2'β-Fluoro-Tricyclo Nucleic Acids (2'F-tc-ANA): Thermal Duplex Stability, Structural Studies, and RNase H activation

Alena Istrate,^[a] Adam Katolik,^[a] Andrei Istrate,^[a] and Christian J. Leumann^{*[a]}

Abstract: We describe the synthesis, thermal stability, structural and RNase H activation properties of 2'β-fluoro-tricyclo nucleic acids (2'F-tc-ANA). Three 2'F-tc-ANA nucleosides (T, ⁵MeC and A) were synthesized starting from a previously described fluorinated tricyclo sugar intermediate. NMR analysis and quantum mechanical calculations indicate that 2'F-tc-ANA nucleosides prefer sugar conformations in the East and South regions of the pseudorotational cycle. UV-melting experiments revealed that non-consecutive insertions of 2'F-tc-ANA units in DNA reduce the affinity to DNA and RNA complements. However, an oligonucleotide with 5 contiguous 2'F-tc-ANA-T insertions exhibits increased affinity to complementary RNA. Moreover, a fully modified 10-mer 2'F-tc-ANA oligonucleotide paired to both DNA (+1.6°C/mod) and RNA (+2.5°C/mod) with significantly higher affinity compared to corresponding unmodified DNA, and similar affinity compared to corresponding tc-DNA. In addition, CD spectroscopy and molecular dynamics simulations indicate that the conformation of the 2'F-tc-ANA/RNA duplex is similar to that of a DNA/RNA duplex. Moreover, in some sequence contexts, 2'F-tc-ANA promotes RNase H-mediated cleavage of a complementary RNA strand. Taken together, 2'F-tc-ANA represents a nucleic acid analogue which offers the advantage of high RNA affinity while maintaining the ability to activate RNase H, and can be considered a prospective candidate for gene silencing applications.

Introduction

Antisense oligonucleotides (AONs) are short synthetic oligonucleotides that inhibit or modulate expression of a specific gene by Watson-Crick binding to cellular RNA targets. AONs act through a number of different mechanisms. Some AONs bind to an mRNA of a gene of interest, inhibiting its expression either by blocking access of the cellular translation machinery or by inducing its enzymatic degradation.^[1] Alternatively, AONs can target a complementary region of a specific pre-mRNA and modulate its splicing, typically to correct a dysfunctional protein.^[2] Currently, more than 60 potential antisense drugs are under clinical investigation^[3], and three drugs are approved by the U.S. FDA: a 2'-O-methoxyethyl oligonucleotide Nusinersen for the treatment of spinal muscular atrophy^[4], a second-generation 2'-O-methoxyethyl chimeric oligonucleotide Mipomersen for the treatment of Familial hypercholesterolemia^[5], and morpholino

oligomer Eteplirsen for the treatment of Duchenne muscular dystrophy (DMD).^[6] However, a widespread therapeutic application of antisense oligonucleotides is still limited by poor tissue distribution, insufficient biostability and issues of toxicity.

Fluorinated oligonucleotide analogues captured significant interest over the past 15 years. Notably, the 2'-deoxy-2'-fluoro-RNA (F-RNA, Figure 1) and the 2'-deoxy-2'-fluoro-arabino nucleic acids (F-ANA, Figure 1) were both successfully applied to antisense^[7] and siRNA^[8] therapeutic constructs. More recently, fluoro cyclohexenyl nucleic acids (F-CeNA^[9]), N-methanocarba nucleic acid (F-NMC^[10]), 3'-fluorohexitol nucleic acids (FHNA and Ara-FHNA^[11]) and others^[12] were all tested in terms of oligonucleotide-based silencing applications.

Figure 1. Selected structures of modified nucleic acids.

Development of such analogues is particularly attractive because the presence of an additional fluorine atom greatly impacts the conformation of the associated modified nucleosides.^[12d, 13] Notably, the fluorine of F-RNA promotes an RNA-like C3'-endo conformation, which pre-organizes the encompassing oligonucleotide for duplex formation with RNA, affording improved affinity towards its RNA complement relative to the corresponding unmodified DNA-RNA duplex.^[14] In addition to the conformational pre-organization, enthalpic factors strongly contribute to the increased affinity. Presumably, the highly electronegative fluorine polarizes the nucleobases, which strengthens their Watson-Crick hydrogen bonds and enhances base stacking.^[8a, 15] In contrast to F-RNA, the 2'-fluorine substituent of F-ANA-modified oligomers is in the β orientation, which promotes these nucleosides to adopt either South or East sugar puckers.^[16] As a consequence, the F-ANA/RNA duplex conformation resembles that of a DNA/RNA duplex, and thus F-ANA can direct RNase H to cleave a complementary RNA strand, a feature which extends the scope of this analogue's therapeutic applications.^[17]

[a] Dr. A. Istrate, Dr. A. Katolik, Dr. A. Istrate, Prof. Dr. C. J. Leumann
Department of Chemistry and Biochemistry
University of Bern
Freiestrasse 3, CH-3012 Bern, Switzerland
E-mail christian.leumann@dcb.unibe.ch

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In addition to conformational modulation, the 2'-F modification often enhances metabolic stability and reduces immunostimulation of the associated oligonucleotides.^[18] Moreover, fluorinating biologically relevant molecules is a common means to change their interactions with protein targets.^[19] Indeed, a recent study has demonstrated that F-RNA antisense oligonucleotides can direct the recruitment of protein complex ILF2/3 to induce exon skipping, while corresponding 2'-O-(2-methoxyethyl) oligonucleotides are unable to do that.^[20] Overall, introduction of fluorine substituent(s) constitutes an effective approach to improve the therapeutic potential of nucleic acid analogues.

