SYNTHESIS AND HYBRIDIZATION PROPERTIES OF RNA CONTAINING 8-CHLOROADENOSINE

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ABSTRACT

8-Chloroadenosine (8-Cl-Ado) has shown potential as a chemotherapeutic agent for the treatment of multiple myeloma and certain leukemias. 8-Cl-Ado treatment leads to a decrease in global RNA levels and incorporation of the analog into cellular RNA in malignant cells. To investigate the effects of 8-Cl-Ado modifications on RNA structure and function, an 8-Cl-Ado phosphoramidite and controlled-pore glass support were synthesized and used to introduce 8-Cl-Ado at internal and 3′- terminal positions, respectively. RNA oligonucleotides containing 8-chloroadenine (8-Cl-A) residues were synthesized and hybridized with complementary RNA strands. Circular dichroism spectroscopy of the resulting RNA duplexes revealed that the modified nucleobase does not perturb the overall A-form helix geometry. The thermal stabilities of 8-Cl-Ado modified duplexes were determined by UV thermal denaturation analysis and were compared with analogous natural duplexes containing standard and mismatched base pairs. The 8-Cl-Ado modification destabilizes RNA duplexes by $\sim 5\,\text{kcal/mole}$, approximately as much as a U:U mismatched base pair. The duplex destabilization of 8-Cl-A may result from perturbation of Watson-Crick base pairing induced by conformational preferences of 8-halogenated nucleosides.

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Key Words: Nucleoside; RNA synthesis; RNA duplex stability; Phosphoramidite; 8-Chloroadenosine

INTRODUCTION

Nucleoside analogs represent a growing class of medicinal agents that have been used extensively in anti-viral and cancer chemotherapy. By resembling the natural building blocks of nucleic acids, these analogs are taken up by cells, activated by phosphorylation, and incorporated into DNA or RNA by polymerases. Structurally altered nucleosides frequently inhibit enzymes or inactivate nucleic acid replication, repair, and processing pathways. Nucleoside analogs have been used for decades in combating viral infections. For example, the azidothymidine derivative, AZT (**1**, Fig. 1), which acts by inhibiting HIV reverse transcriptase (HIV-RT), served as an early treatment for HIV infections and currently is used in therapeutic cocktails.[1] Halogenated nucleosides, such as fludarabine (**2**, Fig. 1) and gemcitabine (**3**, Fig. 1), also have been employed for the treatment of various forms of cancer. These analogs are taken up by cells and converted to the corresponding 5′-triphosphate derivatives, the apparent cytotoxic agents.[2,3]

The halogenated nucleoside, 8-chloroadenosine (8-Cl-Ado, Fig. 1, **4**), recently has shown potential for the treatment of multiple myeloma (MM) and other leukemias. Early experiments demonstrated that 8-chloroadenosine 3′,5′-cyclic monophosphate (8-Cl-cAMP) induced apoptosis in several cancer cell lines.[4–9] However, the anti-proliferative effects of 8-Cl-cAMP were dependent on its enzymatic conversion into 8-Cl-Ado.[4,5,8,10–12] More recently, 8-Cl-Ado has been shown to induce apoptosis in drug resistant MM and leukemia cell lines.[11] Investigations of cellular nucleotide metabolism have demonstrated that MM cells treated with 8-Cl-Ado rapidly accumulate the 5′-triphosphate derivative, 8-Cl-ATP, with a concomitant depletion of natural ATP levels.[12] Furthermore, RNA levels in treated myeloma cells decrease to 50% of untreated control after 12 h, while DNA levels remain largely unaltered.[12]

The emerging picture of 8-Cl-Ado action includes cellular uptake of 8-Cl-Ado, enzymatic conversion to 8-Cl-ATP, and subsequent depletion of
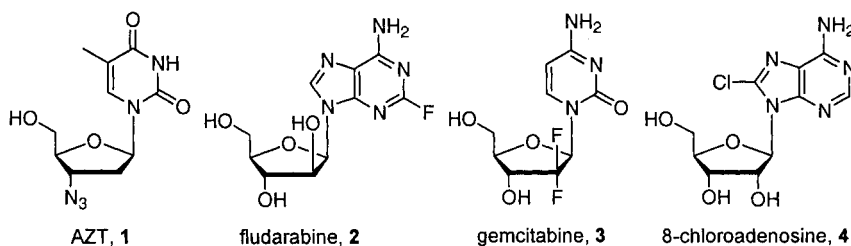

Figure 1.   Nucleoside analogs used in chemotherapeutic approaches.

cellular RNA and ATP levels. However, the molecular mechanism for apoptosis induction remains unclear. To gain a better understanding of the RNA-targeted mechanism of 8-Cl-Ado action, a chemical method for the incorporation of 8-Cl-Ado nucleotides within RNA sequences was developed. Phosphoramidite and controlled-pore glass support derivatives of 8-Cl-Ado were synthesized. These building blocks were used to synthesize RNA oligonucleotides containing 8-Cl-A bases, and the base pairing properties of these modified RNA sequences were assessed by circular dichroism (CD) and UV thermal denaturation spectroscopies. The addition of a chlorine atom at the 8-position of adenine does not perturb global RNA helical structure, but destabilizes RNA duplexes approximately as much as a U:U mismatch in double stranded RNA.

