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# UNA (unlocked nucleic acid): A flexible RNA mimic that allows engineering of nucleic acid duplex stability

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ABSTRACT

UNA (unlocked nucleic acid) monomers are acyclic derivatives of RNA lacking the C2′–C3′-bond of the ribose ring of RNA. Synthesis of phosphoramidite UNA building blocks of the nucleobases adenine, cytosine, guanine, and uracil is described herein together with their incorporation into RNA strands. UNA monomers additively decrease nucleic acid duplex stability and can be positioned strategically to induce either lack of discrimination of mismatches, that is, universal base behavior, or increased discrimination of mismatches, that is, improved hybridization specificity. UNA-modified RNA duplexes are shown to structurally mimic unmodified RNA duplexes by CD spectroscopy.



## 1. Introduction

Chemical modification of the natural nucleotide monomers has been widely explored to engineer nucleic acid binding affinity and biological activity of synthetic oligonucleotides (ONs).[1–4] LNA (locked nucleic acid)[5–9] monomers (Fig. 1) are well known for their affinity increasing effect, and short LNA-containing probes for their excellent binding specificity, that is, capability to discriminate effectively between fully matched and singly mismatched targets.[10] LNA-modified duplexes formed with RNA complementary strands are of the A-type and structurally very similar to the corresponding unmodified RNA duplexes as has been shown by CD and NMR spectroscopy.[11,12]

UNA (unlocked nucleic acid) is an acyclic analogue of RNA in which the bond between the C2′ and C3′ atoms of the ribose ring has been cleaved (Fig. 1). In 1995 we introduced the thymine UNA monomer as a modification in DNA oligonucleotides together with a method for synthesis of the corresponding phosphoramidite derivative.[13] Since then, UNA monomers have been incorporated into DNA-type oligonucleotides using O2′-silylated phosphoramidites.[14] Furthermore UNA-adenylate trimers were synthesized and shown to be stable against phosphordiesterases.[15,16] It should be noted that seco-RNA has been used as a term to describe various acyclic forms of RNA and 2′,3′-seco-RNA as a term to describe what herein is termed as UNA.[13,14] Along with many other acyclic nucleotide modifications, UNA was shown to induce decreased binding affinity towards a complementary DNA strand,[13] but UNA-modified RNA strands or duplexes were not described. UNA has been explored as a constituent in the central DNA segment of gap-mer antisense oligonucleotides, and compatibility with RNase H recognition and RNA cleavage was reported.[14]

Both UNA and LNA can be described as RNA analogues. However, whereas the additional methylene unit linking the O2′ and C4′ atoms of LNA monomers locks the furanose ring into a C3′-*endo* conformation, the acyclic nature of UNA renders this molecule very flexible. Therefore LNA and UNA can be considered ‘locked RNA’ and ‘unlocked RNA’, respectively. These antipodal structural characteristics make UNA and LNA complementary with respect to effect on binding affinity towards DNA targets.[5–7,13]

We have initiated a program to broadly evaluate the influence of UNA monomers on hybridization thermodynamics and nucleic acid duplex structures, and to explore their use within biotechnology. Herein we introduce the synthesis of O2′-benzoylated UNA phosphoramidite derivatives of all four standard RNA nucleobases

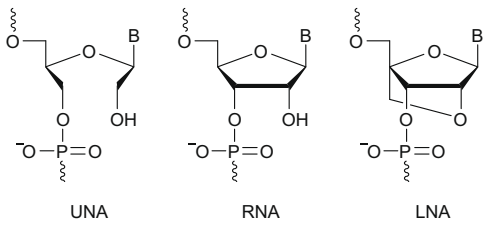


**Figure 1.** Structure of UNA (‘unlocked RNA’), RNA, and LNA (‘locked RNA’) monomers.

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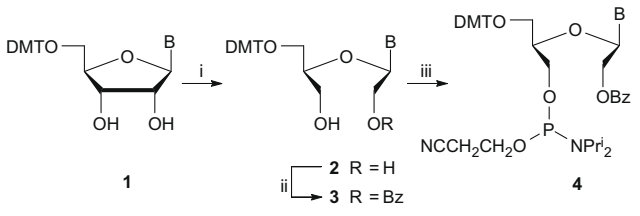
and demonstrate their full compatibility with automated RNA synthesis. We reveal that UNA-modified RNA:RNA duplexes retain A-type structures and that strategic UNA modification of 21-mer RNA strands allow predictable decrease of RNA:RNA or RNA:DNA duplex stabilities and wide-spectrum modulation of hybridization specificity.

## 2. Results and discussion

UNA phosphoramidites were synthesized by an optimized version of the published procedure[13] for synthesis of the thymine monomer. O5′-DMT protected ribonucleosides **1** were treated with sodium periodate to induce oxidative diol cleavage of the bond linking the C2′ and C3′ atoms of the ribose ring. Subsequent reduction by sodium borohydride yielded the acyclic nucleosides **2**. Selective benzoylation of O2′ was optimized relative to the previously published procedure for the thymine derivative by replacing the solvent and lowering the reaction temperature (see caption below Scheme 1). O3′-Benzoylated by-products were detected by analytical TLC, but these did not pose any significant problem during column chromatographic purification as they were formed in only minor amounts. The resulting O2′-benzoylated acyclic nucleosides **3** were eventually converted into the phosphoramidite building blocks **4** by standard phosphitylation (Scheme 1).