In the past we have systematically developed various conformationally restricted nucleic acid analogues.^[21] Recently, one of these analogues, tricyclo-DNA (tc-DNA, Figure 1), has emerged as a particularly promising candidate to potentially treat Duchenne muscular dystrophy.^[22] Our current priorities accentuate on further modifying the tc-DNA backbone to improve its cellular uptake and target affinity. For example, the recently developed the 2'- α -fluorinated analogue of tc-DNA (2'-F-tc-RNA, Figure 1) can significantly stabilize duplexes with RNA targets, enabling the use of shorter and mixed-(DNA/tc-DNA) oligonucleotides for therapeutic applications.^[23] Considering this, herein we explore the properties of an isomer of this compound with the 2'-fluorine in the β -configuration (2'-F-tc-ANA, Figure 1). Specifically, this report addresses the synthesis and structural properties of three 2'-F-tc-ANA (T, ⁵MeC and A) nucleoside analogues, and the biophysical properties of 2'-F-tc-ANA-modified oligodeoxynucleotides.

Results and Discussion

Synthesis of 2'-F-ANA-tc-phosphoramidites

Starting with the previously described 2'-F-ANA-tc-sugar **1**^[23], Vorbrüggen glycosylation conditions^[24] were applied to produce the 2'-F-tc-ANA-T nucleoside **4** (Scheme 1). To this end, the 1'-hydroxyl group of compound **1** was acetylated to furnish the 1',3',5'-triacetylated sugar (**2**), a standard glycosyl donor for nucleoside synthesis. Initial studies on the nucleosidation of compound **2** under standard Vorbrüggen conditions resulted in an anomeric mixture of 2'-F-ANA-tc-T nucleosides, with the α -anomer being the major product ($\alpha:\beta = 2:1$). In addition, harsh conditions ($\sim 80^\circ\text{C}$, 1 day) were required for the reaction to proceed. This low reactivity was not unexpected and was attributed to the fluorine substituent, which likely disfavors the formation of a carbocation at the anomeric center. We then reasoned that the nucleosidation of the corresponding bromoacetal (**3**) may lead to a more favorable anomeric product ratio. Indeed, the treatment of **3**, that was obtained from **2** by treatment with HBr in AcOH, with *in situ* persilylated thymine smoothly afforded the nucleoside mixture (**4**) in a 1:2.5 $\alpha:\beta$ -ratio and an 88% yield over three steps (Scheme 1). These two anomers were separable only after removal of the acetyl groups of **4** yielding the free nucleosides **5a** and **5b**. Next, the β -nucleoside **5b** was tritylated by standard conditions to afford compound **6** that was finally phosphitylated to yield the phosphoramidite building block **7** (65% over two steps), ready for use in oligonucleotide synthesis.

Scheme 1. Synthesis of the 2'-F-ANA-tc-T building block **7**.

To obtain the 2'-F-ANA-tc-⁵MeC nucleoside (**8**, Scheme 2), a standard two step procedure^[25] was applied, which converted the thymine nucleobase in compound **4** to 5-methylcytosine. Selective benzylation of the amino group in **8** then furnished the nucleoside **9**, at which stage the α - and β -anomers were successfully separated by column chromatography. The β -nucleoside **9** was subsequently converted into the desired phosphoramidite **11**, as described for the 2'-F-ANA-tc-T phosphoramidite **7**.

Scheme 2. Synthesis of the 2'-F-ANA-tc-⁵MeC building block **11**.

The previously obtained bromoacetal **3** (Scheme 1) also served as the starting material for the synthesis of the 2'-F-ANA-tc-A nucleoside (**12**, Scheme 3). Nucleophilic substitution at C(1) upon treatment of **3** with the sodium salt of N⁶-benzoyladenine proved to be an efficient way for the 2'-F-ANA-tc-A synthesis. Following optimization, the reaction afforded an anomeric mixture of 2'-F-ANA-tc-A nucleosides **12** in a 58% yield, with the major isomer being the desired β -nucleoside ($\alpha:\beta$ ratio = 1:4). The β -configuration at the anomeric center as well as the N9-attachment of the base was unambiguously assigned by ¹H- and ¹³C-HMBC NMR spectroscopy in **13a** and **13b** (see Supporting Information)

that were isolated in pure form from **12** after basic removal of the acetyl groups. Finally, DMTr-protection and phosphorylation of nucleoside **13b** provided access to phosphoramidite **15**, which was then used for oligonucleotide synthesis.

Scheme 3. Synthesis of the 2'F-ANA-tc-A building block **15**.

Remarkably, all of the 2'F-ANA-tc phosphoramidites (**7**, **11** and **15**) showed ^{31}P -NMR spectra with unusual $^4J_{\text{PF}}$ couplings ($^4J_{\text{PF}} = 4 - 11$ Hz). Such long-range couplings in saturated systems are typically too small to be detected. Similar $^4J_{\text{PF}}$ couplings have been observed for 2'F-ANA-A phosphoramidites, but this was not the case for any other F-ANA phosphoramidite.^[16a] This difference is possibly a consequence of higher conformational restriction of 2'F-ANA-tc-unit compared to standard F-ANA sugar.

X-ray structures of 2'F-ANA-tc-T nucleoside

The structure of 2'F-ANA-tc-T nucleoside was confirmed by X-ray diffraction analysis, using single crystals of the fully deprotected nucleoside **5b** (Figure 2, Table 1, Table S1). The asymmetric unit of the **5b** crystal comprises 4 independent molecules with rather similar conformation. The furanose ring of all four molecules exhibits an O4'-*endo* conformation, with pseudorotation phase angles (P) in the 92-101° range. In all cases the base is in the *anti*-arrangement ($\chi = -136^\circ$ to -166°) and the torsion angle γ is in the *antiperiplanar* range (156° to 160°). The maximum torsion angle ν_{max} resides between 46° and 50° . With respect to the structure of tc-T which in the crystal coexists in the C2'-*endo* and O4'-*endo* conformations (2 independent molecules in the asymmetric unit, Table 1^[21e]), the fluorine substituent drives the furanose unit into the O4'-*endo* conformation exclusively. The reported structure of 2'-deoxy-2'-fluoro-arabino thymidine (F-ANA-T) units in DNA duplexes corroborates this hypothesis, as