RESULTS AND DISCUSSION

Synthesis of 8-Cl-Ado Phosphoramidite and Solid-Support

The introduction of 8-Cl-Ado into RNA was based on standard automated RNA synthesis with $2'$-O-silyl nucleoside phosphoramidites.[13] Thus, 8-Cl-Ado phosphoramidite (**10**, Sch. 1) and solid-support derivative
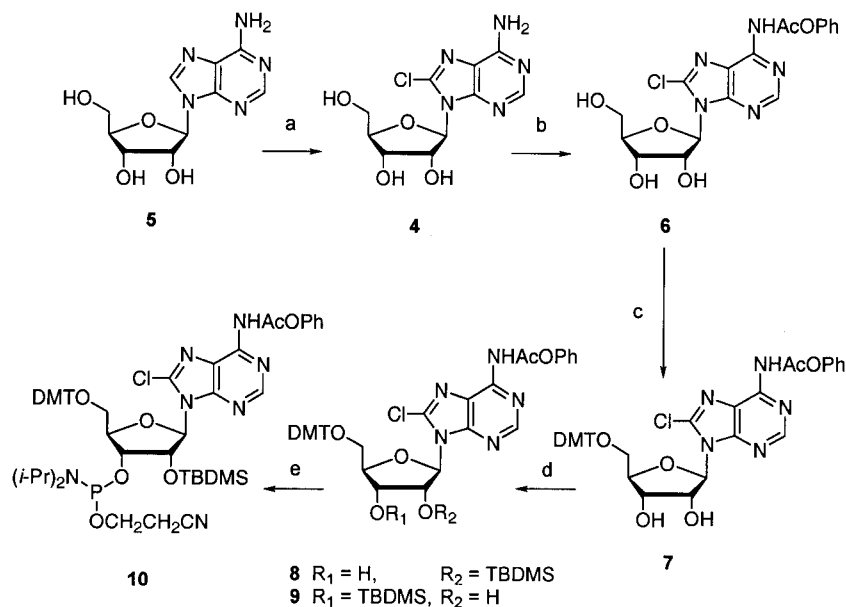
Scheme 1. Synthesis of 8-chloroadenosine phosphoramidite **10**. Reagents and conditions: (a) HCl, MCPBA, DMA, 25°C, 2.5 h, 39%. (b) (CH$_3$)$_3$Si-Cl, pyridine then phenoxyacetylchloride, 1,2,4-triazole, pyridine, CH$_3$CN, 55°C, 7 h, 70%. (c) DMTCl, DMAP, pyridine, 25°C, 12 h, 58%. (d) TBDMSCl, AgNO$_3$, Et$_3$N, pyridine, THF, 25°C, 2.5 h, 16% (**8**) and 38% (**9**). (e) **8** and [(i-Pr)$_2$N]$_2$POClCH$_2$CH$_2$CN, N-methylimidazole, 2,4,6-collidine, THF, 25°C, 4 h, 50%.
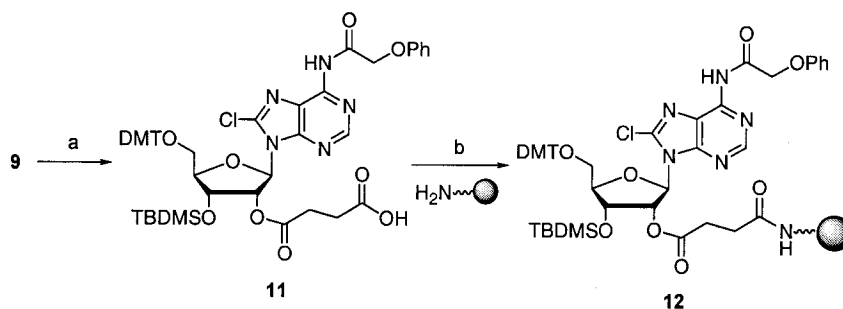
Scheme 2. Synthesis of an 8-chloroadenosine-derivatized controlled-pore glass support. Reagents and conditions: (a) Succinic anhydride, DMAP, 12 h, 25°C, 71% (b) DCC, 4-nitrophenol, pyridine, dioxane, DMF, 12 h, 25°C. Final loading: 22 μmol/g.

(**12**, Sch. 2) were synthesized. Phosphoramidite **10** utilized standard 4,4′-dimethoxytrityl (DMT) and *tert*-butyldimethylsilyl (TBDMS) protecting groups for the sugar hydroxyls, and a phenoxyacetyl (PAC) group for blocking of the exocyclic amine. Isomeric 3′-*O*-silyl nucleoside **9** was used for construction of a controlled-pore glass (CPG) support modified with 8-Cl-Ado (**12**).

The 8-Cl-Ado phosphoramidite building block **10** was prepared as described in Sch. 1. 8-Chloroadenosine (**4**) was synthesized by oxidative chlorination of adenosine (**5**) using a modification of a literature procedure.[14] 8-Cl-Ado (**4**) was subjected to transient trimethylsilyl protection,[15] and reacted with phenoxyacetyl chloride in the presence of 1,2,4-triazole to produce the phenoxyacetyl-protected nucleoside **6**.[16] The *N*-phenoxyacetyl nucleoside **6** was readily converted to the DMT derivative **7** in 58% yield.[17] The tritylated intermediate **7** then was silylated with TBDMS-Cl in the presence of silver nitrate to form the 2′-silyl (**8**) and 3′-silyl (**9**) isomers. The 3′-silyl isomer **9** (38% yield) was formed preferentially over the 2′-silyl isomer **8** (16% yield[18,19]). To complete the synthesis, compound **8** was converted to the desired phosphoramidite **10** by standard methods.[13]

An 8-Cl-Ado-derivatized solid support (**12**) was synthesized as outlined in Sch. 2. The 3′-silyl nucleoside byproduct (**9**, Sch. 1) was reacted with succinic anhydride to produce the carboxylic acid derivative **11**. The resulting succinic acid **11** was activated as a nitrophenol ester and coupled to 1000 Å alkyl amine-derivatized CPG using previously described methods.[20] Unreacted CPG amine sites then were capped using acetic anhydride. The final loading of the 8-Cl-Ado nucleoside on support **12** was 22 μmol/g as determined by dimethoxytrityl cation release.

RNA Oligonucleotide Synthesis, Deprotection, and Characterization

A series of RNA oligonucleotides containing single or multiple 8-Cl-Ado substitutions were synthesized and are listed with their complementary
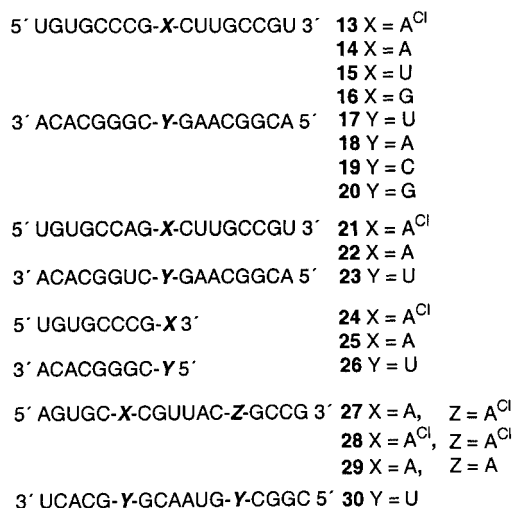
```
5´ UGUGCCCG-X-CUUGCCGU 3´    13 X = A^Cl
                             14 X = A
                             15 X = U
                             16 X = G
3´ ACACGGGC-Y-GAACGGCA 5´    17 Y = U
                             18 Y = A
                             19 Y = C
                             20 Y = G

5´ UGUGCCAG-X-CUUGCCGU 3´    21 X = A^Cl
                             22 X = A
3´ ACACGGUC-Y-GAACGGCA 5´    23 Y = U

5´ UGUGCCCG-X 3´             24 X = A^Cl
                             25 X = A
3´ ACACGGGC-Y 5´             26 Y = U

5´ AGUGC-X-CGUUAC-Z-GCCG 3´  27 X = A,    Z = A^Cl
                             28 X = A^Cl, Z = A^Cl
                             29 X = A,    Z = A
3´ UCACG-Y-GCAAUG-Y-CGGC 5´  30 Y = U
```

Figure 2. 8-Chloroadenosine-modified and standard RNA oligonucleotide sequences.

strands in Fig. 2. Sequences containing internal 8-Cl-Ado residues were prepared by standard solid-phase synthesis using phosphoramidite **10**, which was inserted into RNA oligonucleotides with 99% coupling yields. Three oligonucleotides were synthesized with one internal 8-Cl-A base (**13, 21, 27**), and one sequence identical to **27** was prepared with two internal 8-Cl-Ado modifications (**28**). A 3′-terminally 8-Cl-Ado modified sequence (**24**) was synthesized, which contained the same sequence as **13** upstream of the modified base, to compare the effects of terminal 8-Cl-Ado substitution in an identical sequence context. For comparison, unmodified RNA sequences were prepared with an adenosine residue substituting for the 8-Cl-Ado nucleotide. After synthesis, modified RNA oligonucleotides were deprotected using standard methods.[13] Oligonucleotides were deblocked and cleaved from the resin by treatment with 3:1 aqueous ammonia in ethanol and subsequently were desilylated with tetrabutylammonium fluoride (TBAF) in THF.[21] RNA oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE) and characterized using MALDI-TOF mass spectrometry. Experimentally determined masses were in excellent agreement with calculated masses for each RNA sequence.