The phosphoramidites **4** were used on an automated nucleic acid synthesizer to obtain UNA-modified RNA oligonucleotides **ON2**–**ON5**, **ON7** and **ON8** (Table 1). Standard conditions for RNA (O2′-TBDMS method) synthesis were used, and the stepwise coupling yield of unmodified RNA as well as UNA monomers was >99%. Following standard deprotection, purification and work-up, the composition of the synthesized ONs was confirmed by MALDI-MS analysis and the purity (>80%) was confirmed by ion-exchange HPLC. It is noteworthy that the incorporation of UNA monomers proved to be fully compatible with RNA synthesis and cleavage conditions, and that no strand cleavage was observed despite the O2′-acyl protecting group applied on the UNA monomers. The latter observation can be explained by the flexible structure of UNA monomers leaving no structural preorganization of the deprotected 2′-OH group for nucleophilic attack on the O3′-phosphate moiety.

The influence of UNA monomers on the thermal stability of different duplexes was studied using UV spectroscopy and the duplex melting temperatures ($T_m$ values) were determined at the maximum of the first derivative of the thermal denaturation curves (Table 1).[17] Incorporation of a single UNA monomer into a central position of 21-mer RNA strands (**ON2**, **ON3**, and **ON7**) induced decreases in thermal stability by 5–8 °C for fully complementary duplexes towards DNA or RNA complements. The affinity decreasing effect is slightly less pronounced than for UNA-modified DNA 17-mers when hybridized towards a DNA complement.[13]




**Scheme 1.** Reagents and conditions (yields): (i) (a) $NaIO_4$, 1,4-dioxane/water, (b) $NaBH_4$, 1,4-dioxane/water [$\mathbf{A}^{Bz}$: 86%, $\mathbf{C}^{Ac}$: 71%, $\mathbf{G}^{iBu}$: 68%, U: 82%]; (ii) BzCl, 2 equiv DBU (for U: 10 equiv pyridine), DCM, −70 °C [$\mathbf{A}^{Bz}$: 73%, $\mathbf{C}^{Ac}$: 64%, $\mathbf{G}^{iBu}$: 63%, U: 79%]; (iii) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, DIPEA, acetonitrile [$\mathbf{A}^{Bz}$: 71%, $\mathbf{C}^{Ac}$: 44%, $\mathbf{G}^{iBu}$: 78%, U: 68%]. The following protected base derivatives were applied in compounds **1**–**4**: $\mathbf{A}^{Bz}$ = 6-*N*-benzoyladenin-9-yl, $\mathbf{C}^{Ac}$ = 4-*N*-acetylcytosin-1-yl, $\mathbf{G}^{iBu}$ = 2-*N*-isobutyrylguanin-9-yl, U = uracil-1-yl, DMT = 4,4′-dimethoxytrityl.

Relative to the corresponding unmodified duplexes, our preliminary thermodynamic analysis indicate favorable entropy changes and unfavorable enthalpy changes for duplex formation in the presence of one UNA monomer.[18] These results may seem surprising in light of the flexible noncyclic structure of UNA monomers though the examples of entropically disfavored duplex formation have been reported for the conformationally locked LNA monomers.[19,20] However, whereas a favorable enthalpy change renders the formation of LNA-containing duplexes overall more energetically favorable than the formation of unmodified duplexes, our thermodynamic data confirm overall energetically more unfavorable formation for UNA-containing duplexes in line with the decreased $T_m$ values observed.

We studied the discrimination of mismatches for the singly UNA-modified RNA strands **ON2**, **ON3**, and **ON7** against a mismatch at the central position no. 11 (Table 1; $\Delta T_m$ values caused by the presence of a mismatch are shown in brackets). With the mismatches positioned directly opposite to a UNA monomer (UNA-G in **ON2** and UNA-A in **ON7**) only modest (**ON2**) or negligible (**ON7**) discrimination was observed. These data reflect the relatively weaker base pairing of UNA relative to RNA monomers, but it should be noted that the data implicate Watson–Crick base pairing for UNA monomers. Mismatch discrimination only slightly less than for the unmodified RNA strand (**ON1**) was observed for **ON3** with mismatches neighboring the UNA-U monomer in position 10. This demonstrates that the flexibility of a UNA base is only to a very limited extent transmitted to a neighboring base positioned at the 3′-side of a UNA monomer.

We next determined $T_m$ values of duplexes formed between 21-mer RNA strands containing two UNA monomers (**ON4**, **ON5**, and **ON8**) and DNA/RNA targets (Table 1). For the fully matched duplexes, decreases in thermal denaturation temperatures of 7–12 °C per UNA modification were observed. The stability-decreasing effect of a UNA monomer is thus approximately additive for two incorporations and independent of the nature of the complementary strand.

**ON5** and **ON8** are UNA-modified RNA strands having four RNA monomers positioned between each of the two UNA monomers and the central RNA-G monomer ('distal UNA design'), whereas the two UNA monomers of **ON4** are neighboring the central RNA-G monomer ('proximity UNA design'). These designs can be used to modulate hybridization specificity of UNA-modified RNA strands relative to unmodified reference strands (here **ON1** and **ON6**). **ON4** reveals that lack of mismatch discrimination can be induced by the 'proximity UNA design'. This design is thus an alternative to the so-called universal bases, which in most cases do not induce such thermally uniform hybridization.[21–25] It is likely the flexibility of the UNA monomers, and the associated decrease in stability centrally in the duplexes, which renders mismatched pairing at the central RNA-G monomer of no importance for overall duplex stability. On the contrary, for the 'distal UNA-design',[26] improved mismatch discrimination was observed for all mismatches against both DNA and RNA complements.[17] The observed improvements in mismatch discrimination are interesting, and we consider this novel 'distal UNA design' an advancement towards enabling high base pairing specificity in the central segment of ~20-mer long oligonucleotides. This design originates from the assumption that the introduction of local flexibility, and thus local duplex destabilization, may lead to superior ability to sense the presence of a single mismatch at a predesigned position of an ON because of what can be described as cooperativity between mismatches and sites of low duplex stability.

