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Replacement of the Phosphorodiester Linkages of RNA with Guanidinium Linkages: The Solid-Phase Synthesis of Ribonucleic Guanidine

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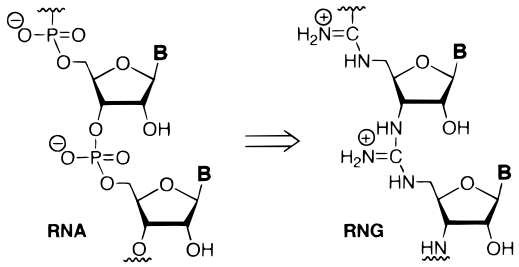
ABSTRACT



Replacement of the negatively charged phosphodiester linkages of RNA with positively charged guanidinium linkages provides the polycationic ribonucleic guanidine (RNG). RNG is anticipated to bind strongly to target DNA/RNA through the specific interactions of nucleobases and the attractive electrostatic interactions of backbones. Preparation of building blocks and the solid-phase synthesis of RNG are reported. Both trimeric and pentameric uridyl RNG have been synthesized.

Putative drugs consisting of oligonucleotide analogues capable of inhibiting cellular processes at the transcriptional or translational level via base pair interactions with DNA or RNA are known as antisense and antigene agents, respectively.[1] Numerous structural analogues of DNA/RNA designed to be effective antisense/antigene agents have been reported.[2] Replacement of the negatively charged phosphodiester linkages with neutral linkages eliminates the electrostatic repulsion which exists in natural duplex DNA; thus increased binding affinity can be expected. Oligonucleotides linked by methylphosphonate,[3] methylenemethylimino (MMI),[4] and amides[5] are representative of this strategy. An alternative approach involves replacing the sugar-phosphate backbone entirely. An example is peptide nucleic acid (PNA).[6] In forming complexes with DNA and RNA, PNA maintains sequence specificity and the formed complexes are more stable than native DNA/RNA duplexes and triplexes. Furthermore, these oligos with modified backbones are expected to have nuclease resistance. This is a key factor in antisense/antigene design strategy. On the other hand, incorporation of positive charges into oligonucleotides has been correctly anticipated to increase binding of analogues to negative DNA/RNA strands. Aminoalkyl linkers attached through the

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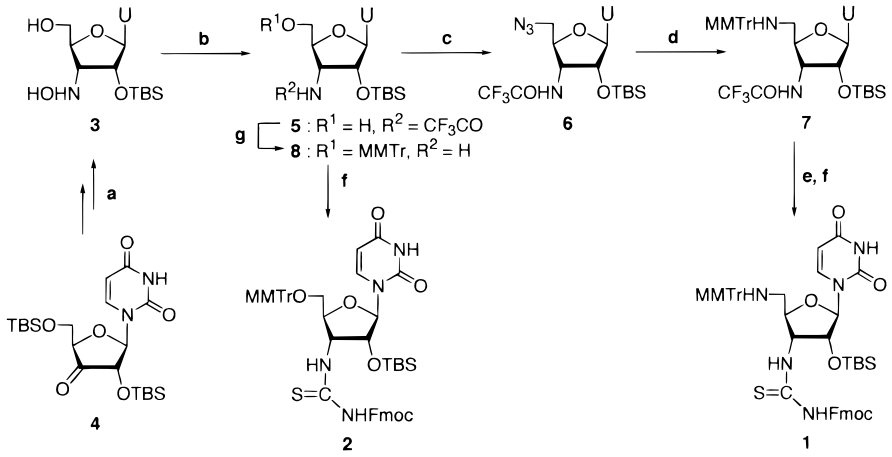
Scheme 1[a]



[a] Reagents and conditions: (a) ref 14; (b) (1) H_2, 10% Pd/C, 90% aqueous AcOH, rt, (2) CF_3CO_2Et, Et_3N, MeOH, rt, 94%; (c) (1) TsCl, pyridine, rt, (2) NaN_3, NH_4Cl, DMF, 70 °C, 76%; (d) (1) H_2S (gas), 60% aq.pyridine, rt, (2) MMTrCl, pyridine, rt, 85%; (e) NH_3/MeOH, rt, 76%; (f) Fmoc-NCS, CH_2Cl_2, rt, 93% for **1**, 96% for **2**; (g) (1) MMTrCl, pyridine, rt, (2) NH_3/MeOH, rt, 87%.

bases,[7] the sugar moieties,[8] and the phosphate backbones[9] have been synthesized and used for biological and biochemical studies.