Spectroscopic Analysis of 8-Cl-Ado-Modified RNA Duplex Structure and Stability

Circular dichroism (CD) spectroscopy and UV thermal denaturation analysis were used to assess the effect of 8-Cl-Ado substitution on the

stability of RNA-RNA duplexes. 8-Cl-Ado modified strands (**13, 21, 24, 27** and **28**) were annealed to the corresponding complementary strands (Fig. 2). The properties of the modified duplexes were compared with natural RNA-RNA duplexes constructed from unmodified RNA oligonucleotides (Fig. 2). Spectroscopic analyses were performed on 2 μM solutions of each RNA oligonucleotide in 10 mM phosphate buffer, 150 mM NaCl, 0.1 mM EDTA, at a final pH of 7.0.

Circular dichroism (CD) spectroscopy indicated that RNA duplexes containing internal 8-Cl-A substitutions adopt A-form RNA helices, characteristic of natural RNA duplexes. Representative CD spectra for modified and natural RNA duplexes are shown in Fig. 3A. As illustrated in Fig. 3A, the complex between unmodified 17-mer RNA strands **29–30** (squares) produced a CD spectrum with a positive absorption peak centered on 262 nm and a strong negative peak below 250 nm, which is diagnostic of A-form RNA duplexes.[22] Hybridization of **27**, which contained a single substitution of an adenosine residue with 8-Cl-Ado, with **30** (Fig. 3A, circles), produced an RNA duplex with a CD spectrum similar to that of the natural **29–30** duplex. The introduction of two 8-Cl-Ado nucleotides into the RNA duplex (**28–30** Figure 3A, triangles) resulted in a CD spectrum similar to the native RNA duplex (**29–30**). The CD spectrum of a shorter RNA duplex (**24–26**), where the 8-Cl-Ado occupied a 3′-terminal position, resembled the spectrum of **25–26**, which adopts A-form RNA structure (not shown).[23] Thus, CD spectroscopy suggests that 8-Cl-A bases at internal or terminal positions do not significantly alter the global RNA A-form helical structure.

Although RNA duplexes containing single or multiple 8-Cl-Ado substitutions adopt a normal RNA helical conformation, these nucleobase modifications destabilize duplex RNA. To assess the relative thermal stabilities of 8-Cl-Ado substitutions, samples used for CD spectroscopy were subjected to UV thermal denaturation analysis. As shown in Fig. 3B, the UV absorbances of modified duplexes **27–30** and **28–30** were monitored at 260 nm as the temperature was increased at a rate of 0.5°C/min. The results were compared to the unmodified control sequences, **29–30**. All samples showed a similar hyperchromicity change ($\sim$25%) over the range of 30 to 90°C in 10 mM phosphate buffer, 150 mM NaCl, 0.1 mM EDTA, pH 7.0. The melting temperature (T_m) for each RNA duplex was calculated from the first derivative of the melting profile, and the results are given in Table 1. The unmodified RNA duplex **29–30** (Fig. 3B, squares) displayed a T_m of 75.3°C. Substitution of a single adenine base with 8-Cl-A (**27–30**, Fig. 3B, circles) produced an RNA duplex that melts at 73.1°C under identical conditions. The destabilization of $\sim$2–3°C for a single 8-Cl-Ado substitution was additive: the insertion of a second 8-Cl-A destabilized duplex **28–30** (Fig. 3B, triangles) by 5°C relative to the natural duplex, **29–30**.

To assess the effect of neighboring sequences on the duplex stability of 8-Cl-Ado-containing RNAs, two alternate sequences (**13** and **21**), each
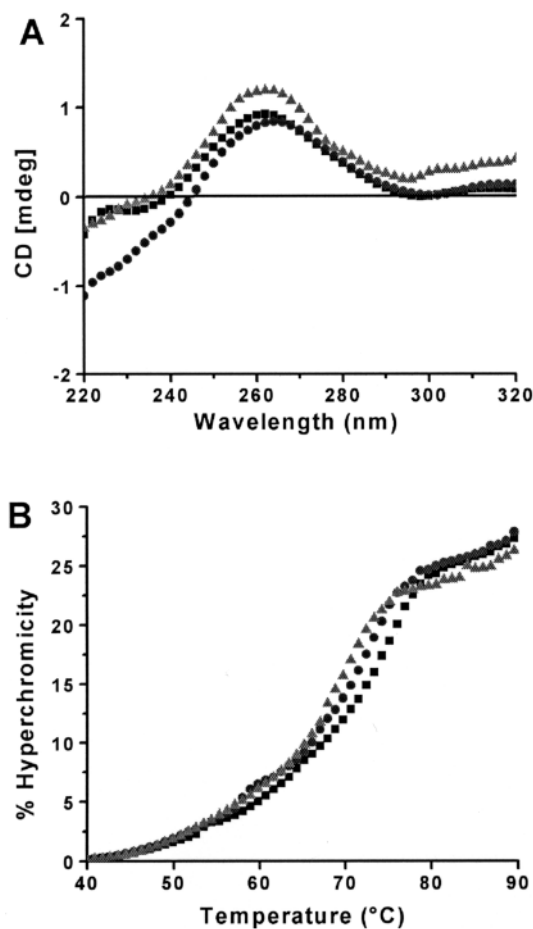
Figure 3. Spectroscopic characterization of modified RNA duplexes containing single (**27–30** circles) and double (**28–30**, triangles) 8-Cl-Ado modifications and comparison with an un-modified RNA-RNA hybrid (**29–30**, squares). **A.** Circular dichroism spectroscopy. **B.** UV thermal denaturation curves at $0.5°C/min$. Conditions: $2\,\mu M$ of each RNA strand in $10\,mM$ phosphate buffer, $150\,mM$ NaCl, $0.1\,mM$ EDTA, pH 7.0.

containing a single 8-Cl-Ado positioned centrally in a 17-mer duplex, were studied using UV thermal denaturation methods. As summarized in Table 1, the natural RNA sequence containing an A:U base pair (**14–17**) displayed a T_m of $83.6°C$, while the analogous 8-Cl-Ado analog duplex (**13–17**) showed a T_m of $77.9°C$, corresponding to a $5.7°C$ decrease in thermal stability. A related sequence exhibited a similar trend, with 8-Cl-Ado modified duplex **21–23** showing a T_m value of $4.4°C$ lower than that of the natural duplex (**22–23**). The effect of a $3'$-terminal 8-Cl-Ado substitution on duplex stability also was evaluated. Comparison of a duplex with a $3'$-terminal 8-Cl-A:U pair (**24–26**) with one containing a $3'$-terminal A:U pair (**25–26**) indicated that

Table 1.   Melting Temperature Comparisons of Control and 8-Chloroadenosine Containing Duplexes[a]