The nearly identical CD spectra of the UNA-modified RNA duplexes **ON3**:RNA and **ON4**:RNA and the unmodified **ON1**:RNA duplex are in the 200–310 nm range (Fig. 2) and reveal that one or two UNA monomers leave the bulk part of the overall helical

**Table 1**
Thermal stability of RNA:DNA and RNA:RNA duplexes containing one or two UNA monomers and effects of mismatches[a]

| RNA strand | $T_m$ value (°C) | |
|---|---|---|
| | DNA complement | RNA complement |
| 5′-UGC ACU GUA UGU CUG UAC CAU (**ON1**) | 58 [−11, −9, −5] | 71 [−10, −8, −6] |
| 5′-UGC ACU GUA U**G**U CUG UAC CAU (**ON2**) | 50 [−4, −2, −3] | 64 [−4, −2, −3] |
| 5′-UGC ACU GUA **U**GU CUG UAC CAU (**ON3**) | 51 [−6, −7, −5] | 66 [−8, −7, −6] |
| 5′-UGC ACU GUA **UGU** CUG UAC CAU (**ON4**) | 41 [−1, ±0, −1] | 56 [−1, −1, −1] |
| 5′-UGC AC**U** GUA UGU CUG **U**AC CAU (**ON5**) | 41 [−17, −13, −7] | 47 [−12, −10, −9] |
| 5′-ACU UGU GGC CAU UUA CGU CGC (**ON6**) | 61 [−6, −7, −6] | 74 [−6, −6, −6] |
| 5′-ACU UGU GGC C**A**U UUA CGU CGC (**ON7**) | 53 [±0, −2, −1] | 66 [−1, −1, −1] |
| 5′-ACU UG**U** GGC CAU UUA **C**GU CGC (**ON8**) | 41 [−12, −10, −10] | 57 [−7, −7, −7] |

[a] Thermal denaturation temperatures ($T_m$ values/°C) of unmodified or modified RNA duplexes measured as the maximum of the first derivative of the melting curve ($A_{260}$ vs temperature) recorded in medium salt buffer (100 mM NaCl, 0.1 mM EDTA, 10 mM $NaH_2PO_4$, 5 mM $Na_2HPO_4$, pH 7.0), using 1.0 μM concentrations of the two complementary strands. $T_m$ values are average of at least two measurements. $\Delta T_m$ values for mismatches at position 11 (central G or A monomer) of the RNA strands **ON1**–**ON5** are shown in brackets; the $\Delta T_m$ values in brackets are shown in the following order: [G:A mismatch, G:G mismatch and G:T/U mismatch or A:A mismatch, A:C mismatch, and A:G mismatch] and are calculated relative to the fully matched duplexes (G:C or A:T/U match). A = adenin-9-yl monomer, C = cytosin-1-yl monomer, G = guanine-9-yl monomer, U = uracil-1-yl monomer; UNA monomers are bold letters.

geometry unperturbed. All the three CD spectra show the characteristic features of an A-type helical environment with negative bands of rather low intensities at ~210 and ~235 nm, and a positive band of strong intensity at ~265 nm. The intensity of the bands is only slightly less intense for UNA-modified RNA duplexes. These spectra reveal that a UNA monomer structurally can be considered as an RNA mimic, which suggests applicability of UNA-modified RNA ONs for biological and biotechnological applications. One such application is UNA-modified siRNA for which highly potent silencing with improved selectivity has been recently reported.[27]

## 3. Conclusion

Synthesis of UNA phosphoramidite derivatives suitable for highly efficient incorporation of UNA monomers of the four natural bases into RNA strands has been accomplished. UNA monomers destabilize duplexes formed between UNA-modified RNA strands and complementary RNA or DNA, and two UNA monomers can be used to decrease or increase mismatch discrimination depending on their sites of incorporation. UNA has herein been introduced as an RNA mimicking novel tool for engineering of nucleic acid duplex stability and base pairing specificity.

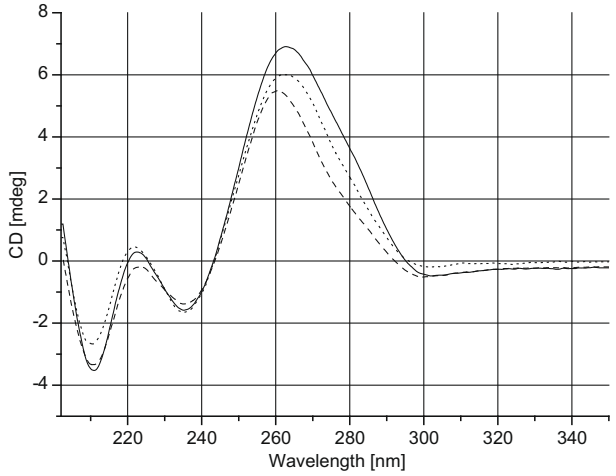



**Figure 2.** CD spectra of unmodified (**ON1**:RNA, solid line) and UNA-modified RNA duplexes (**ON3**:RNA, dashed line; **ON4**:RNA, dotted line).

## 4. Experimental

### 4.1. General

Reactions under anhydrous conditions were carried out under an atmosphere of nitrogen. After column chromatographic purifications, the fractions containing product were pooled and evaporated to dryness under reduced pressure. After drying organic phases over $Na_2SO_4$, filtration was performed. Solvents were of HPLC grade, of which acetonitrile, DCM and pyridine were dried over molecular sieves (4 Å). TLC was run on Merck Silica 60 $F_{254}$ aluminum sheets. $^1$H NMR spectra were recorded at 300 MHz, $^{13}$C NMR spectra at 75.5 MHz and $^{31}$P NMR spectra at 121.5 MHz ($\delta$H: DMSO-$d_6$ 2.50; $\delta$C: DMSO-$d_6$ 39.4). Chemical shifts are reported in ppm relative to either tetramethylsilane or the deuterated solvent as a internal standard for $^1$H NMR and $^{13}$C NMR, and relative to 85% $H_3PO_4$ as an external standard for $^{31}$P NMR. Assignments of NMR spectra, when given, are based on 2D NMR experiments (the assignments of methylene protons/methylene carbons may be interchanged). Coupling constants ($J$ values) are given in Hz. ESI-HRMS and MALDI-HRMS were recorded in positive ion mode on an Ion Spec Fourier transform mass spectrometer.