the F-ANA-T also exists in the O4'-*endo* conformation.^[16b] Moreover, the solution phase structure of an oligonucleotide hairpin comprising a F-ANA/RNA stem revealed that, as before, the F-ANA units adopt the O4'-*endo* sugar pucker.^[26] Based on stereoelectronic effect alone, one could expect that 2'F in the β orientation would steer the furanose into a C2'-*endo* conformation. However, a C2'-*endo* conformation would trigger the fluorine to sterically clash with the H6 of an *anti*-oriented thymine base.^[16b] Thus, as with F-ANA-T^[16b], the observed O4'-*endo* conformation is most likely explained by fluorine-induced steric rather than stereoelectronic effect.

Figure 2. ORTEP plot (50% probability level) of nucleoside **5b** (one out of the four molecules in the asymmetric unit): (a) side view; (b) top view.

Next, the generalized Karplus equation^[27] was applied to analyze the conformation of **5b** in solution. Consistent with the X-ray structure, the observed $^3J_{\text{H1}',\text{H2}'}$ coupling constant in the ^1H -NMR of **5b** ($^3J_{\text{H1}',\text{H2}'} = 4.1$ Hz) corresponds to an H-C(1') and H-C(2') torsion angle, characteristic of an Eastern conformation (Figure S1). However, a 4.1 Hz coupling constant can also be observed in the case of a conformational equilibrium between Northern (59%) and Southern (41%) conformers (as calculated with the empirical equation $S(\%) = 10 \times ^3J_{\text{H1}',\text{H2}'}^{[28]}$). Normally, the $^3J_{\text{H2}',\text{H3}'}$ and $^3J_{\text{H3}',\text{H4}'}$ coupling constants of nucleosides can be used to describe the sugar pucker in more detail which is precluded in this case due to the lack of a 3'-hydrogen in tc-nucleosides.

To further study the sugar pucker preferences of 2'F-ANA-tc-T nucleoside, a potential energy profile versus pseudorotation phase cycle parameters (P and ν_{max}) was obtained using quantum mechanical (QM) methods. To perform these calculations, the Gaussian 09^[29] software was applied at the restricted Hartree-Fock (RHF) level of theory using the 6-311G* basis set. Regarding the 2'F-ANA-tc-T nucleoside, the calculated low-energy regions correspond to Eastern ($P \sim 90^\circ$) and Southern ($P \sim 160^\circ$) sugar conformations (Figure 3, a). By contrast, the tc-T nucleoside prefers to adopt a Northern ($P \sim 350^\circ$) and, to a lesser extent, a Southern ($P \sim 160^\circ$) conformation (Figure 3, b). Thus, in summary, introducing a 2' β -F substituent steers the tricyclo-sugar towards Southern and Eastern conformations, while disfavoring the RNA-like Northern conformation.

Table 1. Selected torsion angles and sugar pucker parameters for tc-T and 2'F-ANA-tc-T nucleosides.

Nucleoside	γ	δ	χ	P	V_{max}
tc-T Mol A ^[a]	125°	152.5°	-130.4°	172.2°	36.7°
tc-T Mol B ^[a]	154.8°	98.7°	-120.3°	94.4°	36°
2'F-ANA-tc-T Mol A	156.1°	99.2°	-135.8°	96.9°	49.7°
2'F-ANA-tc-T Mol B	160.4°	96.8°	-164.9°	92.1°	46.4°
2'F-ANA-tc-T Mol C	157.3°	98.2°	-155.5°	94.8°	47.7°
2'F-ANA-tc-T Mol D	157.3°	102.7°	-165.6°	100.9°	48.1°

[a] Data taken from ^[21e].

Figure 3. Energy profile versus pseudorotation phase angle for (a) 2'F-tc-ANA-T and (b) tc-T nucleosides.

T_m-measurements

To study the effect of insertions of 2'F-ANA-tc-units on the thermal stability of associated duplexes, we incorporated building blocks **7**, **11** and **15** into oligodeoxynucleotides and evaluated them in UV-melting experiments with DNA and RNA complements. These studies (Table 2) clearly reveal that 2'F-ANA-tc-nucleosides do not mix well with the DNA backbone. Specifically, DNA oligomers with single or double insertions of 2'F-ANA-tc-T (**ON1-ON5**) or 2'F-ANA-tc-A (**ON7-ON10**) units significantly destabilized duplexes with complementary DNA and RNA (ΔT_m up to -4.4 °C/mod). It is noteworthy that the lowest destabilization occurs when a 2'F-ANA-tc-T nucleoside is located 5' to a purine nucleoside (**ON2**, **ON4**). This is in agreement with earlier observations in the F-ANA series where the 2' fluorine participates in a non-conventional pseudo hydrogen bond with the H8 of a 3'-adjacent purine nucleoside.^[30] This causes an additional binding contribution, which reduces the observed destabilization.

The destabilizing nature for single, non-consecutive substitutions has been observed before for standard tc-nucleosides and has been attributed to unfavorable energetic contributions arising from the heterojunctions in the backbone.^[31] However, this was not the case for the 2'F-tc-RNA-T modification, which is probably due to the preference of 2'F-tc-RNA-T for a Northern conformation.^[23] As expected, introducing two

consecutive 2'F-ANA-tc-modifications resulted in a much weaker destabilization (**ON5**, **ON10**). In addition, in a duplex having five consecutive 2'F-ANA-tc-T modifications (**ON6**), the destabilization is much weaker against DNA (-1.2°C/mod) and turns into a moderate stabilization of +0.5°C/mod against RNA as complement.