Duplex	Base Pair	T_m (°C)[a]	Duplex	Base Pair	T_m (°C)[a]
Internal modification					
13–17	A^{Cl}:U	77.9 ± 0.7	**14–17**	A:U	83.6 ± 0.9
13–18	A^{Cl}:A	73.8 ± 0.3	**14–18**	A:A	75.2 ± 1.0
13–19	A^{Cl}:C	71.5 ± 0.3	**14–19**	A:C	77.1 ± 0.2
13–20	A^{Cl}:G	74.4 ± 0.8	**14–20**	A:G	76.2 ± 0.2
15–17	U:U	77.5 ± 0.4	**16–17**	G:U	82.5 ± 0.1
15–19	U:C	75.2 ± 0.6	**16–18**	G:A	77.1 ± 0.4
21–23	A^{Cl}:U	74.9 ± 0.2	**16–20**	G:G	77.9 ± 0.1
27–30	A:U/A^{Cl}:U	73.1 ± 0.2	**22–23**	A:U	79.3 ± 0.1
28–30	A^{Cl}:U/ A^{Cl}:U	70.3 ± 0.5	**29–30**	A:U	75.3 ± 0.3
Terminal modification					
24–26	A^{Cl}:U	58.8 ± 0.4	**25–26**	A:U	62.9 ± 0.2

[a]Melting temperatures (T_m's) were determined from the average of three independent experiments. Ranges ($\pm$) are given as the standard deviation of the three runs. Conditions: 2 mM of each RNA strand in 10 mM sodium phosphate, 0.1 mM EDTA, 150 mM NaCl, pH 7.0.

the chlorinated analog destabilizes the duplex by 4.1°C in the context of a 9-mer double helical construct.

The propensity of 8-Cl-A bases to pair with other nucleobases (A, G, C) was assessed and compared with the destabilization induced by mismatch pairs within natural RNA duplexes (Table 1). Of the four natural nucleobases, 8-Cl-A formed the most stable pair with uracil ($T_m = 77.9$°C, **13–17**). 8-Cl-A paired equally well with guanine ($T_m = 74.4$°C, **13–20**) and adenine ($T_m = 73.8$°C, **13–18**), and least well with cytosine ($T_m = 71.5$°C, **13–19**). In contrast, in the same sequence context (**14**), the natural adenine base formed the most stable pair with uracil (**17**), followed by cytosine (**19**), guanine (**20**), then adenine (**18**). Finally, a series of duplexes were constructed that contained the same sequence as **13** or **14**, but introduced natural base mismatches at the central base pair. Interestingly, the 8-Cl-A:U base pair (**13–17**, $T_m = 77.9$°C) was more destabilizing than a G:U wobble base pair (**16–17**, $T_m = 82.5$°C). The 8-Cl-A:U (**13–17**) pair destabilized duplexes to the same extent as a U:U mismatch (**15–17**, $T_m = 77.5$°C) or a G:purine mismatch (G:A, **16–18**, $T_m = 77.1$°C or G:G, **16–20**, $T_m = 77.9$°C). However, the 8-Cl-A:U base pair (**13–17**) was thermally more stable than the mismatched U:C base pair (**15–19**), $T_m = 75.2$°C.

Taken together, the CD spectroscopy and UV thermal denaturation studies revealed that the substitution of adenosine with 8-Cl-Ado in RNA leads to a destabilization of RNA duplexes by 2–6°C in 17 mer RNA oligonucleotides, which corresponds to an ~ 5 kcal/mole ($\Delta\Delta G°_{298}$)

destabilization per internal 8-Cl-Ado substitution. The magnitude of helix stability perturbation depends on the flanking sequence context (compare **13–17** with **27–30**) and the location of the 8-Cl-Ado relative to the duplex termini, consistent with extensive thermodynamic data on mismatches in duplex RNA.[24,25]

Characterization of the stabilities of RNA duplexes containing 8-Cl-Ado provides insight into the effects of potential nucleoside therapeutic agents on nucleic acid structure. Few 8-modified ribonucleotides have been introduced into RNA duplexes and characterized by biophysical methods.[26,27] However, DNA oligonucleotides containing C-8 purine deoxy nucleotide modifications, including 8-methoxy-,[28] 8-bromo-,[29] and 8-oxo-2'-deoxyadenosine,[30] and the guanosine derivatives, 8-methyl-,[31] 8-bromo-,[32] and 8-oxo-2'-deoxyguanosine[33] have been synthesized. Biophysical characterization of these modified DNAs revealed that C-8 purine substitution generally resulted in DNA duplex destabilization in Watson-Crick pairing contexts.[28–33] However, 8-modified purines generally stabilized DNA complex formation in cases where *syn* glycosidic torsion angles were preferred, such as G-quadruplexes[32] or A:G mismatches.[28] In this context, it is interesting that in our system, converting an A:U base pair (**14–17**, $T_m = 83.6°C$) to an A:G base pair (**14–20**, $T_m = 76.2°C$) lowers the T_m by 7.4°C in a 17 mer RNA context. In contrast, converting an 8-Cl-A:U pair to an 8-Cl-A:purine pair is less disruptive: the melting temperature of a 8-Cl-A:G "mismatch" (**13–20**, $T_m = 74.4°C$) is only 3.5°C lower than the 8-Cl-A:U pair (**13–17**, $T_m = 77.9°C$) in the same sequence context.

The structural basis for duplex destabilization by 8-modified bases correlates with the preference of C-8-substituted purines to adopt a *syn* glycosidic torsion angle rather than the *anti* conformation found in A-form and B-form duplexes. X-ray structural analysis[34] and CD studies[35] of 8-bromoadenosine and its 5'-monophosphate derivative demonstrated that substitution of the C-8 hydrogen of adenosine with a sterically-demanding bromine atom forces the nucleobase to adopt exclusively the *syn* conformation. The *syn* preference of 8-bromoguanosine (8-Br-G) was demonstrated for the dinucleotide, Cp-8-Br-G, using ^{1}H-NMR,[27] and for 8-substituted adenosine derivatives of sequence ApUpG, using UV and CD spectroscopy.[26] Thus, we currently believe that RNA helix destabilization caused by 8-Cl-Ado substitution is the result of an altered *syn* glycosidic bond torsion angle induced by the chlorine substitution at C-8. The outcome of a *syn* glycosidic torsion angle preference would be the presentation of a different hydrogen bonding face to potential pairing partners, and potentially, perturbation of the sugar pucker of 8-Cl-Ado nucleosides. This model is consistent with the relatively lower destabilizing effect of a 8-Cl-Ado:purine mismatch compared with a A:A or A:G mismatch. Although a chlorine atom (van der Waals radius 1.8 Å) is somewhat smaller than a bromine atom (1.95 Å), both are more sterically demanding than a hydrogen atom

(1.2 Å).[36] While theoretical calculations suggest that the *syn* conformer of 8-chloroadenosine is favored significantly over the *anti* conformer,[37] structural verification of these predicted effects has not been reported. As a result, NMR structural analyses of 8-Cl-Ado and RNA containing this chlorinated analog are underway in our laboratory.