### 4.2. 5′-*O*-(4,4′-Dimethoxytrityl)-2′,3′-secouridine (2U)[28]

5′-*O*-(4,4′-Dimethoxytrityl)uridine (**1U**, 10.35 g, 18.94 mmol) was at rt dissolved in a stirred mixture of dioxane (250 ml) and water (50 ml). To this solution was added $NaIO_4$ (4.47 g, 20.90 mmol) dissolved in water (50 ml). The resulting mixture was stirred for 1 h during which time a white precipitate was formed. Additional dioxane (200 ml) was added and the suspension was stirred for additional 15 min, whereupon the suspension was filtered through a glass filter and the filter cake was washed with dioxane (100 ml). The filtrates were combined, $NaBH_4$ (797 mg, 21.1 mmol) was added, and the resulting mixture was stirred for 30 min. The mixture was neutralized by the addition of buffer (pyridine:AcOH 1:1, v/v, ~10 ml). After evaporation of the resulting mixture to a volume of approximately 150 ml, DCM (100 ml) was added and the mixture was washed with sat. aq. $NaHCO_3$ (2 × 100 ml). The organic phase was separated and evaporated to dryness under reduced pressure. The crude product was purified by column chromatography (40% acetone in petroleum ether) affording the desired nucleoside **2U** as a white foam (8.53 g, 82%). $R_f$: 0.2 (10% MeOH in DCM). $^1$H NMR (DMSO-$d_6$): $\delta$ 11.34 (br s, NH), 7.62 (d, 1H, $J$ = 8.0, H6), 7.34–7.15 (m, 9H, Ar), 6.85 (d, 4H, $J$ = 8.0, Ar), 5.80 (t, 1H, $J$ = 5.8, H1′), 5.52 (d, 1H, $J$ = 8.0, H5), 5.12 (t, 1H, $J$ = 5.9, 2′OH), 4.74 (t, 1H, $J$ = 5.4, 3′OH), 3.72 (s, 6H, $OCH_3$), 3.55–3.47 (m, 3H, H2′/H4′), 3.40 (t, 2H, $J$ = 5.2,

H3′), 3.01–2.90 (m, 2H, H5′). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 163.2, 157.9, 151.4, 144.8, 141.1 (C6), 135.4, 129.5, 127.7, 127.5, 126.5, 113.0, 101.6 (C5), 83.6 (C1′), 79.3 (C2′/C4′), 63.5 (C5′), 61.1 (C2′/C4′), 60.5 (C3′), 54.9 ($OCH_3$). ESI-MS (M+Na$^+$): $m/z$ 571.2; calcd: 571.2051.

### 4.3. 2′-*O*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secouridine (3U)[28]

Nucleoside **2U** (3.01 g, 5.50 mmol) was coevaporated with anhydrous toluene (15 ml) and dried for 12 h in vacuo. The resulting residue was dissolved in anhydrous DCM (150 ml) along with anhydrous pyridine (4.4 ml) and the mixture was cooled to −78 °C. Benzoyl chloride (700 μl, 6.0 mmol) was added to the mixture over 15 min and stirring was continued for 1 h at −78 °C. The mixture was allowed to warm to rt and EtOH (5 ml) was added. The resulting mixture was washed successively with sat. aq. $NaHCO_3$ (3 × 100 ml) and brine (100 ml). The combined aqueous phase was extracted with DCM (100 ml) and the organic phases were combined and evaporated to dryness under reduced pressure. The resulting residue was purified by column chromatography (3.5% MeOH in DCM) affording the product **3U** as a white foam (3.44 g, 79%). $R_f$: 0.3 (5% MeOH in DCM). $^1$H NMR (DMSO-$d_6$): $\delta$11.43 (s, 1H, NH), 7.93–7.87 (m, 2H, Ar), 7.80 (d, 1H, $J$ = 8.0, H6), 7.70–7.63 (m, 1H), 7.56–7.48 (m, 2H, Ar), 7.35–7.17 (m, 10H, Ar), 6.89–6.81 (m, 4H, Ar), 6.20 (t, 1H, $J$ = 5.7, H1′), 5.56 (d, 1H, $J$ = 8.0, H5), 4.83 (t, 1H, $J$ = 5.2, OH-3′), 4.71–4.45 (m, 2H), 3.73 (s, 6H, $OCH_3$), 3.70–3.62 (m, 1H, H4′), 3.50–3.40 (m, 2H, H3′), 3.11–2.96 (m, 2H, H5′). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 165.01, 163.08, 157.99, 151.10, 144.77, 140.61, 135.55, 135.45, 133.64, 129.55, 129.12, 129.02, 128.84, 127.74, 127.61, 126.59, 113.11, 102.16, 85.45, 80.94, 79.67, 63.47, 60.59, 54.98, 54.90. ESI-MS (M+Na$^+$): $m/z$ 675.2; calcd: 675.2313.

