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2′-Fluorination of Tricyclo-DNA Controls Furanose Conformation and Increases RNA Affinity

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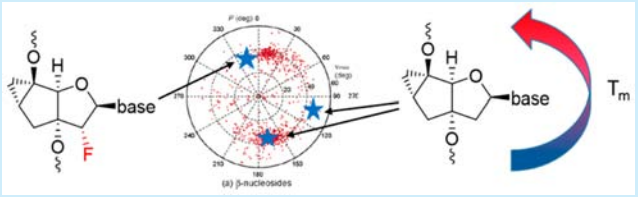
Supporting Information

ABSTRACT: The synthesis of 2′-fluoro tricyclo-DNA pyrimidine nucleosides with fluorine in the ribo-configuration and their incorporation into oligodeoxynucleotides was accomplished. Unlike the parent tc-T nucleoside, the 2′F-RNA-tc-T unit occurs in the 2′-exo conformation in the crystal. Specifically, F-RNA-tc-T was found to stabilize duplexes with RNA by +2 to +4 °C in T_m/mod. F-RNA-tc-nucleosides mix well with the DNA backbone and thus open up possibilities of using shorter and mixed-(DNA/tc-DNA) backbone oligonucleotides for therapeutic applications.



Therapeutic oligonucleotides bind to cellular RNA obeying the Watson−Crick rules, thereby controlling RNA function via a variety of antisense mechanisms.[1,2] From all oligonucleotide modifications that have been synthesized and biophysically characterized over the last three decades, the class of conformationally restricted oligonucleotide analogues undeniably shows the highest increase in affinity to complementary RNA and, in general, also shows a high degree of biostability. The best characterized examples for which in vivo data are available are the locked nucleic acid (LNA),[3−7] the hexitol nucleic acid (HNA),[8−11] and tricyclo(tc)-DNA.[12−17] Tc-DNA has recently been shown to exhibit very potent exon skipping properties in the context of Duchenne muscular dystrophy in the mdx and dko mouse models in all relevant tissues including heart, diaphragm, and brain after systemic delivery.[18]

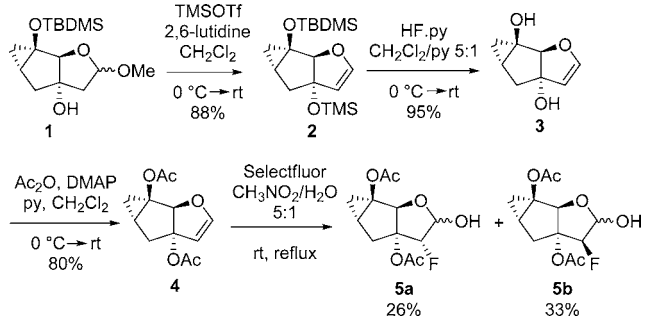
Fluorinated oligonucleotide analogues such as the 2′-deoxy-2′-fluoro-arabino nucleic acids (F-ANA),[19−21] the 2′-deoxy-2′-fluoro-RNA (F-RNA),[22] the fluorohexitol nucleic acids (F-HNA),[23,24] and other modifications[25−28] have recently attracted interest due to the electrostatic impact that the fluorine substituent exerts on the furanose conformation, leading in some cases to stronger RNA binding. In addition, they exhibit unique medicinal chemical properties arising from the polar hydrophobic properties of organofluorine compounds.[29−31] A striking example, highlighting the differential binding of fluorinated oligonucleotides to proteins, is the recruitment of an ILF 2/3 complex by a fluorinated antisense oligonucleotide to RNA transcripts in cellulo and in vivo, leading to alternative splicing patterns that are not observed with classical 2′-O-alkylated oligonucleotides.[32] Furthermore, the nuclear magnetic properties of ^{19}F offer additional and unique possibilities for structure determination as well as for in vivo imaging.

We reasoned that a fluorine substituent in the 2′-position of tc-nucleosides might exert additional steric and stereoelectronic control, driving the furanose unit within the tricyclic sugar scaffold toward the N-type conformation. From this, higher affinity to RNA can be expected. In addition, fluorine substitution may reinforce the favorable characteristics of tc-DNA such as high biostability and broad tissue distribution and could thus improve the therapeutic potential of tc-DNA. Here we present the synthesis and structural properties of the sugar component of F-tc-RNA and F-tc-ANA as well as two novel tc-nucleoside analogues (F-RNA-tc-T and F-RNA-tc-^{5Me}C) and the DNA and RNA binding properties of correspondingly modified oligodeoxynucleotides.

