′ phosphoramidate ribooligonucleotides (5′-amino-RNA, Fig. 1B) with 5′-*O*-nucleotides replaced by 5′-*N*-nucleotides.[11]

For large scale preparations, it was more convenient to perform some reactions directly on the crude from the previous reaction, which did not affect the overall yield. High-yields and pure compounds were obtained and confirmed by gravimetric analysis, TLC, HRMS, and ^1H NMR spectroscopy. Protection of the 2′- and 3′-hydroxyl groups of the pentose ring was performed before running the Mitsunobu reaction on the 5′-hydroxyl group. To a stirred suspension of N^4-benzoylcytedine (30.0 mmol) in acetone (250 mL), *p*-toluenesulfonic acid monohydrate (3.0 mmol) and an excess of 2,2-dimethoxypropane (60 mL) were added. After 24 h reflux, the mixture was filtered to remove the unreacted starting material. The filtrate was concentrated under vacuum and the residue was dissolved with EtOAc, washed with 5% $NaHCO_3$ and brine. The solvent was removed under reduced pressure, and ether was added to induce further precipitation of compound **1** in a 93% yield. Compound **2** was produced by Mitsunobu reaction according to the previously published method.[12,13] To a suspension of **1** (22.2 mmol), PPh_3, (27.75 mmol) and tetrachlorophthalimide (26.64 m mol) in anhydrous THF (250 mL), diisopropyl azodicarboxylate (26.64 mmol) was added dropwise over 15 min and the mixture was refluxed for 15 h. The solvent was removed under reduced pressure and the crude mixture was purified by silica gel column chromatography (50% EtOAc in hexanes) to afford **2** in an 80% yield. The 2′-, 3′-hydroxyl groups were then deprotected by stirring compound **2** (17.0 mmol) in aqueous trifluoroacetic acid (75%, 100 mL) at RT for 2 h. Compound **3** was obtained in an 89% yield. The low solubility of **3** in organic solvents allowed the precipitation and isolation of the pure compound from the reaction mixture. More convenient was direct conversion of the crude of **2** to a pure precipitate of compound **3**. Protection of the 2′-hydroxyl group with TBDMS was performed to allow for further reactions on the 3′-hydroxyl group. To a solution of **3** (9.3 mmol) in dry DMF (80 mL), pyridine (37.1 mmol) and $AgNO_3$ (11.77 mmol) were added. After stirring for 10 min, *tert*-butyldimethylsilyl chloride (11.61 mmol) was added and the reaction mixture was stirred at RT for 24 h. The mixture was filtered through Celite, washed with ethanol, and the filtrate was concentrated in a vacuum. The crude product was purified by silica gel column chromatography (40% EtOAc in hexanes) to give compound **4**.[14] This reaction failed when THF was used as a solvent, likely due to the low solubility of **3** in THF. The 5′-amino nucleoside **5** was generated upon treatment of **4** (2.0 mmol) with ethylenediamine (2.4 mmol) in anhydrous THF (25 mL). The crude of **5** was directly converted to pure compound **6** in an overall yield of 82%. Compound **6** was purified by silica gel column chromatography pre-washed with 2% Et_3N (33% ethyl

acetate in hexanes). Esterification of the 3′-OH with succinic anhydride afforded the loading monomer **7**. Succinic anhydride (3.0 mmol) was added to a mixture of **6** (0.6 mmol) and Et_3N (6.0 mmol) in anhydrous CH_2Cl_2 (15 mL) and the mixture was stirred for 20 h. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2, washed with 5% citric acid, then H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated in a vacuum. The crude mixture was purified by silica column chromatography pre-washed with 2% Et_3N (45% EtOAc in hexanes) to afford compound **7** in a 76% yield.[15]

A common initial step in the solid-phase synthesis of oligonucleotides is to covalently attach the 3′ moiety of a modified pentose sugar on the first nucleoside to controlled pore glass (CPG), which contains long chain alkylamines.[16] The modifications we have incorporated enable our loading monomer to be joined to a solid support[17] and form guanidinium linkages with additional synthetic nucleotides.[18]

In conclusion, we have confirmed the synthesis of N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*N*-(4-monomethoxytritylamino)-3′-*O*-succinyl-5′-deoxycytidine by means of high resolution mass-spectroscopy and proton nuclear magnetic resonance.[19] Our method provides an efficient means of producing an RNG (and also a P3′→ N5′ phosphoramidate) monomer with high-yields and high purity.