### 4.4. 2′-*O*-Benzoyl-3′-*O*-(2-cyanoethoxy(diisopropylamino)phosphino)-5′-O-(4,4′-dimethoxytrityl)-2′,3′-secouridine (4U)[28]

Nucleoside **3U** (679 mg, 1.04 mmol) was coevaporated with DCE (3 × 6 ml) and dried for 12 h in vacuo. The residue was dissolved at rt in 20% DIPEA in MeCN (6.5 ml) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite [P(Cl)($OCH_2CH_2CN$)(-N(iPr)$_2$)] (0.66 ml, 3.02 mmol) was added and stirring was continued for 40 min. The mixture was then poured into DCE (10 ml) and washed with sat. aq. $NaHCO_3$ (10 ml), and the separated aqueous phase was back-extracted with DCE (10 ml). The organic phases were pooled and evaporated to dryness to afford at residue which was purified by column chromatography (0–20% EtOAc in toluene) to give nucleoside **4U** as a white solid material (600 mg, 68%). $R_f$: 0.6 (50% EtOAc in toluene). $^{31}$P NMR (MeCN): $\delta$ 147.8. ESI-MS (M+Na$^+$): $m/z$ 875.3; calcd: 875.3391.

### 4.5. 6-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secoadenosine (2A$^{Bz}$)

6-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)adenosine (**1A$^{Bz}$**; 7.02 g, 10.42 mmol) was at rt dissolved in dioxane (150 ml) and water (25 ml). To this solution was added $NaIO_4$ (2.73 g, 11.77 mmol) dissolved in water (25 ml). The resulting mixture was stirred for 1 h during which time a white precipitate was formed. Additional dioxane (100 ml) was added and the suspension was stirred for additional 15 min, whereupon the suspension was filtered through a glass filter and the filter cake was washed with dioxane (50 ml). The filtrates were combined, $NaBH_4$ (435 mg, 11.5 mmol) was added and the resulting mixture was stirred for 30 min. The mixture was then neutralized by the addition of buffer (pyridine:AcOH 1:1, v/v, ∼10 ml). After evaporation of the resulting mixture to a volume of approximately 100 ml, DCM (100 ml) was added and the mixture was washed with sat. aq. $NaHCO_3$ (2 × 100 ml). The organic phase was evaporated to dryness under reduced pressure to give a residue which was purified by column chromatography (0–10% *i*PrOH in DCM) to give nucleoside **2A$^{Bz}$** as a white solid material (6.07 g, 86%). $R_f$: 0.22 (5% *i*PrOH in DCM). $^1$H NMR (DMSO-$d_6$): $\delta$ 11.23 (br s, 1H), 8.76 (s, 1H), 8.68 (s, 1H), 8.07 (d, 2H, $J$ = 7.0, Ar), 7.69–7.50 (m, 3H, Ar), 7.25–6.91 (m, 9H, Ar), 6.81–6.78 (m, 4H, Ar), 6.06 (t, 1H, $J$ = 6.1, H1′), 5.29 (t, 1H, $J$ = 5.8, 2′OH), 4.84 (t, 1H, $J$ = 5.4, 3′OH), 4.19–4.02 (m, 2H, H2′), 3.90–3.80 (m, 1H, H4′), 3.69 (s, 6H, $OCH_3$), 3.49 (t, 2H, $J$ = 5.3, H3′), 2.93–2.74 (m, 2H, H5′). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 165.6, 157.9, 152.8, 151.6, 150.2, 144.6, 143.1, 135.7, 135.4, 132.4, 129.4, 128.5, 128.4, 127.7, 127.5, 126.4, 125.2, 113.0, 84.5 (1′C), 79.6 (4′C), 63.6 (5′C), 61.4 (2′C), 60.9 (3′C), 54.9 ($OCH_3$). ESI-HRMS (M+Na$^+$): $m/z$ 698.2613; calcd: 698.2585.

### 4.6. 6-*N*-Benzoyl-2′-*O*-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secoadenosine (3A$^{Bz}$)

Nucleoside **2A$^{Bz}$** (2.01 g, 2.98 mmol) was coevaporated with anhydrous MeCN (2 × 30 ml) and dried for 12 h in vacuo. The residue was dissolved in anhydrous DCM (150 ml) along with anhydrous DBU (900 mg, 5.96 mmol) and the resulting mixture was cooled to −78 °C. A 0.5 M solution of benzoyl chloride (6.56 ml, 3.28 mmol) was added over 15 min and the resulting mixture was stirred for 1 h at −78 °C and was subsequently allowed to reach rt, whereupon EtOH (5 ml) was added. The mixture was then washed successively with sat. aq. $NaHCO_3$ (3 × 150 ml) and brine (150 ml) and the combined aqueous phase was back-extracted with DCM (100 ml). The organic phases were combined and evaporated to dryness under reduced pressure. The resulting residue was purified by column chromatography (0–100% EtOAc in petroleum ether) affording the product nucleoside **3A$^{Bz}$** as a white foam (1.69 g, 73%). $R_f$: 0.49 (EtOAc). $^1$H NMR (DMSO-$d_6$): $\delta$ 11.28 (s, 1H, NH), 8.82 (s, 1H), 8.76 (s, 1H), 8.06 (d, 2H, $J$ = 7.1, Ar), 7.83–7.78 (m, 2H), 7.55–7.40 (m, 6H, Ar), 7.25–6.89 (m, 10, Ar), 6.82–6.74 (m, 4H), 6.51 (t, 1H, $J$ = 6.1, H1′), 5.12–4.93 (m, 2H, H2′), 4.90 (t, $J$ = 5.3, 3′OH), 3.89 (br s, 1H, H4′), 3.72 (s, 6H, $OCH_3$), 3.54 (m, 2H, H3′), 2.81 (m, 2H, H5′). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 165.0, 157.9, 152.4, 150.5, 144.6, 142.9, 135.6, 133.6, 132.4, 129.0, 128.8, 128.4, 127.5, 125.2, 113.0, 85.1, 81.5 (C1′), 79.9 (C4′), 63.8 (C2′), 63.5, 60.8 (C5′), 54.9 ($OCH_3$). ESI-HRMS (M+Na$^+$): $m/z$ 802.2848; calcd: 802.2847.