EXPERIMENTAL SECTION

General Methods

All reactions were performed in oven dried (140°C) glassware using anhydrous Aldrich Sure-Seal[TM] solvents and N_2 as an inert atmosphere. Unless otherwise noted, reagents were obtained from Aldrich and used without further purification. Amine-derivatized 1000 Å long chain alkyl amine controlled pore glass (LCAA-CPG) was obtained from Chem Genes Corporation. EM Science Kieselgel 60 F254 plates (0.25 mm) were used for thin-layer chromatography. Compounds on TLC were visualized using a short-wave UV lamp and stained with *p*-anisaldehyde/sulfuric acid/acetic acid. Silica gel (0.040–0.063 mm, EM Science) was used for flash column chromatography. ^{1}H- and ^{13}C-NMR spectra were recorded at 500 and 125 MHz respectively, on a Varian 500 instrument, unless otherwise noted, and are referenced in parts per million (ppm) in the following format: chemical shift (multiplicity, integration, coupling constant in Hertz, proton assignment). Proton assignments for ^{1}H spectra were determined using ^{1}H -^{1}H NMR COSY data. ^{31}P-NMR data were recorded at 162 MHz on a Varian 400 spectrometer and were referenced to an external standard of 85% aqueous phosphoric acid. High resolution data were obtained using analytically pure materials.

8-Chloroadenosine (4). *m*-Chloroperoxybenzoic acid (MCPBA, 25 g, 77%) was suspended in 120 mL of CH_2Cl_2 and stirred for 4 h at 25°C. The water layer was removed, and the CH_2Cl_2 layer was dried over $MgSO_4$ and filtered. To the filtrate solution was added 110 mL of anhydrous dimethylacetamide (DMA), and the resulting solution was concentrated in vacuo to 120 mL (final concentration 0.9 M MCPBA/DMA). A hydrochloric acid solution was prepared by bubbling HCl gas through 100 mL of anhydrous DMA at 0°C for 10 min. The solution then was diluted with 100 mL of anhydrous DMA to give a 1.5 M HCl/DMA solution. Adenosine (5 g, 18.7 mmol) was taken up in 25 mL of anhydrous DMA, and 21 mL (31.5 mmol) of 1.5 M HCl/DMA solution was added. The reaction was stirred at 0°C for 5 min, then 35 mL (32 mmol) of a 0.9 M MCPBA/DMA solution was added dropwise over 30 min while the reaction was kept between $0 - 10$°C. The reaction was stirred under nitrogen, gradually warming to

25°C over 2.5 h. The reaction was quenched by dropwise addition of a solution of 3.5 g of $NaHCO_3$ in 7.5 mL of H_2O. The reaction was concentrated in vacuo at 45°C to 25 mL The resulting syrup was co-evaporated twice with 60 mL 2:1 xylenes/EtOH, then 60 mL MeOH and 167 mL of H_2O was added to the syrup. The resulting solution then was filtered, extracted with Et_2O (3 × 100 mL), and the aqueous layer was concentrated in vacuo to an orange solid. The product was purified by column chromatography (10% $MeOH/CHCl_3$) to give 3.8 g of crude product, which was recrystallized from absolute EtOH to give 2.2 g of 8-chloroadenosine (**4**), (17.6 mmol), 39% yield). mp 189–191°C (lit.[14] 188–190°C),^{1}H NMR (DMSO-d_6) δ: 8.15 (s, 1H, H2), 7.59 (s, 2H, NH_2), 5.84 (d, 1H, $J_{1\&',2'} = 6$ Hz, H1′), 5.53 (d, 1H, $J_{2'OH,2'} = 6$ Hz 2′OH), 5.48 (q, 1H, $J_{5'OH,5'a} = 8$ Hz, $J_{5'OH,5b'} = 8.4$ Hz, 5′OH), 5.29 (d, 1H, $J_{3'OH,3'} = 4.4$ Hz, 3′OH), 5.04 (q, 1H, $J_{1',2'} = 6$ Hz, $J_{2',3'} = 11.6$ Hz, H2′), 4.18 (s, 1H, H3′), 3.97 (d, 1H, $J = 1.6$ Hz, H4′), 3.67 (m, 1H, H5′a), 3.52 (m, 1H, H5′b). ^{13}C NMR (DMSO-d_6) δ: 56.7, 71.5, 71.9, 87.4, 89.9, 118.6, 137.7, 150.3, 153.3, 155.9. HRMS-ESI (m/z): [M + H]$^+$ calcd, 302.0656; found, 302.0656.

N-6-Phenoxyacetyl-8-chloroadenosine (6). To a stirred solution of **4** (0.600 g, 1.99 mmol) in 10 mL anhydrous pyridine, 1.89 mL (14.9 mmol) of chlorotrimethylsilane was added by syringe. The resulting white suspension was stirred for 30 min at 25°C. In a separate flask, 0.206 g (2.98 mmol) of 1,2,4-triazole and 0.55 mL (3.98 mmol) phenoxyacetyl chloride were dissolved in 5 mL each of anhydrous pyridine and anhydrous CH_3CN. The phenoxyacetyl chloride solution was added dropwise over 2 h to the 8-chloroadenosine solution while the temperature was gradually raised to 55°C over 1 h. The reaction was stirred for another 5 h at 55°C, then cooled to 25°C. The reaction was quenched by addition of 2 mL of water and 1.5 mL of 28% NH_4OH (aq). The solution then was evaporated to a syrup. Water (15 mL) and CH_2Cl_2 (15 mL) were added to the syrup, the layers were separated, and the aqueous layer was back-extracted with CH_2Cl_2 (4 × 15 mL). The combined CH_2Cl_2 layers were washed with water (2 × 20 mL, dried over Na_2SO_4, filtered and concentrated to an orange foam. The product was purified by column chromatography (3–7% $MeOH/CHCl_3$) to give 0.611 g (71% yield) of pale orange foam (**6**). ^{1}H NMR (DMSO-d_6) δ: 11.09 (s, 2H, NH_2), 8.71 (s, 1H, H2), 7.30 (t, 2H, $J = 7.75$ Hz, O-Ph), 6.96 (m, 3H, O-Ph), 5.93 (d, 1H, $J_{1',2'} = 6$ Hz, H1′), 5.53 (d, 1H, $J_{2',2'OH} = 5.5$ Hz, 2′OH), 5.29 (d, 1H, $J_{3',3'OH} = 4.5$ Hz, 3′OH), 5.15 (q, 1H, $J_{1',2'} = 6$ Hz, $J_{2',3'} = 11.5$ Hz, H2′), 5.00 (s, 2H, -CH_2-OPh), 4.97 (t, 1H, $J_{5'a,5'OH} = 5$ Hz, $J_{5'b,5'OH} = 6.5$ Hz, 5′OH), 4.27 (q, 1H, $J_{3',3'OH} = 4.5$ Hz, $J_{3',4'} = 8$ Hz, H3′), 3.97 (q, 1H, $J_{3',4'} = 8$ Hz, $J_{4',5'} = 4.75$ Hz, H4′), 3.68 (m, 1H, H5′a), 3.53 (m, 1H, H5′b). ^{13}C NMR (DMSO-d_6) δ: 167.4, 157.8, 152.2, 148.3, 141.5, 129.5, 122.3, 121.1, 114.6, 94.0, 86.3, 67.1, 25.2. HRMS-FAB (m/z): [M + Na]$^+$ calcd, 458.0843; found, 458.0842.