Unlike single and double incorporations of 2'F-ANA-tc-T and 2'F-ANA-tc-A nucleosides, incorporations of 2'F-ANA-tc-⁵MeC stabilized duplexes with complementary RNA in a sequence-specific manner, and did not affect the stability of duplexes with complementary DNA (**ON11-ON13**). Previously we showed that the 5-methyl group on cytosine contributes to a slight stabilization (~ 1.0°C/mod) in this sequence context.^[23] Thus, even if the fluorine substituent slightly destabilizes the duplex, this effect is compensated by the stabilization imparted by the 5-methylcytosine nucleobase.

Although incorporating single 2'F-ANA-tc-units mostly destabilize duplexes with complementary DNA and RNA, increasing the number of consecutive modifications seems to reduce this effect. Thus, we expected that a fully modified oligonucleotide will exhibit high affinity to complementary DNA and RNA. Indeed, the fully modified 2'F-tc-ANA oligonucleotide (**ON14**) stabilized duplexes with both DNA (+1.6°C/mod) and

Table 2. T_m data of 2'F-ANA-tc-modified oligodeoxynucleotides in complex with complementary DNA and RNA. Duplex concentration: 2 μ M in 10 mM NaH₂PO₄, 150 mM NaCl, pH 7.0. Underlined letters indicate 2'F-ANA-tc-residues; italic letters indicate tc-residues. 2'F-ANA-tc-⁵MeC and tc-⁵MeC modification is designated as **C** and **C**, respectively.

Entry	Sequence (5'→3')	vs DNA		vs RNA	
		T_m (°C)	$\Delta T_m/\text{mod}$	T_m (°C)	$\Delta T_m/\text{mod}$
ON1	d(AACTG <u>T</u> CACG)	39.6	-4.4	39.6	-4.4
ON2	d(AAC <u>T</u> GTCACG)	41.0	-3.0	41.8	-2.2
ON3	d(AAC <u>T</u> G <u>T</u> CACG)	35.6	-4.2	38.1	-3.0
ON4	d(CCC <u>T</u> A <u>T</u> ACCC)	38.8	-0.4	45.0	-1.2
ON5	d(CCCAA <u>T</u> TCCC)	37.9	-2.2	44.1	-0.1
ON6	d(GCA <u>T</u> <u>T</u> <u>T</u> <u>T</u> ACCG)	41.2	-1.2	46.2	+0.5
ON7	d(CCCTA <u>T</u> ACCC)	36.0	-3.6	43.4	-4.0
ON8	d(CCCTAT <u>A</u> CCC)	35.9	-3.7	44.2	-3.2
ON9	d(CCCTA <u>T</u> A <u>T</u> ACCC)	32.4	-3.6	40.7	-3.3
ON10	d(CCC <u>A</u> A <u>T</u> TCCC)	39.1	-1.6	41.9	-1.3
ON11	d(AACTGT <u>C</u> ACG)	44.1	+0.1	43.9	-0.1
ON12	d(AAC <u>T</u> GTCACG)	44.0	0	47.1	+3.1
ON13	d(AAC <u>T</u> GT <u>C</u> ACG)	44.2	+0.1	46.9	+1.4
ON14	<u>CCCTATACCC</u>	55.2	+1.6	72.1	+2.5
ON15	<u>CCCTATACCC</u>	61.5	+2.2	73.6	+2.6

RNA (+2.5°C/mod) complements, compared to corresponding unmodified duplexes. The increase in RNA affinity of 2'F-tc-ANA oligonucleotide (**ON14**) is similar to that of the corresponding tc-DNA oligonucleotide (**ON15**), and corresponds to a ΔT_m of 25°C for a 10-mer duplex. Encouraged by this result, we decided to gain more insight into the conformation of the **ON14/DNA** and **ON14/RNA** duplexes.

We therefore explored the conformational preferences of a fully modified 2'F-tc-ANA oligonucleotide in duplex with DNA and RNA using MD simulations and CD spectroscopy. Figure 4 displays selected snapshots of the 100 ns MD trajectories for **2'F-tc-ANA/RNA** and **2'F-tc-ANA/DNA** duplexes (for details see Supporting Information). From these calculated trajectories, we extracted and analyzed the preferred sugar conformations and backbone torsion angles of both the modified and unmodified strands within each duplex (Figure 5). Interestingly, the 2'F-tc-ANA strand exhibits a nearly identical conformation, regardless of whether it is hybridized with DNA (Figure 5, c, e) or with RNA (Figure 5, g, i). Analysis of the backbone torsion angles revealed that the presence of a fused ring system mainly affects the torsion angles β and γ in 2'F-tc-ANA. More specifically, the angle β predominantly adopts values close to 90° in contrast to the 180° observed in A- and B-type duplexes.^[32] Moreover, the value of the γ angle is restricted to a 120-170° range within this system, which is consistent with the X-ray structures of 2'F-ANA-tc-T and tc-T nucleosides. As expected, the modified ribose units of both duplexes are constrained to the Southern and Eastern conformations within the pseudorotation cycle (C2'-endo, C1'-exo and O4'-endo), which resembles the standard DNA sugar pucker. The conformation of the unmodified strands in 2'F-tc-ANA/RNA and 2'F-tc-ANA/DNA duplexes closely resembles the conformation of canonical A- and B-type helices, respectively (Figure 5). Notably, the conformation of 2'F-tc-ANA/RNA duplex is reminiscent to that of DNA/RNA hybrid duplexes (which is also corroborated by CD measurements, Figure S2).

RNase H cleavage

The ability of RNase H to cleave the RNA strand of DNA/RNA hybrid duplexes is hallmark of many antisense therapeutic strategies.^[1, 14] To be a substrate of RNase H, the conformation of AON/RNA duplexes should resemble that of DNA/RNA hybrid duplexes. Specifically, the sugars of the antisense strand should be able to adopt a DNA-like conformation. Yet, most of the oligonucleotide modifications synthesized to date mimic RNA and therefore lack the ability to activate RNase H.