### 4.7. 3′-*O*-(2-Cyanoethoxy(diisopropylamino)phosphino)-6-*N*,2′-*O*-dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secoadenosine (4A$^{Bz}$)

Nucleoside **3A$^{Bz}$** (1.69 g, 2.17 mmol) was coevaporated with anhydrous MeCN (2 × 20 ml) and was dried for 12 h in vacuo. The residue was dissolved in 20% DIPEA in MeCN (40 ml) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.0 ml, 4.48 mmol) was added at rt under stirring. After 40 min, additional 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.2 ml, 0.86 mmol) was added and stirring was continued for 3 h, whereupon EtOH (5 ml) was added. The resulting mixture was washed with sat. aq. $NaHCO_3$ (3 × 50 ml) and the aqueous phases were combined and extracted with DCM (50 mL). The organic phases were pooled and evaporated to dryness under reduced pressure to give a residue which was purified by column chromatography (0–100% EtOAc in petroleum ether) to afford the desired nucleoside **4A$^{Bz}$** as a white foam (1.52 g, 71%). $R_f$: 0.75 (5% MeOH in DCM). $^{31}$P NMR (MeCN): $\delta$ 148.8. ESI-HRMS (M+Na$^+$): $m/z$ 1002.3885; calcd: 1002.3926.

### 4.8. 4-*N*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secocytidine (2C$^{Ac}$)

4-*N*-Acetylcytidine (11.75 g, 41.18 mmol) was coevaporated with anhydrous pyridine (50 ml) and then dissolved in anhydrous pyridine (160 ml). 4,4′-Dimethoxytrityl chloride (DMT-Cl, 16.76 g, 49.42 mmol) was added as a solid material and the resulting mixture was stirred for 2 h at rt. The mixture was then washed with sat. aq. $NaHCO_3$ (3 × 50 ml) and the organic phase was evaporated to dryness under reduced pressure. This residue was dissolved in a mixture of dioxane (500 ml) and water (100 ml), and $NaIO_4$ (10.62 g, 49.5 mmol) dissolved in water (100 ml) was added. The mixture was stirred for 1 h during which time a white precipitate was formed. Additional dioxane (400 ml) was added and stirring was continued for 15 min. The precipitate was filtered off and washed with dioxane (200 ml). The filtrates were combined, $NaBH_4$ (1720 mg, 45.5 mmol) was added, and stirring was continued for 30 min. To the mixture was added a buffer (AcOH:pyridine, 1:1, 10 ml) and the resulting mixture was evaporated to a volume of approximately 300 ml. EtOAc (150 ml) was added and washing was performed with sat. aq. $NaHCO_3$ (3 × 200 ml). The organic phase was evaporated to dryness under reduced pressure and the residue was purified by column chromatography (0–10% MeOH in EtOAc) to afford nucleoside **2C$^{Ac}$** as a white solid material (17.24 g, 71%). $R_f$: 0.19 (5% MeOH in $CHCl_3$). $^1$H NMR (DMSO-$d_6$): $\delta$ 10.94 (s, 1H, NH), 8.08 (d, 1H, $J$ = 7.4), 7.31–7.12 (m, 12H), 6.95–6.75 (m, 4H, Ar), 5.96 (t, 1H, $J$ = 4.8, H1′), 5.13 (t, 1H, $J$ = 5.9, 2′OH), 4.74 (t, 1H, $J$ = 5.5, 3′OH), 3.73 (s, 6H, $OCH_3$), 3.63 (m, 3H, H2′/H4′), 3.43 (m, 2, H3′), 3.00 (m, 2H, H5′), 2.13 (s, 3H, $CH_3$). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 170.9, 162.3, 158.0, 155.4, 146.1, 144.6, 135.6, 129.6, 127.7, 126.6, 113.1, 95.4, 85.5, 84.7 (C1′), 79.4, 63.8 (C5′), 61.7, 60.5 (C3′), 55.0 ($OCH_3$), 24.3 ($CH_3$). ESI-HRMS (M+Na$^+$): $m/z$ 612.2298; calcd: 612.2316.

### 4.9. 4-*N*-Acetyl-2′-*O*-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secocytidine (3C$^{Ac}$)

Nucleoside **2C$^{Ac}$** (3.03 g, 5.14 mmol) was coevaporated with anhydrous toluene (2 × 30 ml) and was dried for 12 h in vacuo. The resulting residue was dissolved in anhydrous DCM (150 ml) along with anhydrous DBU (1.5 ml, 10.3 mmol) and the resulting mixture was cooled to −78 °C. Benzoyl chloride (656 μl, 5.65 mmol) was slowly added over 5 min and stirring was continued for 1 h at −78 °C. The mixture was then allowed to warm to rt, whereupon EtOH (4 ml) was added. The mixture was washed with sat. aq. $NaHCO_3$ (2 × 150 ml) and the organic phase was evaporated to dryness under reduced pressure. The resulting residue was purified by column chromatography (0–5% MeOH in $CHCl_3$) affording product nucleoside **3C$^{Ac}$** as a white foam (2.08 g, 64%). $R_f$: 0.24 (5% MeOH in $CHCl_3$). $^1$H NMR (DMSO-$d_6$): $\delta$ 10.98 (s, 1H, NH), 8.24 (d, $J$ = 7.4, 1H), 7.93–7.85 (m, 2H), 7.69–7.60 (m, 1H), 7.54–7.44 (m, 2H), 7.33–7.13 (m, 12H), 6.89–6.79 (m, 4H), 6.34 (t, $J$ = 5.0, 1H, H1′), 4.83 (t, $J$ = 5.4, 1H, 3′OH), 4.67–4.48 (m, 2H, H2′), 3.72 (s, 6H, $OCH_3$), 3.70–3.62 (m, 1H, H4′), 3.55–3.39 (m, 2H, H3′), 3.11–2.93 (m, 2H, H5′), 2.12 (s, 3H, $CH_3$). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 171.0, 165.0, 162.5, 157.1, 145.5, 144.6, 135.48, .133.6, 129.6, 128.8, 127.7, 127.6, 126.6, 113.1, 95.8, 85.6, 82.0 (C1′), 79.6 (C4′), 79.2 63.9 (C5′), 63.7 (C2′), 60.5 (C3′), 54.9 ($OCH_3$), 24.3 ($CH_3$). ESI-HRMS (M+Na$^+$): $m/z$ 716.2589; calcd: 716.2759.