We have recently reported the synthesis and DNA/RNA binding properties of deoxyribonucleic guanidine (DNG), wherein the negatively charged phosphodiester linkages of DNA have been entirely replaced by positively charged guanidinium linkages.[10] DNG is anticipated to, and does, bind strongly to target DNA/RNA because the repulsive electrostatic effects in duplex DNA are replaced by close attractive electrostatic interactions. Pentameric thymidyl oligomer of DNG have been shown to bind to poly(dA) and poly(rA) with unprecedented high affinity and with base-pair specificity to provide both double- and triple-stranded helices.[10c] Computational molecular modelings suggested that the DNG·DNA duplex primarily retained a B-DNA conformation while the DNG·RNA duplex adopted an A-type structure.[10b]

The interesting differences and similarities between the nature of DNA and RNA, in addition to their possible differential antisense/antigene properties, prompted us to explore the properties of DNG versus its RNA analogue, ribonucleic guanidine (RNG). In this paper, we report the preparation of building blocks and the solid-phase method for the synthesis of RNG.[11]

To synthesize RNG on solid support, certain nucleoside monomers with *N*-protected thiourea substitution are needed. Previously, we found that a thiourea with an electron-withdrawing protecting group on one nitrogen is a sufficient precursor for the mercury(II)-mediated formation of activated carbodiimide. The carbodiimide, which is electronically activated by an electron-withdrawing group, then reacts with the terminal amino nucleoside to create an internucleoside guanidinium linkage. At the same time, the protecting group now acts to protect the resulting guanidinium linkage.[10d,12] This protecting group must remain throughout the RNG synthesis; it can be removed by mild conditions at the very last stage. The 9-fluorenylmethyloxycarbonyl (Fmoc) group was chosen as suitable for *N*-protection because it can be cleaved with mild base treatment and has good acid stability.[13]

The Fmoc-protected thiourea nucleoside for the internal building block **1** was obtained from known 3′-deoxy-3′-(hydroxylamino)uridine derivative **3** (Scheme 1). Compound **3** was prepared from 3′-ketouridine derivative **4** according to a recently reported method,[14] involving a stereoselective reduction of the 3′-oxime moiety with $NaBH(OAc)_3$. Reduction of **3** by catalytic hydrogenation in acetic acid gave the 3′-amino derivative, which was subsequently protected with a trifluoroacetyl group to provide **5**, and upon the conversion of the 5′-hydroxyl group to azide, **6** was obtained. Reduction of **6** with hydrogen sulfide in aqueous pyridine followed by protecting with the MMTr group provided **7**. The trifluoro-

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(12) Barawkar, D. A.; Linkletter, B. A.; Bruice, T. C. *Bioorg. Med. Chem. Lett*. **1998**, *8*, 1517.

(13) In the solid-phase synthesis of DNG, the trichloroethyloxycarbonyl (Troc) group was used for *N*-protection of thiourea instead of the Fmoc group (ref 10d). However, using Fmoc has advantages in preparation of corresponding isothiocyanate, and in the removal step, which can be readily cleaved by NH_4OH treatment. We have also tried some acyl groups for the *N*-protection of thiourea. In these cases, reaction of *N*-acyl isothiocyanate with 3′-amino nucleoside gave not only desired 3′-thiourea nucleoside but also 3′-*N*-acylated nucleoside as a side product. Formation of *N*-acyl compound was increased with the increase of electronegativity of the acyl group on isothiocyanate.