Correspondingly, we explored the ability of 2'F-tc-ANA to effectively direct the RNase H to cleave the complementary RNA strands. Reportedly, RNase H requires a minimum 5-6 DNA-RNA base pairs to cleave the RNA portion.^[33] We thus designed a chimeric oligonucleotide with six consecutive 2'F-tc-ANA-T nucleosides, which were flanked on each end by short stretches of DNA (Figure 6, a) to use as a model antisense strand. The complementary sense strand was also chimeric, and contained six consecutive units of ribo-A flanked on each end by short wings of 2'-OMe RNA nucleotides. In this system, the 2'F-tc-ANA-T stretch was complementary to a ribo-A stretch while the DNA stretch was paired with the 2'-OMe units. This architecture was intended to ensure that RNase H cannot cleave those portions of the sense strand which are complementary to the DNA. A duplex

of a similar design was previously used for assessing whether cyclohexenyl nucleic acids (CeNA) recruit RNase H.^[34]

Figure 4. Snapshots of the MD trajectories illustrating the overall conformation of **ON14/DNA** (a) and **ON14/RNA** (b) duplexes.

Next, this chimeric duplex was subjected to *E. coli* RNase H in parallel with a corresponding DNA/RNA positive control and an RNA/RNA negative control. Remarkably, some cleavage of RNA opposing the 2'F-tc-ANA stretch was reproducibly happening. The rate of this cleavage was expectedly compromised compared to the native DNA/RNA hybrid (Figure 6, a), and curiously, while the native hybrid produced two different hydrolysis products, the synthetic construct created only the lower band, which corresponds to a cleavage site that is closer to the 5'-end. As expected the RNA/RNA control duplex did not activate RNase H.

We next asked whether the 2'F-tc-ANA can universally activate RNase H, irrespective of sequence. To this end, the fully modified mix base **ON14** was annealed to its complementary RNA and exposed to RNase H (Figure 6, b). This was done concomitantly with a DNA/RNA positive control, an RNA/RNA negative control, and another tc-DNA/RNA negative control. Curiously, in this sequence context, no RNA cleavage was observed for the 2'F-tc-ANA modified **ON14** oligomer, while the positive control was fully cleaved.

Clearly, the hybrid-type conformation of a substrate duplex is crucial but not satisfactory to activate RNase H. Reportedly, the catalytic domain of the Human RNase H1 establishes contacts with seven consecutive residues within the antisense strand^[35], and the enzyme manifests a unique conformational preference for each of these nucleotide positions.^[36] Furthermore, the fully modified 2'F-tc-ANA strand is likely very rigid, and some degree of flexibility is required as the enzyme's active site bends the antisense strand so as to correctly position the cleaved phosphate within the binuclear catalytic site, as concluded from NMR^[37] and computational studies.^[38] In this context, antisense molecules comprising entirely of F-ANA cannot effectively activate RNase H, but acquiring some flexibility, through flexible linkers, leads to triggering a very potent RNase H response.^[39] With that said, more RNase H experiments concerning the 2'F-tc-ANA modification are underway.

Figure 5. Backbone torsion angle distribution for canonical A-type (a) and B-type (b) duplexes.^[32] Backbone torsion angles distribution (c, d, g, h) and preferred sugar pucker (e, f, i, j) for **ON14/DNA** and **ON14/RNA** duplexes extracted from 100 ns molecular dynamics trajectory.

conformation and thereby mimics RNA in duplexes with DNA and RNA (unpublished data), introducing the 2'β-F substituent steers the conformation into the Southern and Eastern regions of the pseudorotation cycle. Moreover, unlike their parent tc-nucleosides, 2'F-tc-ANA-A and 2'F-tc-ANA-T units are destabilizing towards complementary DNA and RNA, when incorporated in a non-consecutive manner within a DNA strand. However, an oligonucleotide with 5 contiguous 2'F-tc-ANA-T substitutions stabilizes its duplex with complementary RNA. Furthermore, with particular relevance to therapeutic applications, a fully modified 10-mer 2'F-tc-ANA oligomer paired to RNA with much higher affinity (+2.5°C/mod) than the corresponding unmodified DNA. Moreover, the results of CD spectroscopy and molecular dynamics simulations indicate that the conformation of the 2'F-tc-ANA/RNA duplex is similar to that of a DNA/RNA duplex. Because of these properties, in certain circumstances, 2'F-tc-ANA oligomers can recruit RNase H to cleave their complementary RNA strands. Overall, 2'F-tc-ANA oligonucleotides combine the advantages of high RNA affinity while maintaining the ability to activate RNase H, and can be considered a prospective candidate for gene silencing applications. Further studies on biological properties of 2'F-tc-ANA-modified oligonucleotides are underway.

Experimental Section

Synthesis of phosphoramidites

For detailed synthetic procedures and characterization of the phosphoramidites **7**, **11** and **15**, as well as the corresponding intermediates see Supporting Information.

Methods and instruments

UV-melting curves. UV-melting curves were recorded on a Varian Cary Bio100 UV/vis spectrophotometer. Absorbances were monitored at 260 nm, and the heating rate was set to 0.5°C/min. A cooling-heating-cooling cycle in the temperature range 15–80°C or 15–90°C was applied. T_m values were obtained from the maximum of the first derivative curves and reported as the average of at least three ramps. To avoid evaporation of the solution, the sample solutions were covered with a layer of dimethylpolysiloxane. All measurements were carried out in NaCl (150 mM), Na₂HPO₄ (10 mM) buffer at pH 7.0 with a duplex concentration of 2 μM.

CD Spectroscopy. CD spectra were recorded with Spectra Manager v1.54 software on a Jasco J-715 spectropolarimeter equipped with a Jasco PFO-350S temperature controller, using the same buffer conditions and oligonucleotide concentrations as for UV melting curves. All CD spectra were collected at 20°C and 90°C between 210 to 320 nm at a 50 or 100 nm/min rate, baseline-corrected with a blank spectrum (NaCl (150 mM), Na₂HPO₄ (10 mM) buffer at pH 7.0) and smoothed. The reported spectra correspond to the average of at least three scans.