5′-*O*-Dimethoxytrityl-*N*-6-phenoxyacetyl-8-chloroadenosine (7). Nucleoside **6** (0.241 g, 0.552 mmol) was co-evaporated with anhydrous THF (2 × 5 mL), then dried in vacuo for 12 h. The dried starting material was dissolved in 1.4 mL anhydrous pyridine and 4,4-dimethylaminopyridine (DMAP, 0.0120 g, 0.16 mmol) was added. The reaction was stirred for 5 min, then 4,4′-dimethoxytritylchloride (DMTCl, 0.387 g, 1.14 mmol) was added in three equal portions over 3 h, and the reaction was stirred for 16 h. The reaction was quenched with 2 mL saturated aqueous $NaHCO_3$, 5 mL H_2O, and 5 mL CH_2Cl_2. The layers were separated and the aqueous layer was back-extracted with CH_2Cl_2 (4 × 5 mL). The combined CH_2Cl_2 layers were washed successively with 10 mL each of 10% aqueous $NaHCO_3$, H_2O, and brine. The organic layer then was dried over Na_2SO_4, filtered, and concentrated in vacuo to a brown oil. The residue was coevaporated with 10 mL toluene and purified by column chromatography (5% MeOH/$CHCl_3$) to give 0.238 g (58% yield) of golden foam.[7] ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.08 (s, 1H, NH), 8.57 (s, 1H, H2), 7.29 (m, 4H, DMT, O-Ph), 7.15 (m, 7H, DMT), 6.96 (m, 3H, O-Ph), 6.78 (m, 4H, DMT), 5.97 (d, 1H, $J_{1',2'} = 4.5$ Hz, H1′), 5.59 (d, 1H, $J_{2',2'OH} = 4.5$ Hz, 2′OH), 5.30 (d, 1H, $J_{3',3'OH} = 5.5$ Hz, 3′OH), 5.25 (q, 1H, $J_{2',2'OH} = 4.5$ Hz, $J_{2',3'} = 10$ Hz, H2′), 5.01 (s, 2H, -C*H*$_2$-OPh), 4.54 (q, 1H, $J_{3',3'OH} = 5.5$ Hz, $J_{2',3'} = 10$ Hz, H3′), 4.11 (q, 1H, $J_{3',4'} = 9.5$ Hz, $J_{4',5'} = 4.75$ Hz, H4′), 3.67 (s, 6H, -OCH$_3$), 3.25 (dd, 1H, $J_{4',5'a} = 3.5$ Hz, $J_{5'a,5'b} = 10.5$ Hz, H5′a), 3.16 (dd, 1H, $J_{4',5'b} = 5.5$ Hz, $J_{5'a,5'b} = 10.5$ Hz, H5′b). HRMS-FAB (m/z): [M + Na]$^+$ calcd, 760.2149; found, 760.2147.

2′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-dimethoxytrityl-*N*-6-phenoxyacetyl-8-chloroadenosine (8). Compound **7** (0.595 g, 0.806 mmol) was coevaporated with anhydrous THF (3 × 2 mL) and dried in vacuo for 12 h. The starting material then was dissolved in 8 mL anhydrous, THF, and subsequently, anhydrous pyridine (0.240 mL, 2.98 mmol) and Et$_3$N (0.110 mL, 0.806 mmol) were added by syringe. Silver nitrate (0.164 g, 0.967 mmol) was added, and the reaction was stirred at 25°C for 15 min in the absence of light. *tert*-Butyldimethylsilylchloride (TBDMSCl, 0.158 g, 1.04 mmol) then was added, and the reaction was stirred for 2.5 h. Then, Et$_3$N (0.17 mL, 1.5 equiv), AgNO$_3$ (0.137 g, 1 equiv), and TBDMS-Cl (0.103 g, 0.85 equiv) were added sequentially, and the reaction was stirred for another 20 min. The reaction was diluted with 15 mL CH_2Cl_2 and filtered through Celite. The Celite was rinsed with 50 mL CH_2Cl_2 and the combined CH_2Cl_2 layers were washed with a 5% aqueous NaHCO$_3$ solution (2 × 25 mL). The aqueous layer was back-extracted with CH_2Cl_2 (2 × 25 mL), and the combined organics were dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to a yellow oil. The product was purified by column chromatography (3% EtOAc/$CHCl_3$) to give (**8**) (0.110 g, 16% yield) and (**9**) (0.259 g, 38% yield) as pale yellow foams. Compound (**8**): ^{1}H NMR (DMSO-d_6) δ: 11.10 (s, 1H, NH), 8.54

(s, 1H, H2), 7.30 (m, 4H, DMT, O-Ph), 7.20 (m, 7H, DMT), 6.96 (m, 3H, O-Ph), 6.79 (m, 4H, DMT), 5.96 (d, 1H, $J_{1',2'} = 6.5$ Hz, H1'), 5.29 (t, 2H, $J_{1',2'} = 6.5$ Hz, $J_{2',3'} = 6$ Hz, H2', 3'OH), 5.00 (s, 2H, -CH_2-OPh), 4.45 (q, 1H, $J_{2',3'} = 6$ Hz, $J_{3',4'} = 10.5$ Hz, H3'), 4.13 (q, 1H, $J_{3',4'} = 10.5$ Hz, $J_{4',5'} = 5.5$ Hz, H4'), 3.69 (s, 6H, -OCH_3), 3.29 (dd, 1H, $J_{4',5'a} = 5$ Hz, $J_{5'a,5'b} = 10.5$ Hz, H5'a), 3.18 (dd, 1H, $J_{4',5'b} = 6$ Hz, $J_{5'a,5'b} = 10.5$ Hz, H5'b), 0.725 (s, 9H, t-Bu), -0.05 (s, 3H, Si-CH_3), -0.18 (s, 3H, Si-CH_3). ^{13}C NMR (DMSO-d_6) δ: 158.7, 158.5, 152.7, 152.6, 149.1, 149.0, 145.6, 142.0, 140.9, 136.2, 136.2, 130.4, 130.3, 130.2, 129.6, 128.4, 128.3, 128.1, 127.3, 127.1, 122.6, 121.8, 115.2, 113.7, 113.5, 90.2, 86.0, 84.6, 80.6, 79.87, 73.3, 70.6, 67.8, 63.5, 26.2, 18.5, -4.1, -4.7. HRMS-FAB (m/z): [M + H]$^+$ calcd, 852.3195; found, 852.3195.

3'-*O*-*tert*-Butyldimethylsilyl-5'-*O*-dimethoxytrityl-*N*-6-phenoxyacetyl-8-chloroadenosine (9).