Synthesis of Monomers

A reasonable plan for the synthesis of the fluorinated tricyclo sugar involved the electrophilic fluorination of a suitably protected enol ether that should be easily accessible from the already known methyl glycoside **1** (Scheme 1).[33] From this procedure, we expected to have access to both sugars containing fluorine in the 2′-ribo and arabino configuration. TMSOTf-induced elimination of the methoxy group in **1** under

Scheme 1. Synthesis of the F-ANA- and F-RNA tc-Sugar Units 5



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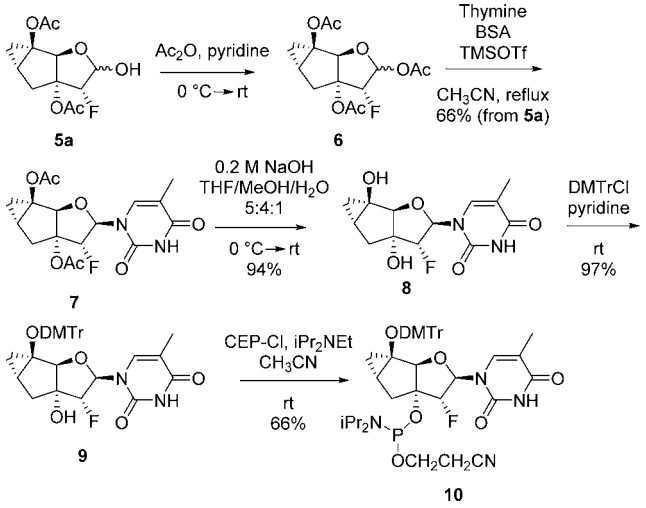
concomitant silylation of the tertiary hydroxyl group gave enol ether **2** in 88% yield. Since the silyl groups in **2** were considered to not withstand the conditions for fluorination, it was decided to change them into acetyl protecting groups via standard desilylation with HF·pyridine (→ **3**) followed by acetylation with Ac_2O to give intermediate **4** in 76% yield over two steps. Compound **4** was then subjected to fluorination with Selectfluor resulting in the two sugar building blocks with the fluoro substituent in either the ribo (**5a**) or the arabino (**5b**) configuration, roughly as a 1:1 mixture in 59% combined yield. The relative configuration of the fluorine substituents was unambiguously assigned by ^{1}H NMR–NOE experiments (see the Supporting Information).

The corresponding building block **10** with the fluorine in ribo-configuration was prepared starting from tricyclo sugar **5a** (Scheme 2). Compound **5a** was activated for nucleosidation by

Scheme 2. Synthesis of the F-RNA-tc-T Building Block 10

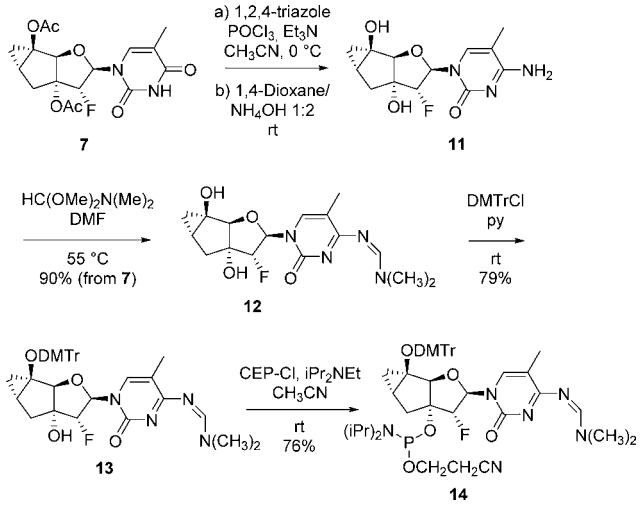


acetylation at the anomeric center (→ **6**) and subsequently converted into nucleoside **7** in high selectivity (α/β = 1:12) and good yield (66% over two steps). Full conversion of the starting material needed comparably harsh conditions (CH_3CN, reflux, 3 days). We therefore believe that the high β-stereoselectivity is the consequence of thermodynamic reaction control. Indeed, reaction in CH_2Cl_2 (reflux, 1 day) produced only 33% of nucleosides in an α/β ratio ratio of 1:2. The synthesis of building block **10** was then completed by standard removal of the acetyl protecting groups (→ **8**) followed by dimethoxytritylation (→ **9**) and phosphitylation. Final separation of the residual α-anomer was performed on the stage of the tritylated compound **9**.