Molecular dynamics simulations. The initial structures of duplexes employed in MD simulations were generated using the Nucleic Acid Builder (a molecular manipulation language, currently distributed as a part of the AmberTools16 program package^[41]). MD simulations were carried out using the GROMACS-5.0.7 package^[42] and the Amber 94 force field^[43]. The force field parameters for modified units were obtained from QM calculations using the Gaussian 09 software and the R.E.D. Tools program package^[44]. To prepare the system for an MD simulation, the oligonucleotide was placed in to a cubic cell of 1.0x1.0x1.0 nm, then the cell was filled with water molecules of TIP3P model. The total charge of the obtained system was -18, so 18 Na⁺ ions were added to neutralize it. At the next stage, the potential energy was minimized using the steepest descent algorithm. After energy minimization, the system was equilibrated

in two phases: 1) under constant number of particles, volume, and temperature (isothermal-isochoric ensemble); 2) under constant number of particles, pressure, and temperature (isothermal-isobaric ensemble). During equilibration, the "position restrained" algorithm was employed in order to prevent the system from collapsing. The resulting system was used for producing 100 ns MD trajectories. The calculated MD trajectories were analyzed using programs included in the GROMACS package, the *do_x3dna* package^[45] and in house written *bash* and *awk* scripts. All calculations were performed on the UBELIX cluster.

Oligonucleotide synthesis and purification

Syntheses of oligonucleotides were performed on the 1.3 μmol scale of a LKB Gene Assembler Plus (*Pharmacia*) DNA synthesizer using standard solid-phase phosphoramidite chemistry. Natural DNA phosphoramidites were purchased from SAFC (Prologo reagents). Oligomers were assembled on nucleoside preloaded dmf-dG-Q-CPG 500 (29 μmol/g) or universal Glen UnySupport CPG 500 (47 μmol/g) solid support (*Glen Research*). Natural phosphoramidites (dT, dC^{4Bz}, dA^{6Bz}, dG^{2dmf}) were coupled as a 0.1 M solution in CH₃CN, modified phosphoramidites as 0.15 M solution in CH₃CN (tc-T, tc-^{5MeC}4Bz) or dichloroethane (all others). The coupling step was 4 min for natural phosphoramidites. An extended coupling time of 12 min for tricyclonucleosides was necessary to achieve >94% coupling efficiency (trityl assay). As a coupling reagent, 5-(ethylthio)-1H-tetrazole (0.25 M in CH₃CN) was used. Capping was performed with a solution of DMAP (0.5 M in CH₃CN, Cap A) and a solution of 25% Ac₂O and 12.5% *sym*-collidine in CH₃CN (Cap B). Oxidation was performed with a solution of 20 mM I₂ and 0.45 M *sym*-collidine in 2:1 CH₃CN/H₂O. Detritylation after coupling of natural phosphoramidites was carried out using a solution of 3% dichloroacetic acid in dichloroethane.

Deprotection of the oligonucleotides and detachment from the solid support was carried out using standard conditions (concentrated aq NH₃ for 16 h at 55 °C). The fully-modified oligonucleotides were additionally deprotection with 0.4 M NaOH, 90 min, r.t in MeOH/H₂O 1:1. After deprotection, the solutions were centrifuged, the supernatants were collected, and the remaining beads were washed with 3x0.5 ml of H₂O. The combined supernatants were filtered and concentrated to dryness on Speed-Vac (*UniEquip*, UniVapo 150 H). Crude oligonucleotides were purified by ion-exchange HPLC (DNA Pac-PA200, *Dionex*) on ÄktaTM basic 10/100 system (*Amersham Pharmacia Biotech*). As mobile phases, the following buffers were prepared: (A) 25 mM Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) in H₂O, pH 8.0; (B) 25 mM Trizma, 1.25 M NaCl in H₂O, pH 8.0; or (A) 0.01 M NaOH in H₂O, pH 12.0; (B) 0.01 M NaOH, 1.5 M NaCl in H₂O, pH 12.0. Linear gradients of B in A were used (typically 0–40% B in A over 50 min), with a 1 ml/min flow rate and detection at 260 nm. Purified oligonucleotides were desalted over Sep-Pak Classic C18 Cartridges (*Waters*), quantified at 260 nm with a Nanodrop spectrophotometer, and analyzed by ESI-mass spectrometry. Solutions of oligonucleotides in H₂O were then stored at -18°C. Natural DNA and RNA oligonucleotides were purchased from Microsynth, and repurified by HPLC or polyacrylamide gel electrophoresis and desalted, if needed.

RNase H degradation experiments

The RNase H assays were carried out with: >100K cps labeled sense strand, 0.1 μM unlabeled sense strand, 2.5 μM antisense strand, 0.2 or 0.4 U/μL *E. Coli* RNase H (Thermo Scientific EN0201), 20 mM Tris-HCl (pH 7.8), 40 mM KCl, 8 mM MgCl₂, 1 mM DTT, 20 μL total volume, 37°C, shaking for 2 or 4h. The reactions were stopped by adding 20 μL Formamide Loading dye (see Supporting Information for further details).