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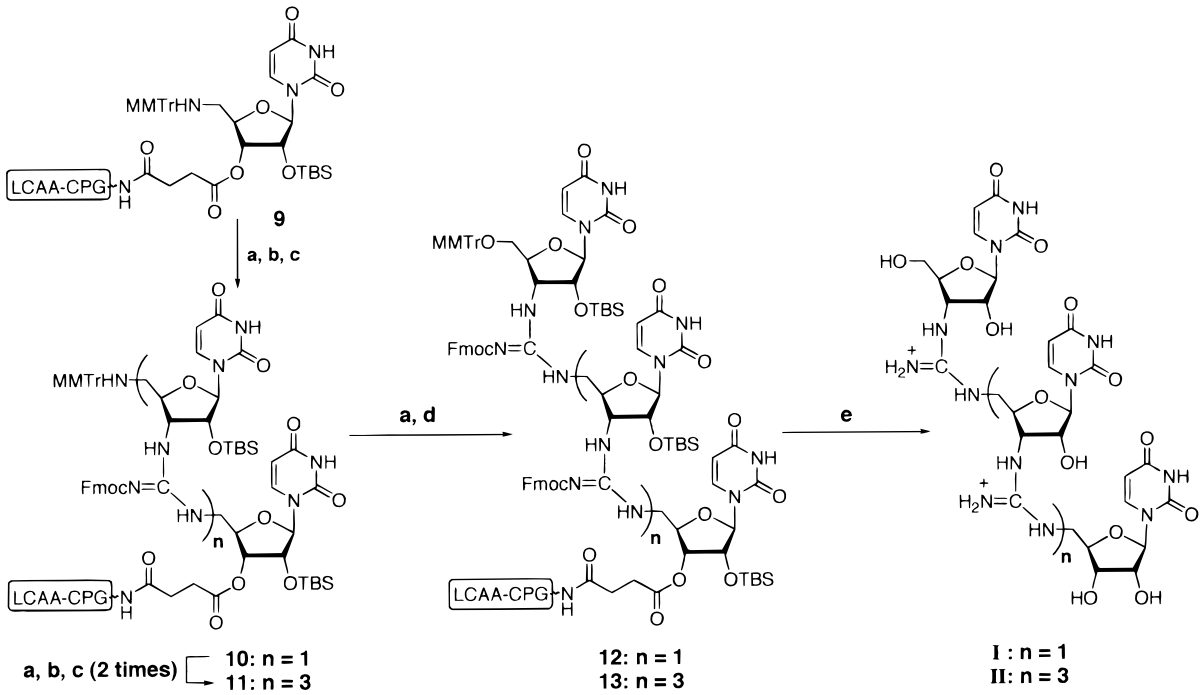
Scheme 2[a]



[a] Reagents and conditions: (a) deblocking 3% TCA in CH_2Cl_2; (b) coupling (1) **1**, $HgCl_2$, DIEA, DMF, (2) 20% thiophenol in DMF; (c) capping CF_3CO_2Et, DIEA, DMF; (d) coupling of 5′-end monomer (1) **2**, $HgCl_2$, DIEA, DMF, (2) 20% thiphenol in DMF; (e) deprotection and cleavage (1) 3% TCA in CH_2Cl_2, (2) NH_4OH/EtOH (3/1), (3) 1 M TBAF in THF.

acetyl protecting group was removed, and the resulting 3′-amino group was allowed to react with fluorenylmethyloxycarbonyl isothiocyanate (Fmoc-NCS)[15] in DCM to give the desired building block **1**.[16] To place a hydroxyl group at the 5′-end of the RNG, a building block with a 5′-hydroxyl group was required. Needed compound **2** was obtained from intermediate **5** by protection of the 5′-hydroxyl moiety with an MMTr group followed by conversion of the 3′-position to *N*-Fmoc-protected thiourea.[17]

Long chain alkylamine controlled pore glass (LCAA-CPG) was chosen for the solid support. The attachment of the 3′-end nucleoside onto LCAA-CPG followed the standard procedure for DNA/RNA synthesis.[7b] Selective silylation of 5′-monomethoxytritylaminouridine was accomplished using TBSCl, $AgNO_3$, and pyridine in THF to give the 2′-OTBS derivative. This compound was condensed with LCAA-CPG via 3′-succinate to provide solid-support-attached 3′-end monomer **9** (23.5 μmol/g).

A typical solid-phase synthesis is outlined in Scheme 2. Both trimeric (**I**) and pentameric (**II**) uridyl RNG were synthesized on a 2.0 μmol scale: 85 mg of LCAA-CPG support containing the 3′-end nucleoside (**9**) was placed in a solid-phase synthesis vessel and treated with 3% trichloroacetic acid in DCM (2.0 mL, 10 s, 3 times) to remove the MMTr group. Next, there was added **1** (27.3 mg, 0.030 mmol) in DMF (0.5 mL) followed by a solution of mercury(II) chloride (60 mM in DMF, 0.5 mL) and *N*,*N*-diisopropylethylamine (120 mM in DMF, 0.5 mL). The mixture was agitated at room temperature for 4 h. Preliminary study indicated that this single coupling reaction gave an average coupling yield of 77% as determined from the released MMTr cation. This relatively low coupling yield is probably due to the bulkiness of the adjacent 2′-OTBS group of incoming nucleoside monomer, hindering the attack of the terminal 5′-amino group to carbodiimide. The coupling reaction was repeated and resulted in an average of 90% in

(15) Kearney, P. C.; Fernandez, M.; Flygare, J. A. *J. Org. Chem.* **1998**, *63*, 196.

