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Synthesis and Biophysical Characterization of RNAs Containing 2′-Fluorinated *Northern* Methanocarbacyclic Nucleotides

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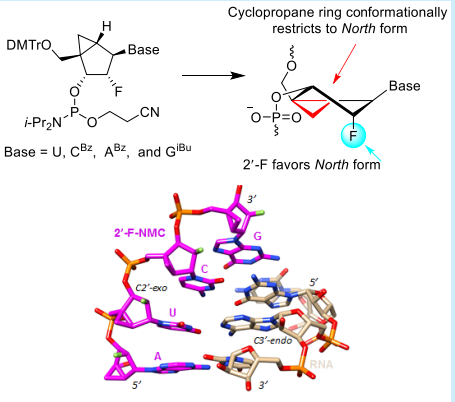
Supporting Information

ABSTRACT: 2′-Fluorinated *Northern* methanocarbacyclic (2′-F-NMC) nucleosides and phosphoramidites, based on a bicyclo[3.1.0]hexane scaffold bearing all four natural nucleobases (U, C, A, and G), were synthesized to enable exploration of this novel nucleotide modification related to the clinically validated 2′-deoxy-2′-fluororibonucleotides (2′-F-RNA). Biophysical properties of the 2′-F-NMC-containing oligonucleotides were evaluated. A duplex of 2′-F-NMC-modified oligonucleotide with RNA exhibited thermal stability similar to that of the parent RNA duplex, 2′-F-NMC-modified oligonucleotides had higher stability against 5′- and 3′-exonucleolytic degradation than the corresponding oligonucleotides modified with 2′-F-RNA, and 2′-F-NMC-modified oligonucleotides exhibited higher lipophilicity than the corresponding RNA oligonucleotides as well as those modified with 2′-F-RNA.



Therapeutics based on RNA interference (RNAi) have great potential for treating human diseases. Both US FDA and EMA have recently approved the first small interfering RNA (siRNA) drug, ONPATTRO (patisiran), for treatment of polyneuropathy caused by hereditary transthyretin-mediated amyloidosis.[1] The oligonucleotides used in oligonucleotides based therapeutics require use of unnatural nucleotide building blocks to stabilize the agents against nuclease degradation, to enhance cell-membrane permeability, and to limit immune responses.[2] The 2′-deoxy-2′-fluororibonucleotide (2′-F-RNA, Figure 1A) and 2′-*O*-methyl (2′-*O*Me) modifications have been used to modify siRNAs,[3−5] antisense oligonucleotides,[6,7] aptamers,[8] microRNAs,[9] and ribozymes.[10] Incorporation of 2′-F-RNA and 2′-*O*-Me residues stabilizes an A-form RNA duplex.[4,11] Specifically, 2′-F ribo-substitution preorganizes the sugar into a C3′-*endo* or *North* conformation and hence reduces the entropic penalty for the formation of the A-form duplex, and increases base stacking and Watson−Crick hydrogen-bond stabilities due to its electron-withdrawing power.[11] Furthermore, 2′-F-RNA-modified siRNAs have reduced immune stimulation and improved activity *in vitro* and *in vivo* compared to unmodified siRNA,[4] and are in clinical development. The 2′-F-RNA-modified oligonucleotides are, however, more sensitive to nucleolytic degradation than other 2′-modified oligonucleotides; moreover, these monomers are recognized, albeit poorly, by human RNA polymerases at high concentrations.[12,13] Hence, chemically modified building blocks that retain the advantages of 2′-F-RNA with additional features to overcome these limitations have the potential to improve pharmacological properties of oligonucleotides. A number of fluorine-containing building blocks have been

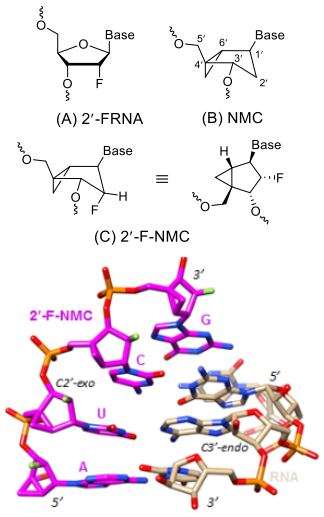


Figure 1. (A−C) Structures of (A) 2′-F-RNA, (B) NMC, and (C) 2′-F-NMC. (D) Model of a hybrid duplex between 2′-F-NMC and RNA constructed using the program UCSF Chimera; 2′-F-NMC residues have an idealized C2′-*exo* pucker with an axial 2′-fluorine.