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- [1] C. F. Bennett, E. E. Swayze, *Annu. Rev. Pharmacol. Toxicol.* **2010**, *50*, 259-293.
- [2] a) R. Kole, A. R. Krainer, S. Altman, *Nature reviews. Drug discovery* **2012**, *11*, 125-140; b) P. Jarver, L. O'Donovan, M. J. Gait, *Nucleic acid therapeutics* **2014**, *24*, 37-47.
- [3] K. Sridharan, N. J. Gogtay, *Br. J. Clin. Pharmacol.* **2016**, *82*, 659-672.
- [4] D. R. Corey, *Nat. Neurosci.* **2017**, *20*, 497-499.
- [5] F. J. Raal, R. D. Santos, D. J. Blom, A. D. Marais, M. J. Charng, W. C. Cromwell, R. H. Lachmann, D. Gaudet, J. L. Tan, S. Chasan-Taber, D. L. Tribble, J. D. Flaim, S. T. Crooke, *Lancet* **2010**, *375*, 998-1006.
- [6] K. R. Lim, R. Maruyama, T. Yokota, *Drug Des Devel Ther* **2017**, *11*, 533-545.
- [7] a) A. Kalota, L. Karabon, C. R. Swider, E. Viazovkina, M. Elzagheid, M. J. Damha, A. M. Gewirtz, *Nucleic Acids Res.* **2006**, *34*, 451-461; b) N. Souleimanian, G. F. Deleavey, H. Soifer, S. Wang, K. Tiemann, M. J. Damha, C. A. Stein, *Mol Ther Nucleic Acids* **2012**, *1*, e43; c) A. M. Kawasaki, M. D. Casper, S. M. Freier, E. A. Lesnik, M. C. Zounes, L. L. Cummins, C. Gonzalez, P. D. Cook, *J. Med. Chem.* **1993**, *36*, 831-841.
- [8] a) M. Manoharan, A. Akinc, R. K. Pandey, J. Qin, P. Hadwiger, M. John, K. Mills, K. Charisse, M. A. Maier, L. Nechev, E. M. Greene, P. S. Pallan, E. Rozners, K. G. Rajeev, M. Egli, *Angewandte Chemie International Edition* **2011**, *50*, 2284-2288; b) T. Dowler, D. Bergeron, A. L. Tedeschi, L. Paquet, N. Ferrari, M. J. Damha, *Nucleic Acids Res.* **2006**, *34*, 1669-1675.
- [9] P. P. Seth, J. Yu, A. Jazayeri, P. S. Pallan, C. R. Allerson, M. E. Østergaard, F. Liu, P. Herdewijn, M. Egli, E. E. Swayze, *The Journal of Organic Chemistry* **2012**, *77*, 5074-5085.
- [10] M. E. Jung, T. A. Dwight, F. Vigant, M. E. Østergaard, E. E. Swayze, P. P. Seth, *Angewandte Chemie International Edition* **2014**, *53*, 9893-9897.
- [11] M. Egli, P. S. Pallan, C. R. Allerson, T. P. Prakash, A. Berdeja, J. Yu, S. Lee, A. Watt, H. Gaus, B. Bhat, E. E. Swayze, P. P. Seth, *J. Am. Chem. Soc.* **2011**, *133*, 16642-16649.
- [12] a) S. Martínez-Montero, G. F. Deleavey, N. Martín-Pintado, J. F. Fakhoury, C. González, M. J. Damha, *ACS Chem. Biol.* **2015**, *10*, 2016-2023; b) S. Martínez-Montero, G. F. Deleavey, A. Dierker-Viik, P. Lindovska, T. Ilina, G. Portella, M. Orozco, M. A. Parniak, C. González, M. J. Damha, *The Journal of Organic Chemistry* **2015**, *80*, 3083-3091; c) P. P. Seth, P. S. Pallan, E. E. Swayze, M. Egli, *ChemBioChem* **2013**, *14*, 58-62; d) M. E. Østergaard, T. Dwight, A. Berdeja, E. E. Swayze, M. E. Jung, P. P. Seth, *The Journal of Organic Chemistry* **2014**, *79*, 8877-8881.
- [13] a) H. Ikeda, R. Fernandez, J. J. Barchi, X. Huang, V. E. Marquez, A. Wilk, *Nucleic Acids Res.* **1998**, *26*, 2237-2244; b) N. Martín-Pintado, G. F. Deleavey, G. Portella, R. Campos-Olivas, M. Orozco, M. J. Damha, C. González, *Angewandte Chemie International Edition* **2013**, *52*, 12065-12068.
- [14] Glen F. Deleavey, Masad J. Damha, *Chem. Biol.* **2012**, *19*, 937-954.
- [15] P. S. Pallan, E. M. Greene, P. A. Jicman, R. K. Pandey, M. Manoharan, E. Rozners, M. Egli, *Nucleic Acids Res.* **2011**, *39*, 3482-3495.
- [16] a) C. J. Wilds, M. J. Damha, *Nucleic Acids Res.* **2000**, *28*, 3625-3635; b) Berger, V. Tereshko, H. Ikeda, V. E. Marquez, M. Egli, *Nucleic Acids Res.* **1998**, *26*, 2473-2480.
- [17] a) F. Li, S. Sarkhel, C. J. Wilds, Z. Wawrzak, T. P. Prakash, M. Manoharan, M. Egli, *Biochemistry* **2006**, *45*, 4141-4152; b) M. J. Damha, C. J. Wilds, A. Noronha, I. Brukner, G. Borkow, D. Arion, M. A. Parniak, *J. Am. Chem. Soc.* **1998**, *120*, 12976-12977.
- [18] J. K. Watts, G. F. Deleavey, M. J. Damha, *Drug Discovery Today* **2008**, *13*, 842-855.
- [19] a) J. C. Biffinger, H. W. Kim, S. G. DiMaggio, *ChemBioChem* **2004**, *5*, 622-627; b) E. P. Gillis, K. J. Eastman, M. D. Hill, D. J. Donnelly, N. A. Meanwell, *J. Med. Chem.* **2015**, *58*, 8315-8359.