(16) Physical data for **1**: ^{1}H NMR (400 MHz DMSO-d_6) δ (ppm) 11.67 (s, 1 H), 11.45 (d, 1 H, J = 1.9 Hz), 10.12 (d, 1 H, J = 8.0 Hz), 8.02 (d, 1 H, J = 8.0 Hz), 7.89 (m, 2 H), 7.80 (m, 2 H), 7.45−7.14 (m, 16 H), 6.83 (m, 2 H), 5.69 (d, 1 H, J = 2.6 Hz), 5.60 (dd, 1 H, J = 8.0, 1.9 Hz), 5.06 (ddd, 1 H, J = 6.1, 8.6, 8.0 Hz), 4.67 (dd, 1 H, J = 2.6, 6.1 Hz), 4.42−4.34 (m, 2 H), 4.27 (t, 1 H, J = 7.3 Hz), 4.08 (ddd, 1 H, J = 8.6, 4.2, 4.1 Hz), 3.70 (s, 3 H), 3.05 (t, 1 H, J = 8.1 Hz), 2.53 (ddd, 1 H, J = 4.2, 12.8, 8.1 Hz), 2.36 (ddd, 1 H, J = 4.1, 12.8, 8.1 Hz), 0.82 (s, 9 H), 0.07 (s, 3 H), 0.02 (s, 3 H); ^{13}C NMR (100 MHz DMSO-d_6) δ (ppm) 179.86, 163.00, 157.41, 153.49, 150.23, 146.06, 143.17, 143.13, 141.42, 140.72, 140.68, 137.59, 129.59, 128.26, 127.85, 127.69, 127.12, 126.07, 125.43, 125.39, 120.17, 113.03, 101.84, 91.23, 81.03, 73.70, 69.81, 67.37, 56.72, 54.91, 45.98, 44.65, 25.51, 17.59, −5.14, −5.24; FAB-MS (HR) *m*/*z* 910.3698, calcd for $C_{51}H_{56}N_5O_7SiS$ $(M + H)^+$ 910.3670.

(17) Physical data for **2**: ^{1}H NMR (400 MHz DMSO-d_6) δ (ppm) 11.73 (s, 1 H), 11.48 (d, 1 H, J = 2.0 Hz), 10.09 (d, 1 H, J = 7.3 Hz), 7.90 (m, 2 H), 7.85 (d, 1 H, J = 8.3 Hz), 7.81 (m, 2 H), 7.46−7.20 (m, 16 H), 6.89 (m, 2 H), 5.76 (d, 1 H, J = 2.9 Hz), 5.35 (dd, 1 H, J = 8.3, 2.0 Hz), 5.14 (dt, 1 H, J = 6.0, 7.3 Hz), 4.58 (dd, 1 H, J = 2.9, 6.0 Hz), 4.39 (dd, 1 H, J = 8.0, 10.2 Hz), 4.35 (dd, 1 H, J = 7.4, 10.2 Hz), 4.27 (dd, 1 H, J = 7.4, 8.0 Hz), 4.18, (ddd, 1 H, J = 7.3, 3.6, 2.2 Hz), 3.72 (s, 3 H), 3.44, (dd, 1 H, J = 3.6, 11.0 Hz), 3.37 (dd, 1 H, J = 2.2, 11.0 Hz), 0.80 (s, 9 H), 0.06 (s, 3 H), 0.00 (s, 3 H); ^{13}C NMR (100 MHz DMSO-d_6) δ (ppm) 180.24, 162.91, 158.30, 153.57, 150.27, 144.10, 143.70, 143.18, 140.75, 140.72, 140.18, 134.38, 130.18, 128.02, 127.97, 127.93, 127.19, 128.15, 127.02, 125.49, 120.22, 113.27, 101.83, 90.04, 86.50, 81.03, 73.79, 67.41, 62.63, 55.56, 55.05, 45.97, 44.86, 25.43, 17.60, −5.23, −5.33; FAB-MS (HR) *m*/*z* 911.3535, calcd for $C_{51}H_{55}N_4O_8SiS$ $(M + H)^+$ 911.3510.