### 4.10. 4-*N*-Acetyl-2′-*O*-benzoyl-3′-*O*-(2-cyanoethoxy(diisopropylamino)phosphino)-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secocytidine (4C$^{Ac}$)

Nucleoside **3C$^{Ac}$** (1.49 g, 2.15 mmol) was coevaporated with anhydrous MeCN (2 × 20 ml). The residue was dissolved in 20% DIPEA in MeCN (20 ml) and the mixture was stirred at rt. 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.8 ml, 3.6 mmol) was added to the mixture and stirring was continued for 40 min. Additional 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.4 ml, 1.8 mmol) was added and stirring was continued for 3 h. EtOH (5 ml) was added and the resulting mixture was washed with sat. aq. $NaHCO_3$ (3 × 50 ml) and the aqueous phase was extracted with DCM (50 ml). The organic phases were pooled and evaporated. The residue was purified by column chromatography (0–100% EtOAc in petroleum ether) to afford nucleoside **4C$^{Ac}$** as a white foam (940 mg, 44%). $R_f$: 0.42 (5% MeOH in DCM). $^{31}$P NMR (MeCN): $\delta$ 148.8. ESI-HRMS (M+Na$^+$): $m/z$ 916.3622; calcd: 916.3657.

### 4.11. 5′-*O*-(4,4′-Dimethoxytrityl)-2-*N*-isobutyryl-2′,3′-secoguanosine (2G$^{iBu}$)

2-*N*-Isobutyrylguanosine (11.68 g, 17.8 mmol) was coevaporated with anhydrous pyridine (50 ml). The resulting residue was dissolved in anhydrous pyridine (100 ml) and DMT-Cl (7.26 g, 21.46 mmol) was added as a solid material and the reaction mixture was stirred for 1 h at rt. DMAP (50 mg, 0.40 mmol) was added and the resulting mixture was stirred for additional 12 h. The reaction mixture was then washed with sat. aq. $NaHCO_3$ (3 × 50 ml) and the organic phase was evaporated to yield a white foam. This residue was dissolved in dioxane (250 ml) and water (50 ml). $NaIO_4$ (4.57 g, 21.3 mmol) was dissolved in water (50 ml) and was added to the dissolved nucleoside. The mixture was stirred for 1 h during which time a white precipitate was formed. Additional dioxane (200 ml) was added and stirring was continued for 15 min. The precipitate was filtered off and washed with dioxane (100 ml). The filtrates were collected and $NaBH_4$ (748 mg, 19.77 mmol) was added and the resulting mixture was stirred for 30 min at rt. The mixture was neutralized by the addition of buffer (10 ml, 1:1–AcOH:pyridine). The volume of the resulting mixture was reduced to 150 ml and EtOAc (150 ml) was added. The organic phase was washed with sat. aq. $NaHCO_3$ (3 × 100 ml) and evaporated. The residue was purified by column chromatography using a gradient of 0–10% MeOH in DCM to yield the nucleoside **2G$^{iBu}$** as a white material (8.02 g, 68%). $R_f$: 0.24 (7% MeOH in DCM). $^1$H NMR (DMSO-$d_6$): $\delta$ 12.10 (s, 1H, NH), 11.71 (s, 1H, NH), 8.17 (s, 1H, guanidine H8), 7.25–6.94 (m, 10H, Ar), 6.82–6.74 (m, 4H, ar), 5.72 (t, 1H, $J$ = 6.2, H1′), 5.19 (t, 1H, $J$ = 5.8, 2′OH), 4.79 (t, 1H, $J$ = 5.2, 3′OH), 3.99 (t, 2H, $J$ = 6.1, H2′), 3.83–3.67 (m, 8H, H4′/$OCH_3$), 3.48–3.40 (m, 2H, H3′), 2.93–2.73 (m, 2H, H5′/tertiary *i*-Pr), 1.12 (dd, 6H, $J$ = 6.8, 2.6., 2 × $CH_3$). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 180.2, 157.9, 154.9, 147.8, 144.7, 135.4, 129.3 (Ar), 127.5 (Ar), 127.4 (Ar), 126.4 (Ar), 120.4, 113.0 (Ar), 85.2, 85.1 (C1′), 79.9 (C4′), 63.5 (C5′), 61.7 (C2′), 61.1 (C3′), 54.9 (–$OCH_3$), 34.7 (tertiary *i*-Pr), 18.9 (*i*-Pr), 18.8 (*i*-Pr). MALDI-HRMS (M+Na$^+$): $m/z$ 680.2679; calcd: 680.2691.