Having substantial amounts of nucleoside **7** in hand enabled us to prepare the building block with the base 5-methylcytosine (Scheme 3). To this end, **7** was converted to the triazolide, which after treatment with ammonia gave the 5-methyl tc-cytidine derivative **11** in good yield. The 4-amino group was then dmf protected (→ **12**), and the synthesis of the phosphoramidite building block **14** was completed via dimethoxytritylation (→ **13**) followed by phosphitylation.

Despite several attempts, we were not able to get crystals of the corresponding F-RNA-tc-T nucleoside **8**. However, we were successful in crystallizing the 5′-O-TBS-protected derivative **15** (Figure 1). The furanose unit in **15** occurs in a perfect 2′-exo conformation (N-type conformation) with the

Scheme 3. Synthesis of the F-RNA-tc-^{5Me}C Building Block 14



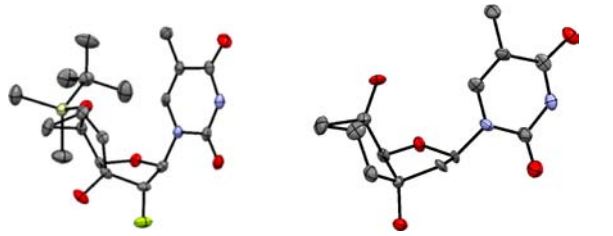


Figure 1. ORTEP plot (50% probability level) of (left) nucleoside **15** and (right) nonfluorinated tc-T[35] (one of the two molecules in the asymmetric unit). Hydrogen atoms are omitted for clarity.

fluorine substituent in the pseudoaxial position. The base is in the high-anti range, and the conformation of the furanose unit closely resembles that of 2′-deoxy-2′-fluororibothymidine (F-RNA-T) in RNA duplexes.[34] In comparison, the parent, nonfluorinated tc-T nucleoside coexists in a 2′-endo (S-type) and a O4′-endo (E-type) conformation in the crystal.[35] The X-ray structure of **15** therefore clearly shows that the fluorine substituent determines the furanose conformation, overriding the effect of the fused carbocyclic unit that does not lead to any significant discrimination of the N- and S-conformation. The absence of a vicinal coupling constant between HC(1′) and HC(2′) in the ^{1}H NMR spectra in all nucleoside derivatives **7**–**14** indicates a torsion angle between these protons of around 90°, which perfectly fits with the N-type conformation also in solution. It compares well with 2′F-RNA nucleosides, for which coupling constants of around or below 2 Hz were typically found.[36]

To determine the effect of fluorine substitution on DNA and RNA affinity we incorporated building blocks **10** and **14** into oligodeoxynucleotides via standard phosphoramidite chemistry (see the Supporting Information). The coupling yields of **10** and **14** were typically in the range of 97%. We then collected T_m data from UV-melting curves and compared them with oligodeoxynucleotides containing standard tc-T, tc-C, or tc-^{5Me}C units.

Oligodeoxynucleotides containing single or double substitutions of F-RNA-tc-T units (Table 1) strongly stabilize duplexes with DNA with ΔT_m values of +1.8 to +3.0 °C/mod and with RNA with ΔT_m values of +1.5 to +4.1 °C/mod. Interestingly, this is not the case for parent tc-T residues. This has been observed before for standard tc-nucleosides and has been

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Table 1. T_m Data of tc-T and F-RNA-tc-T-Modified Oligodeoxynucleotides in Complex with Complementary DNA and RNA[a]

	T_m (°C) vs DNA (ΔT_m/mod)		T_m (°C) vs RNA (ΔT_m/mod)	
sequence, X =	tc-T	F-RNA-tc-T	tc-T	F-RNA-tc-T
d(AACTGXCACG)	44.0 (0.0)	45.8 (+1.8)	43.9 (−0.1)	45.5 (+1.5)
d(AACXGTCACG)	44.0 (0.0)	47.0 (+3.0)	45.4 (+1.4)	48.1 (+4.1)
d(AACXGXCACG)	43.5 (−0.3)	47.7 (+1.9)	44.8 (+0.4)	50.1 (+3.1)
d(GCAXXXXXACCG)	46.5 (−0.1)	47.1 (0.0)	48.7 (+1.0)	49.7 (+1.2)

[a]Duplex concentration: 2 μM in 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.0.