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19. Spectral data for selected compounds: N^4-Benzoyl-2′,3′-*O*-isopropylidenecytidine (**1**) HRMS (ESI) *m*/*z* Calcd for $C_{19}H_{21}N_3O_6$ $(M+Na)^+$ 410.1322. Found 410.1311. ^1H NMR (400 MHz, DMSO-d_6): 11.29 s, 1H (NH); 8.30 d, 1H, *J* = 7.4 (H-6); 8.02 d, 2H, *J* = 6.8 (BzH); 7.62–7.50 m, 3H (BzH); 7.35 d, 1H, *J* = 7.2 (H-5); 5.85 d, 1H, *J* = 1.6 (H-1′); 5.12 t, 1H, *J* = 5.0 (OH-5′); 4.90 dd, 1H, *J* = 4.6, 1.6 (H-3′); 4.76 q, 1H, *J* = 3.0 (H-2′); 4.22 d, 1H, *J* = 3.2 (H-4′); 3.65–3.56 m, 2H (H-5′); 1.48 s, 3H (CH_3); 1.28 s, 3H (CH_3). N^4-Benzoyl-5′-tetrachlorophthalimido-2′,3′-*O*-isopropylidene-5′-deoxycytidine (**2**) HRMS (ESI) *m*/*z* Calcd for $C_{27}H_{20}N_4O_7Cl_4$ $(M+H)^+$ 653.0164. Found 653.0189 (base peak of 4 Cl isotopes). ^1H NMR (400 MHz, $CDCl_3$): 11.62 s, 1H (NH); 7.94 d, 1H, *J* = 7.0 (H-6); 7.67 d, 2H, *J* = 7.0 (BzH); 7.62–7.48 m, 4H (BzH and H-5); 5.54 d, 1H, *J* = 1.6 (H-1′); 5.27 dd, 1H, *J* = 7.2, 1.6 (H-3′); 5.05 q, 1H, *J* = 3.0 (H-2′); 4.48 m, 1H (H-4′); 4.30–4.00 m, 2H (H-5′); 1.54 s, 3H (CH_3); 1.35 s, 3H (CH_3). N^4-Benzoyl-5′-tetrachlorophthalimido-5′-deoxycytidine (**3**) HRMS (ESI) *m*/*z* Calcd for $C_{24}H_{16}N_4O_7Cl_4$ $(M+Na)^+$ 634.9665. Found 634.9667 (base peak of 4 Cl isotopes). ^1H NMR (400 MHz, DMSO-d_6): 11.29 s, 1H (NH); 8.25 d, 1H, *J* = 7.8 (H-6); 8.02 d, 2H, *J* = 7.2 (BzH); 7.66–7.47 m, 3H (BzH); 7.41 d, 1H, *J* = 7.6 (H-5); 5.74 d, 1H, *J* = 3.0 (H-1′); 5.59 br s, 1H (OH-3′); 5.29 br s, 1H (OH-2′); 4.20–4.16 m, 2H (H-5′); 4.13–3.97 m, 3H (H-2′,H-3′,H-4′). N^4-Benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-tetrachlorophthalimido-5′-deoxycytidine (**4**) HRMS (ESI) *m*/*z* Calcd for $C_{30}H_{30}N_4O_7SiCl_4$ $(M+Na)^+$ 749.0530. Found 749.0522 (base peak of 4 Cl isotopes). ^1H NMR (400 MHz, $CDCl_3$): 8.18 d, 1H, *J* = 7.6 (H-6); 8.91 d, 2H, *J* = 7.0 (BzH); 7.65–7.47 m, 4H (BzH and H-5); 5.64 d, 1H, *J* = 1.2 (H-1′); 4.42 dd, 1H, *J* = 1.2, 3.8 (H-2′); 4.32–4.16 m, 1H (H-3′); 4.12–4.07 m, 1H (H-4′); 4.03–3.88 m, 2H (H-5′); 0.92 s, 9H ($SiC(CH_3)_3$); 0.25 s, 3H ($SiCH_3$); 0.17 s, 3H ($SiCH_3$). N^4-Benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-amino-5′-deoxycytidine (**5**) LRMS (ESI) *m*/*z* Calcd for $C_{22}H_{32}N_4O_5Si$ $(M+Na)^+$ 461. Found 461. N^4-Benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*N*-(4-monomethoxytritylamino)-5′-deoxycytidine (**6**) HRMS (ESI) *m*/*z* Calcd for $C_{42}H_{48}N_4O_6Si$ $(M+Na)^+$ 755.3235. Found 755.3242. ^1H NMR (400 MHz, $CDCl_3$): 7.91 d, 1H, *J* = 7.6 (H-6); 7.75 d, 2H, *J* = 7.8 (BzH); 7.61–7.22 m, 16H (ArH, BzH and H-5); 6.87 d, 2H, *J* = 8.8 (ArH); 5.78 d, 1H, *J* = 1.2 (H-1′); 4.25 dd, 1H, *J* = 1.2, 3.6 (H-2′); 4.17–4.13 m, 1H (H-3′); 4.10–4.06 m, 1H (H-4′); 3.80 s, 3H ($MMTr\text{-}OCH_3$); 3.73–3.53 m, 2H (H-5′); 0.94 s, 9H ($SiC(CH_3)_3$); 0.30 s, 3H ($SiCH_3$); 0.17 s, 3H ($SiCH_3$). N^4-Benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*N*-(4-mono-methoxytritylamino)-3′-*O*-succinyl-5′-deoxycytidine (**7**) HRMS (ESI) *m*/*z* Calcd for $C_{46}H_{52}N_4O_9Si$ $(M+Na)^+$ 833.3576. Found 833.3585. ^1H NMR (400 MHz, $CDCl_3$): 8.69 d, 1H, *J* = 7.6 (H-6); 7.97 d, 2H, *J* = 8.4 (BzH); 7.58–7.20 m, 16H (ArH, BzH and H-5); 6.86 d, 2H, *J* = 8.8 (ArH); 5.78 d, 1H, *J* = 1.2 (H-1′); 4.88 dd, 1H, *J* = 4.8, 4.0 (H-3′); 4.43 dd, 1H, *J* = 4.4, 1.2 (H-2′); 4.40–4.36 m, 1H (H-4′); 3.78 s, 3H ($MMTr\text{-}OCH_3$); 3.64–3.25 m, 2H (H-5′); 2.57 s, 4H ($CO(CH_2)_2$); 0.90 s, 9H ($SiC(CH_3)_3$); 0.21 s, 3H ($SiCH_3$); 0.07 s, 3H ($SiCH_3$).