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synthesized and evaluated in the context of oligonucleotide-based therapeutics.[14−29] However, to the best of our knowledge, none of these modifications have been made with all four nucleobases. As nucleotide pharmacology in a therapeutic oligonucleotide like siRNA depends on both sequence and position, it is necessary to have all four nucleosides with same ribosugar modification to evaluate its therapeutic potential.[30]

Conformationally restricted nucleic acids bearing bicyclic or tricyclic scaffolds that exhibit high affinity for complementary RNA are also being widely explored as potential therapeutic oligonucleotide modifications.[31,32] Nucleosides with a carbocyclic bicyclo[3.1.0]hexane system, here referred to as *Northern* methanocarbacyclic (NMC) nucleosides, are constrained to an RNA-like sugar pucker (Figure 1B).[33−38] The bicyclic NMC sugar is predicted to adopt a pseudoboat C2′-*exo* conformation due to the methylene bridge between the C4′ and C6′ positions. Consequently, NMC-modified oligonucleotides form more stable duplexes with RNA than do unmodified DNA.

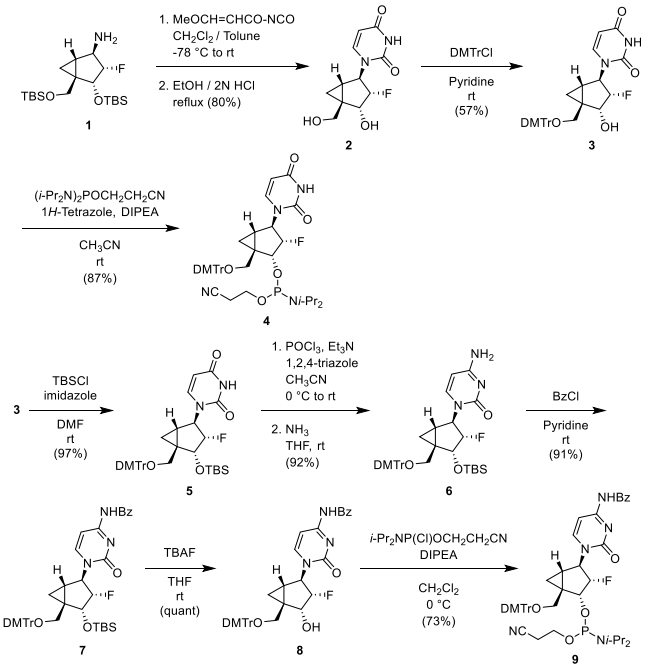
Jung and co-workers recently synthesized thymidine analogs 2′-F-NMC T (Figure 1C) and *ara*-2′-F-NMC T and oligonucleotides containing these building blocks.[18] Deoxyoligonucleotides containing 2′-F-NMC T have higher RNA binding affinity than oligonucleotides containing 2′-deoxy-2′-fluoro uridine or nonfluorinated NMC thymidine.[19] This duplex stabilization is presumed to result from stabilization of Watson−Crick hydrogen-bonding and base-stacking interactions due to the 2′-fluoro incorporation and demonstrates the potential of 2′-F-NMC analogs in oligonucleotide-based therapeutics.

Our laboratory has systematically evaluated the role of chemical modifications in siRNA activity. To assess 2′-F-NMC analogs in terms of their sequence- and position-dependent RNAi activity, we required all RNA nucleobase analogs. Here, we report the synthesis of 2′-F-NMC phosphoramidites bearing the four natural RNA nucleobases (i.e., A, U, G, C) from a common starting material in a convergent approach. We also report the binding affinities to a target RNA and susceptibilities to exonuclease degradation of oligonucleotides containing 2′-F-NMC.

The 2′-F-NMC U and 2′-F-NMC C phosphoramidites were synthesized as depicted in Scheme 1. The starting amine **1** was prepared according to the procedure reported by Jung et al.[18] and was coupled with 3-methoxyacryloyl isocyanate and cyclized under acidic conditions to afford the uridine derivative **2**.[39,40] The 5′-OH group of the nucleoside **2** was protected with 4,4′-dimethoxytriphenylmethyl chloride (DMTrCl) to give compound **3**. Subsequent phosphitylation of the 3′-OH group of **3** gave the desired phosphoramidite **4**. For the synthesis of 2′-F-NMC C, fully protected nucleoside **5** was obtained by *tert*-butyldimethylsilyl (TBS) protection of **3**. Compound **5** was then converted into the cytidine derivative **6** by reacting with 1,2,4-triazole in the presence of Et_3N and $POCl_3$, followed by treatment with aqueous NH_3. The exocyclic amine of **6** was benzoylated using benzoyl chloride (BzCl), and the resulting protected cytidine derivative **7** was treated with tetra-*n*-butylammonium fluoride (TBAF) to obtain alcohol **8**. Phosphitylation of **8** gave the desired phosphoramidite **9**.