efficiency. At this point, a black deposit of mercury sulfide was formed. In order not to reduce further coupling yields, the deposit was removed by treating the CPG resin with a 20% thiophenol solution in DMF (2.0 mL, 10 s, 3 times).[10d] After the resin was washed with DMF, unreacted amino groups were capped using the mixture of ethyl trifluoroacetate/*N*,*N*-diisopropylethylamine (1:1, 120 mM in DMF, 2.0 mL). The deblocking/coupling/capping cycles were repeated two more times for pentameric uridyl RNG oligomer (**II**) before coupling the 5′-end building block **2**.

The MMTr groups on the 5′-end of the completed RNG oligomers (**12**, **13**) were removed prior to the cleavage of oligomers from the CPG support. On the other hand, this MMTr group can be left on throughout the purification steps if desired. Treatment of CPG-bound RNG oligomers with an NH_4OH/EtOH (3/1) mixture resulted in cleavage of oligomers from the CPG support as well as removal of Fmoc groups from guanidinium moieties. TBS groups on 2′-hydroxyl were then removed using a 1.0 M solution of tetrabutylammonium fluoride in THF. This provides fully deprotected RNG oligomers. Finally, the crude RNG oligomers were purified by RP-HPLC using the solvent system of 0.1 M trimethylammoniumacetate buffer/CH_3CN or 1% aqueous AcOH/CH_3CN. Both trimeric and pentameric uridyl oligomers (**I**, **II**) showed a single peak after purification (Figure 1). Furthermore, RNG oligomers were analyzed by electrospray ionization mass spectrometry, and the observed molecular weights supported their structures.[18]

In summary, we have developed a solid-phase method for the synthesis of ribonucleic guanidine (RNG), a polycationic analogue of RNA. Both trimeric and pentameric uridyl oligomers of RNG (**I**, **II**) have been successfully synthesized. The method described herein for the synthesis of building blocks (**1**, **2**), as well as the solid-phase RNG synthesis, is designed to be expandable to all other RNA bases (adenine, guanine, and cytosine). In these cases, exocyclic amino groups of the bases can be protected with standard benzoyl, isobutyryl, or more labile phenoxyacetyl groups, which can be removed concurrently on treatment with NH_4OH/EtOH at the stage of cleavage from the polymer support. Research toward the synthesis of longer sequence RNG containing all four bases is currently in progress. Physicochemical properties of synthesized RNG with complementary DNA and RNA will be published separately.

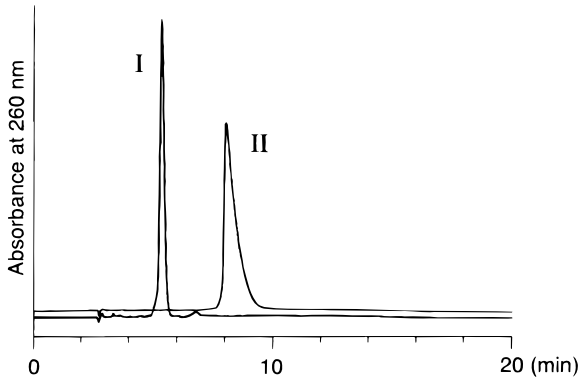


Figure 1. HPLC profiles of purified RNG oligomers **I** and **II**: Alltech Macrosphere RP C8 column, 0−4% acetonitrile in 2% aqueous AcOH over 20 min at 1.3 mL/min.

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(18) For **I**, singly charged [*m*/*z* 779.3, calcd for $C_{29}H_{39}N_{12}O_{14}$ $(M + H)^+$ 779.3] and doubly charged [*m*/*z* 390.2, calcd for $C_{29}H_{40}N_{12}O_{14}$ $(M + 2H)^{2+}$ 390.1] forms were observed. For **II**, the doubly charged [*m*/*z* 657.1, calcd for $C_{49}H_{66}N_{22}O_{22}$ $(M + H)^{2+}$ 657.2] form was observed.