- [20] F. Rigo, Y. Hua, S. J. Chun, T. P. Prakash, A. R. Krainer, C. F. Bennett, *Nat. Chem. Biol.* **2012**, *8*, 555-561.
- [21] a) R. Steffens, C. J. Leumann, *J. Am. Chem. Soc.* **1997**, *119*, 11548-11549; b) M. Tarköy, M. Bolli, B. Schweizer, C. J. Leumann, *Helv. Chim. Acta* **1993**, *76*, 481-510; c) A. I. Haziri, C. J. Leumann, *J. Org. Chem.* **2012**, *77*, 5861-5869; d) J. Lietard, C. J. Leumann, *J. Org. Chem.* **2012**, *77*, 4566-4577; e) B. Dugovic, M. Wagner, C. J. Leumann, *Beilstein J. Org. Chem.* **2014**, *10*, 1840-1847.
- [22] A. Goyenvalle, G. Griffith, A. Babbs, S. E. Andaloussi, K. Ezzat, A. Avril, B. Dugovic, R. Chaussonot, A. Ferry, T. Voit, H. Amthor, C. Buhr, S. Schurch, M. J. A. Wood, K. E. Davies, C. Vailland, C. Leumann, L. Garcia, *Nat Med* **2015**, *21*, 270-275.
- [23] A. Istrate, M. Medvecky, C. J. Leumann, *Org. Lett.* **2015**, *17*, 1950-1953.
- [24] H. Vorbrüggen, K. Krolkiewicz, B. Bennua, *Chem. Ber.* **1981**, *114*, 1234-1255.
- [25] K. J. Divakar, C. B. Reese, *Journal of the Chemical Society, Perkin Transactions 1* **1982**, 1171-1176.
- [26] J.-F. Trempe, C. J. Wilds, A. Y. Denisov, R. T. Pon, M. J. Damha, K. Gehring, *J. Am. Chem. Soc.* **2001**, *123*, 4896-4903.
- [27] C. A. G. Haasnoot, F. A. A. M. de Leeuw, C. Altona, *Tetrahedron* **1980**, *36*, 2783-2792.
- [28] F. A. A. M. de Leeuw, C. Altona, *Journal of the Chemical Society, Perkin Transactions 2* **1982**, 375-384.
- [29] M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, *Gaussian 09*, Wallingford CT, **2009**.
- [30] M. Y. Anzahaee, J. K. Watts, N. R. Alla, A. W. Nicholson, M. J. Damha, *J. Am. Chem. Soc.* **2011**, *133*, 728-731.
- [31] D. Ittig, A.-B. Gerber, C. J. Leumann, *Nucleic Acids Res.* **2011**, *39*, 373-380.
- [32] B. Schneider, S. Neidle, H. M. Berman, *Biopolymers* **1997**, *42*, 113-124.
- [33] S. T. Crooke, K. M. Lemonidis, L. Neilson, R. Griffey, E. A. Lesnik, B. P. Monia, *Biochem. J.* **1995**, *312*, 599-608.
- [34] J. Wang, B. Verbeure, I. Luyten, E. Lescrinier, M. Froeyen, C. Hendrix, H. Rosemeyer, F. Seela, A. Van Aerschot, P. Herdewijn, *J. Am. Chem. Soc.* **2000**, *122*, 8595-8602.
- [35] M. Nowotny, S. A. Gaidamakov, R. Ghirlando, S. M. Cerritelli, R. J. Crouche, W. Yang, *Mol. Cell.* **28**, 264-276.
- [36] M. E. Østergaard, J. Nichols, T. A. Dwight, W. Lima, M. E. Jung, E. E. Swayze, P. P. Seth, *Molecular Therapy - Nucleic Acids*, **7**, 20-30.
- [37] J. T. Nielsen, P. C. Stein, M. Petersen, *Nucleic Acids Res.* **2003**, *31*, 5858-5867.
- [38] A. Noy, F. J. Luque, M. Orozco, *J. Am. Chem. Soc.* **2008**, *130*, 3486-3496.
- [39] M. M. Mangos, K.-L. Min, E. Viazovkina, A. Galarneau, M. I. Elzagheid, M. A. Parniak, M. J. Damha, *J. Am. Chem. Soc.* **2003**, *125*, 654-661.
- [40] A. Goyenvalle, C. Leumann, L. Garcia, *J. Neuromuscul Dis* **2016**, *3*, 157-167.
- [41] D. A. Case, R. M. Betz, D. S. Cerutti, T. E. Cheatham, T. A. Darden, R. E. Duke, T. J. Giese, H. Gohlke, A. W. Goetz, N. Homeyer, S. Izadi, P. Janowski, J. Kaus, A. Kovalenko, T. S. Lee, S. LeGrand, P. Li, C. Lin, T. Luchko, R. Luo, B. Madej, D. Mermelstein, K. M. Merz, G. Monard, H. Nguyen, H. T. Nguyen, I. Omelyan, A. Onufriev, D. R. Roe, A. Roitberg, C. Sagui, C. L. Simmerling, W. M. Botello-Smith, J. Swails, R. C. Walker,

- J. Wang, R. M. Wolf, X. Wu, L. Xiao, P. A. Kollman, *AMBER 2016*, University of California, San Francisco, **2016**.
- [42] D. Van Der Spoel, E. Lindahl, B. Hess, G. Groenhof, A. E. Mark, H. J. Berendsen, *J. Comput. Chem.* **2005**, *26*, 1701-1718.
- [43] W. D. Cornell, P. Cieplak, C. I. Bayly, I. R. Gould, K. M. Merz, D. M. Ferguson, D. C. Spellmeyer, T. Fox, J. W. Caldwell, P. A. Kollman, *J. Am. Chem. Soc.* **1995**, *117*, 5179-5197.
- [44] F.-Y. Dupradeau, A. Pigache, T. Zaffran, C. Savineau, R. Lelong, N. Grivel, D. Lelong, W. Rosanski, P. Cieplak, *Phys. Chem. Chem. Phys.* **2010**, *12*, 7821-7839.
- [45] R. Kumar, H. Grubmüller, *Bioinformatics* **2015**, *31*, 2583-2585.