Table 2. T_m Data of tc-C-, tc-^{5Me}C-, and F-RNA-tc-^{5Me}C-Modified Oligodeoxynucleotides in Complex with Complementary DNA and RNA[a]

	T_m (°C) vs DNA (ΔT_m/mod)			T_m (°C) vs RNA (ΔT_m/mod)		
sequence, X =	tc-C	tc-^{5Me}C	F-RNA-tc-^{5Me}C	tc-C	tc-^{5Me}C	F-RNA-tc-^{5Me}C
d(AAXTGTCACG)	46.0 (+2.0)	47.0 (+3.0)	47.1 (+3.1)	47.0 (+3.0)	48.2 (+4.2)	48.9 (+4.9)
d(AACTGTXACG)	46.0 (+2.0)	47.4 (+3.4)	47.1 (+3.1)	46.0 (+2.0)	46.2 (+2.2)	46.1 (+2.1)
d(AAXTGTXACG)	47.7 (+1.9)	50.8 (+3.4)	50.7 (+3.4)	49.0 (+2.5)	51.1 (+3.6)	52.1 (+4.1)

[a]Duplex concentration: 2 μM in 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.0.

attributed to unfavorable energetic contributions arising from the heterojunctions in the backbone.[37] Multiple consecutive substitutions also lead to stabilizing interactions although on a slightly lower level. A comparison of F-RNA-tc-T with standard tc-T modifications shows an additional stabilization of +2 to +4 °C/mod, solely attributable to the fluorine atom. We reason that this is largely due to additional conformational control of the furanose unit (2′-exo conformation) in F-RNA-tc-T, as it significantly exceeds the classical effect of 2′-fluorine substitution on RNA and DNA affinity, which has been determined to be around +0.6 °C/mod in systems where the fluorine atom (ribo configuration) does not significantly influence the conformation of the underlying sugar unit (e.g., in HNA and NMC).[25] The tendency of F-RNA-tc-T to drive duplexes with complementary DNA or RNA into a A-like conformation is also supported by corresponding CD spectra (Figure S1, Supporting Information).

With F-RNA-tc-^{5Me}C units (Table 2), the increase in T_m relative to standard tc-^{5Me}C units is close to 0 against DNA as complement and between −0.1 and +0.7 °C/mod with RNA as complement. Interestingly, the 5-methyl group on the base C contributes with roughly 1−1.5 °C/mod to thermal stability. It appears thus that for cytosine as base the increase in T_m attributable to the 2′-fluorine substituent is smaller compared to that of the T-series and is comparable to other carbohydrate-modified, fluorinated nucleic acid analogues.[25] This may have its origin in the higher intrinsic preference of tc-C for an N-type conformation compared to tc-T. Indeed, according to the vicinal coupling constants between HC(1′) and HC(2′α,β), the tc-C nucleoside shows the highest propensity to an N-type conformation of all four tc-nucleosides.[17,33]

In summary, substitution of the 2′-position in tc-nucleosides with fluorine exerts similar conformational effects on the furanose structure as in the 2′-deoxy-2′-fluoro nucleosides and thus overrides conformational features imposed by the tricyclic sugar scaffold. Forcing the furanose unit in tc-DNA in a N-type conformation, as for the F-RNA-tc-nucleosides, leads to additional thermal stability and relieves the energetic penalty that comes with heterobackbone junctions in single and multiple substituted oligodeoxynucleotides. The fluorine effect is particularly strong in the thymine nucleoside series, most likely due to more potent conformational correction compared to the cytosine-series. It seems to act in concert with stronger stacking/hydrogen bonding mediated by the trans-diaxial arrangement of the fluorine substituent and the glycosidic bond.[22,26] Thus, replacing tc-T units by F-RNA-tc-T units leads to the so far strongest RNA binders within the tricyclo-DNA family and opens up possibilities of using shorter and mixed-(DNA/tc-DNA) backbone oligonucleotides for therapeutic applications.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, and NMR spectra for all new compounds as well as HPLC traces and MS data of all modified oligodeoxynucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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