### 4.12. 2′-*O*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-isobutyryl-2′,3′-secoguanosine (3G$^{iBu}$)

Nucleoside **2G$^{iBu}$** (2.01 g, 3.05 mmol) was suspended in anhydrous toluene (2 × 30 ml) and evaporated. The resulting residue was dried for 12 h in vacuo and then dissolved in anhydrous DCM (100 ml) along with anhydrous DBU (0.9 ml, 6.1 mmol). The resulting mixture was cooled to −78 °C. Benzoyl chloride (390 μl, 3.36 mmol) was added over 5 min to the reaction mixture and stirring was continued for 1 h at −78 °C. The reaction mixture was then allowed to reach rt, whereupon EtOH (4 ml) was added. The resulting mixture was washed with sat. aq. $NaHCO_3$ (2 × 100 ml), and the organic phases were combined and evaporated. The resulting residue was purified by column chromatography (0–5% MeOH in $CHCl_3$) affording nucleoside **3G$^{iBu}$** as a white foam (1.49 g, 63%).

$R_f$: 0.47 (7% MeOH in DCM). $^1$H NMR (DMSO-$d_6$): $\delta$ 12.10 (s, 1H, NH), 11.72 (s, 1H, NH), 8.32 (s, 1H, guanidine H8), 7.85–7.79 (m, 2H, Ar), 7.65–7.63 (m, 1H, Ar), 7.51–7.45 (m, 2H, Ar), 7.26–6.97 (m, 11H, Ar), 6.81–6.77 (m, 4H, Ar), 6.18 (t, 1H, $J$ = 6.2, H1′), 5.04–4.82 (m, 3H, H2′/3′OH), 3.82 (m, 1H, H4′), 3.72 (s, 6H, 2 × $OCH_3$), 3.49 (t, 2H, $J$ = 5.1, H3′), 3.03–2.74 (m, 3H, H5′/quaternary *i*-Pr), 1.15–1.04 (m, 6H, 2 × $CH_3$). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 180.1, 164.9, 157.8, 154.8, 148.6, 147.9, 144.6, 138.4, 135.5, 135.3, 133.6, 129.3 (Ar), 129.0 (ar), 128.8 (ar), 128.7 (ar), 127.6 (ar), 127.4 (ar), 126.3 (ar), 120.6, 112.9 (ar), 85.1, 82.0 (C1′), 80.1 (C4′), 63.7, 63.3 (C5′), 61.0 (C3′), 54.8 ($OCH_3$), 34.6 (quaternary *i*-Pr), 18.8 ($CH_3$), 18.6 ($CH_3$). ESI-HRMS (M+$Na^+$): *m*/*z* 784.294; 3 calcd: 784.2953.

### 4.13. 2′-*O*-Benzoyl-3′-*O*-(2-cyanoethoxy(diisopropylamino)-phosphino)-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-isobutyryl-2′,3′-secoguanosine (4G$^{iBu}$)

Nucleoside **3G$^{iBu}$** (1.45 g, 1.9 mmol) was coevaporated with anhydrous MeCN (2 × 20 ml). The residue was dissolved in 20% DIPEA in MeCN (20 ml) and the resulting mixture was stirred. 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.65 ml, 2.91 mmol) was added to the reaction mixture and stirring was continued for 40 min. EtOH (5 ml) was added and the resulting mixture was washed with sat. aq. $NaHCO_3$ (3 × 50 ml), and the aqueous phase was extracted with DCM (50 ml). The organic phases were pooled and evaporated. The residue was precipitated by adding a solution of the crude in EtOAc to vigorously stirring petroleum ether to furnish amidite **4G$^{iBu}$** as a white material (607 mg, 33%). $R_f$: 0.3 (33% Acetone in toluene). $^{31}$P NMR (MeCN): $\delta$ 148.6, 148.7. ESI-HRMS (M+$Na^+$): *m*/*z* 984.4028; calcd: 984.4031.

### 4.14. Synthesis of UNA-modified RNA oligonucleotides[29]

The UNA-modified and unmodified RNA oligonucleotides (Table 1) were synthesized on an automated nucleic acid synthesizer using the phosphoramidite approach. The syntheses were performed in 1.0 μmol scale. Standard RNA synthesis conditions of the synthesizer were used for the incorporation of RNA and UNA monomers using DCI as an activator, and the stepwise coupling yield of unmodified RNA as well as UNA monomers were >99% based on the absorbance of the dimethoxytrityl cation released after each coupling step. After detritylation, cleavage from the solid support and deacylation was carried out by using 30% aqueous ammonia solution. Cleaving of protecting groups was achieved by the addition of methylamine to the above solution to furnish a 1:1 mixture (2 h, 65 °C). Analysis by ion-exchange HPLC verified the purity of all oligonucleotides to be >80%, whereas their composition was verified by MALDI-TOF mass spectrometry.

### 4.15. Thermal denaturation experiments

UV-based thermal denaturation experiments were performed on a Perkin–Elmer Lambda 35 UV/vis spectrometer equipped with PTP 6 (Peltier Temperature Programmer) in a medium salt buffer (see caption below Table 1 for details) using 1.0 μM concentration of each strand. The two strands were thoroughly mixed, heated to 90 °C and subsequently cooled to the starting temperature of the experiment (10 °C). Thermal denaturation temperatures ($T_m$ values, °C) were determined as the maximum of the first derivative of the thermal denaturation curve ($A_{260}$ vs temperature; reported $T_m$ values are an average of two measurements within ±1.0 °C).

### 4.16. Circular dichroism spectra

CD spectra were measured in a 5 mm cuvette on a Jasco J-600A spectropolarimeter at 20 °C, in 200–350 nm wavelength range. Oligonucleotide single strand concentrations were calculated based on the absorbance values measured at rt. The samples containing 2 μM solutions of each duplex were prepared in a medium salt buffer (see caption below Table 1 for details), annealed for two minutes at 90 °C and slowly cooled to room temperature before the experiment. The buffer spectrum was subtracted from the duplex spectra. The spectra were smoothed using Savitzky–Golay filter.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.06.045.

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