Isolated from synthesis of **8**. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.11 (s, 1H, NH), 8.65 (s, 1H, H2), 7.29 (t, 2H, $J = 6.4$ Hz, O-Ph), 7.18 (m, 4H, DMT), 7.06 (m, 4H, DMT), 6.95 (m, 3H, O-Ph), 6.75 (m, 3H, DMT), 5.93 (d, 1H, $J_{1',2'} = 3$ Hz, H1'), 5.53 (d, 1H, $J_{2',2'OH} = 4.4$ Hz, H2'OH), 5.34 (d, 1H, $J_{1',2'} = 3$ Hz H3'), 4.99 (s, 2H, -CH_2-OPh), 4.90 (t, 1H, $J_{3',4'} = 4$ Hz, H3'), 4.07 (d, 1H, $J_{3',4'} = 4$ Hz, H4'), 3.69 (s, 3H, -$OCHa_3$), 3.68 (s, 3H, O-CHb_3), 2.93 (dd, 1H, $J_{4',5'a} = 2.6$ Hz, $J_{5'a,5'b} = 10.25$ Hz, H5'a), 0.83 (s, 9H, t-Bu), 0.11 (s, 3H, Si-CHa_3), 0.07 (s, 3H, Si-CHb_3). HRMS-FAB (m/z): [M + H]$^+$ calcd, 852.3195; found, 852.3195.

2'-*O*-*tert*-Butyldimethylsilyl-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-5'-*O*-dimethoxytrityl-*N*-6-phenoxyacetyl-8-chloroadenosine (10).

Nucleoside **8** (0.108 g, 0.127 mmol) was coevaporated with anhydrous THF (2×2 mL), dried in vacuo, and dissolved in 0.39 mL anhydrous THF. 2,4,6-Collidine (0.110 mL, 0.954 mmol) and *N*-methylimidazole (0.005 mL, 0.064 mmol) were added by syringe, followed by dropwise addition of *N*,*N*-diisopropylaminocyanoethylphosphoramidic chloride (0.071 mL, 0.318 mmol) . The reaction was stirred at 25°C for 4 h, then diluted with 5 mL EtOAc, and washed sequentially with 5 mL of cold 5% aqueous NaHCO$_3$ solution, and 5 mL of H$_2$O. The organic layer was dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to a yellow oil. The products were purified by column chromatography (5% hexanes/10% EtOAc/CH$_2$Cl$_2$) to give 0.067 g (50% yield) of isomers **10a** and **10b**. HRMS-FAB (m/z): [M + H]$^+$ calcd, 1052.4274; found, 1052.4278. Compound **10a**. ^{1}H NMR (CD$_3$CN) δ: 9.23 (s, 1H, NH), 8.50 (s, 1H, H2), 7.28 (m, 11H, DMT, O-Ph), 7.03 (m, 3H, O-Ph), 6.79 (m, 4H, DMT), 6.00 (d, 1H, $J_{1',2'} = 5$ Hz, H1'), 5.55 (t, 1H, $J = 5$ Hz, H2'), 4.96 (s, 2H, -CH_2-OPh), 4.73 (m, 1H, $J_{3',4'} = 4.4$ Hz, $J = 10$ Hz, H3'), 4.43 (q, 1H, $J_{3',4'} = 4.4$ Hz, H4'), 3.76 (s, 6H, -OCH_3), 3.68 (m, 4H, O-CH_2CH$_2$-CN, N-CH(CH$_3$)$_2$), 3.51 (dd, 1H, $J_{4',5'a} = 4$ Hz, $J_{5'a,5'b} = 10$ Hz, H5'a), 3.30 (dd, 1H, $J_{4',5'b} = 4.8$ Hz, $J_{5'a,5'b} = 10$ Hz, H5'b), 2.47 (td, 2H, $J = 2$ Hz, 6 Hz,

O-CH$_2$CH$_2$-CN), 1.22 (d, 6H, J = 6.8 Hz, i-Pra), 1.20 (d, 6H, J = 6.4 Hz, i-Prb), 0.80 s 9H, t-Bu), 0.01 (s, 3H, Si-CHa$_3$), − 0.16 (s, 3H, Si-CHb$_3$). ^{13}C NMR (CD$_3$CN) δ: 167.11, 158.87, 158.84, 157.87, 152.40, 152.19, 148.03, 145.42, 142.50, 136.30, 136.16, 130.32, 130.24, 129.96, 128.25, 127.96, 127.0, 122.06, 118.50, 115.00, 113.20, 113.18, 89.97, 86.22, 83.93, 72.89, 72.81, 72.39, 68.19, 43.50, 43.40, 43.06, 25.53, 25.27, 24.49, 24.43, 24.19, 24.14, 20.25, 20.20, 17.80, − 4.97, − 5.63. ^{31}P NMR (CD$_3$CN) δ: 151.19. Compound **10b.** ^{1}H NMR (400 MHz, CD$_3$CN) δ: 9.21 (s, 1H, NH), 8.45 (s, 1H, H2), 7.28 (m, 11H, DMT, O-Ph), 7.02 (m, 3H, O-Ph), 6.79 (m, 4H, DMT), 6.00 (d, 1H, $J_{1',2'}$ = 6 Hz, H1′), 5.44 (t, 1H, J = 5 Hz, H2′), 4.94 (s, 2H, -CH$_2$-OPh), 4.64 (td, 1H, J = 4 Hz, H3′), 4.34 (s, 1H, J = 3.2 Hz, H4′), 3.73 (s, 6H, -OCH$_3$), 3.66 (m, 4H, O-CH$_2$CH$_2$-CN, N-CH(CH$_3$)$_2$), 3.49 (m, 1H, H5′a), 3.38 (m, 1H, H5′b), 2.68 (m, 2H, O-CH$_2$CH$_2$-CN), 1.18 (d, 6H, J = 7 Hz i-Pra), 1.10 (d, 6H, J = 7 Hz, i-Prb), 0.75 (s, 9H, t-Bu), − 0.04 (s, 3H, Si-CHa$_3$), − 0.25 (s, 3H, Si-CHb$_3$). ^{31}P NMR (CD$_3$CN) δ: 149.34.

2′-O-Succinyl-3′-O-*tert*-butyldimethylsilyl-5′-O-dimethoxytrityl-N-6-phe-noxyacetyl-8-chloroadenosine (11). To a stirred solution of **9** (0.177 g, 0.207 mmol) dissolved in 0.90 mL anhydrous pyridine was added DMAP (0.0165 g, 0.135 mmol) and succinic anhydride (0.0332 g, 0.332 mmol) . The solution was stirred under N$_2$ for 12 h, then diluted with 5 mL EtOAc, and washed with 5 mL H$_2$O. The aqueous layer was back-extracted with EtOAc (2 × 5 mL), then the combined organics were washed sequentially with 10 mL satd. aqueous KH$_2$PO$_4$ (pH = 4.13), 10 mL brine, and dried over Na$_2$SO$_4$. The organic layer was concentrated in vacuo to an orange oil, and the product was purified by column chromatography (5% MeOH/CHCl$_3$) to give **11** (0.140 g, 71% yield). ^{1}H NMR (400 MHz, acetonitrile-d_3) δ: 9.25 (s, 1H, NH), 8.76 (s, 1H, H2), 8.759 (s,1H, OH), 7.35 (m, 3H, O-Ph), 7.11 (m, 11H, DMT, O-Ph), 6.75 (m, 4H, DMT, O-Ph), 6.51 (dd, 1H, $J_{1',2'}$ = 1.6 Hz, $J_{2',3'}$ = 5 Hz, H2′), 6.12 (d, 1H, $J_{1',2'}$ = 1.6 Hz, H1′), 5.40 (dd, 1H, $J_{2',3'}$ = 5 Hz, $J_{3',4'}$ = 7.6 Hz, H3′), 4.94 (s, 2H, -CH$_2$-OPh), 4.12 (m, 1H, H4′), 3.75 (s, 3H, -OCHa$_3$), 3.74 (s, 3H, O-CHb$_3$), 3.48 (dd, 1H, $J_{4',5'a}$ = 2.4 Hz, $J_{5'a,5'b}$ = 11 Hz, H5′a), 3.02 (dd, 1H, $J_{4',5'a}$ = 3.4 Hz, $J_{5'a,5'b}$ = 11 Hz, H5′b), 0.84 (s, 9H, t-Bu), 0.15 (s, 3H, Si-CH$_3$), 0.06 (s, 3H, Si-CH$_3$). HRMS-FAB (m/z): [M + Na]$^+$ calcd, 974.3175; found, 974.3174.

Controlled Pore Glass Linked 2′-O-Succinyl-3′-O-*tert*-Butyldimethylsilyl-5′-O-dimethoxytrityl-N-6-phenoxyacetyl-8-chloroadenosine (12). Succiny-lated nucleoside **11** (0.128 g, 0.134 mmol) was dissolved in 0.67 mL anhydrous 1,4-dioxane. To this solution was added anhydrous pyridine (0.028 mL, 0.348 mmol), *p*-nitrophenol (0.021 g, 0.148 mmol), and dicyclohex-ylcarbodiimide (0.083 g, 0.402 mmol) . The solution was stirred for 30 min under N$_2$, and the resulting precipitate was filtered. Then, 1000 Å LCAA-cpg (0.500 g, 54 μmol amino groups) was suspended in 3 mL anhydrous DMF and

0.20 mL Et$_3$N, and the filtrate solution was added. The mixture was shaken for 12 h, and the CPG was filtered and washed with 5 mL each of DMF, MeOH and Et$_2$O, and dried in vacuo. The underivatized amino groups were capped by placing the CPG in a solution containing 4.5 mL anhydrous pyridine, acetic anhydride (0.5 mL, 4.5 mmol), and DMAP (0.055 g, 0.45 mmol). The solution was shaken for 12 h, then the CPG was rinsed with 5 mL each of DMF, MeOH, and Et$_2$O. The final loading of 22 μmol/g was determined by UV quantitation of dimethoxytrityl cation release.

RNA Synthesis, Deprotection, and Purification

All unmodified RNA oligonucleotides were purchased from Dharmacon Research Inc. (Boulder, CO) and were deprotected at 37°C for 30 min with 400 μL of 100 mM acetic acid, adjusted to pH 3.8 with tetramethylethylene-diamine (TEMED). Modified oligonucleotides were synthesized by automated methods on a Pharmacia Gene Assembler on a 1.0 μmol scale using RNA phosphoramidites obtained from Glen Research (Sterling, VA) with labile base protecting groups for the natural nucleosides (Pac-A-CE, Ac-C-CE, *i*Pr-Pac-G-CE, U-CE). Phosphoramidites **10a** and **10b** were coupled using 0.1 M anhydrous CH$_3$CN solutions (coupling time, 10 min). After synthesis, the oligonucleotide was deprotected by treatment with 1 mL 1:3 EtOH/NH$_4$OH for 20 h at 37°C. The EtOH/NH$_4$OH solution was removed using a Speedvac concentrator and the resulting powder was desilylated with 600 μL of 1.0 M TBAF in THF for 24 h at 25°C. The product was dried using a Speedvac concentrator, redissolved in 500 μL double-distilled water (ddH$_2$O), and desalted using a G-25 Sephadex column (NAP, Amersham). The coupling yield for **10** was 99%, as determined by dimethoxytrityl cation release. Synthesis of 3′–terminally modified sequences using solid support **12** proceeded with a 77% coupling yield for the first 3′–nucleotide, and with a yield of 91–96% for subsequent bases. RNA oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE) as described.[38] Purified oligonucleotide solutions were quantitated by UV absorption at 260 nm using extinction coefficients based on nearest-neighbors method.[39] The extinction coefficient of 8-chloroadenosine was assumed to be equal to adenosine.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry

8-Cl-Ado-modified and unmodified RNA oligonucleotides were characterized using a PerSeptive Biosystems, Inc. (Foster City, CA), Voyager-DE™ PRO Biospectrometry™ Workstation MALDI-TOF mass spectrometer. A N$_2$ laser was used (337 nm wavelength, 3 ns pulse), and spectra were

acquired in the negative ion mode averaging 128 shots. A matrix consisting of 2,4,6-trihydroxyacetophenone/ammonium citrate in CH_3CN/H_2O was used, and poly$(dT)_{10}$ and poly$(dT)_{18}$ sequences obtained from Integrated DNA Technologies were used for internal calibration. All masses found were in agreement with calculated masses. 5′-UGU GCC CGA^{Cl} CUU GCC GU-3′ (13): m/z [M]$^-$ calcd, 5387.6; found, 5386.8. 5′-UGU GCC AGA^{Cl} CUU GCC GU-3′ (21): m/z [M]$^-$ calcd, 5411.6; found, 5411.0. 5′-UGU GCC CGA^{Cl}-3′ (24): m/z [M]$^-$ calcd, 2865.2; found, 2865.6. 5′-AGU GCA CGU UAC A^{Cl}GC CG-3′ (27): m/z [M]$^-$ calcd, 5458.7; found, 5458.0. 5′-AGU GCA^{Cl} CGU UAC A^{Cl}GC CG-3′ (28): m/z [M]$^-$ calcd, 5493.2; found, 5493.4.

Circular Dichroism Spectroscopy

CD spectra were recorded using a JASCO J-715 CD spectropolarimeter. Oligonucleotide duplex samples containing 2 μM of each strand were prepared in 10 mM sodium phosphate, 0.1 mM EDTA, 150 mM NaCl, pH = 7.0. Samples were annealed by heating to 95°C for 3 min, followed by gradual cooling to 25°C over 2 h. Spectra were recorded from 200 to 350 nm at 25°C in a 2 mm cuvette. Each spectrum represents a baseline corrected average of four accumulations per scan. All data were corrected by subtracting the buffer reference signal.

UV Thermal Denaturation Analysis

Thermal denaturation data were acquired on a Cary 500 spectrophotometer equipped with a multi-cuvette thermoelectric controller in 10 mm quartz cuvettes. Solutions of 2 μM duplex oligonucleotides in pH 7.0 buffer consisting of 10 mM sodium phosphate, 0.1 mM EDTA, 150 mM NaCl were annealed by heating to 95°C for 5 min and cooling to 25°C over 2 h. The samples were degassed under vacuum for 3 min prior to melting analysis. The absorbance was measured at 260 nm at 0.1°C intervals as the samples were heated from 30°C to 90°C at 0.5°C/min. Data sets for the downward transition from 90°C to 30°C were also collected at 0.5°C/min; all melting profiles were fully reversible to within 1.0°C. Melting temperatures (T_m's) were determined from plots of dA_{260}/dT vs. T as described[40], and are derived from replicate melting experiments using three independent samples.

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