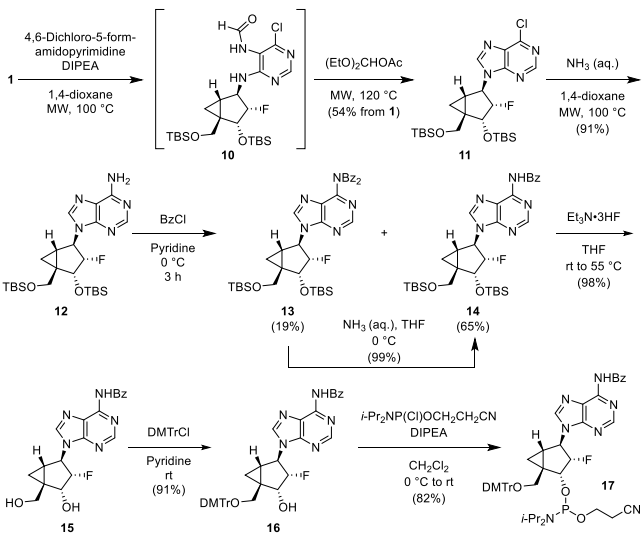
To synthesize the purine analogs, Jung et al. attempted to introduce the *N*-benzoyladenine base into the unsubstituted enone derivative of the NMC scaffold by 1,4-addition. The

Scheme 1. Synthesis of 2′-F-NMC U and C Amidites



yield of the reaction for this adenine precursor was low, and synthesis of 2′-F-NMC purine analogs was not pursued.[18] This prompted us to explore alternate routes. Nencka et al. reported a one-pot build-up procedure leading to 6-chloro- or 2-amino-6-chloropurines, which are, respectively, adenosine and guanosine precursors, from a primary amine.[41] Marquez et al. also achieved excellent yields of bicyclo[3.1.0]hexane carbocyclic nucleosides, which involve 6-chloropurines on a primary amine, using a microwave reactor.[42] Therefore, we evaluated the synthesis of the 2′-F-NMC purine phosphoramidites from the starting amine **1**.[18] As shown in Scheme 2, the reaction of **1** with 4,6-dichloro-5-formamidopyrimidine followed by cyclization of the formamido intermediate **10** in the presence of diethoxymethyl acetate gave the 6-chloropurine derivative **11** in 54% yield over two steps. Ammonolysis of the compound **11** using a microwave reactor

Scheme 2. Synthesis of 2′-F-NMC A Amidite



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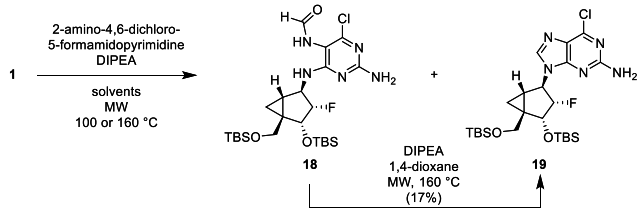
produced the desired adenine nucleoside **12** in 91% yield. The amino group was then protected with BzCl to furnish the dibenzoyl derivative **13** (19%) and monobenzoyl derivative **14** (65%). Compound **13** was readily converted to **14** by treatment with aqueous NH_3 in THF. The silyl protection was then removed by treating **14** with $Et_3N{\cdot}3HF$ at 55 °C, to obtain diol **15** in 98% yield. Subsequent dimethoxytritylation of the 5′-OH followed by phosphitylation of **16** yielded the desired phosphoramidite **17** (82%).

For the synthesis of 2′-F-NMC G, various conditions were evaluated for the preparation of the 2-amino-6-chloropurine intermediate **19** using the microwave-assisted reaction of the amine **1** with 2-amino-4,6-dichloro-5-formamidopyrimidine (Table 1). The reaction in 1,4-dioxane at 100 °C led to only

Table 1. Construction of 2′-F-NMC G Precursor 19

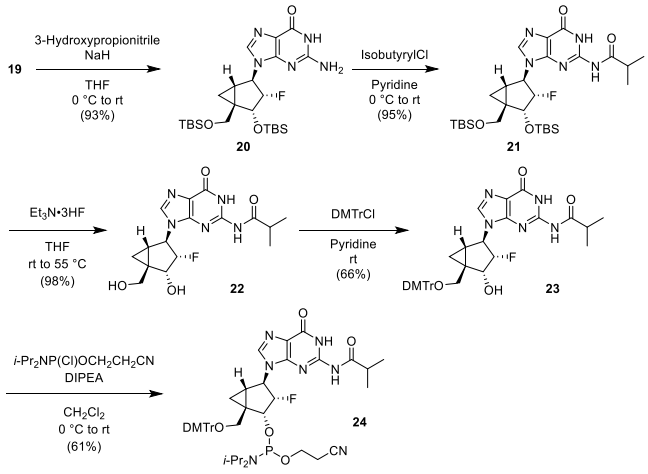


				yield (%)	
entry	solvent	temp (°C)	time (h)	**18**	**19**
1	1,4-dioxane	100	12	88	trace
2	toluene	160	2	70	24
3	*n*-BuOH	160	2	59	40

the intermediate **18** as a mixture of rotamers (entry 1). By heating at 160 °C in toluene, the intermediate **18** and the desired product **19** were obtained in 70% and 24% yield, respectively (entry 2). The yield of **19** was improved to 40% by changing the solvent to *n*-BuOH (entry 3). However, longer reaction times resulted only in substitution of 6-Cl groups by *n*-BuOH. Attempts to cyclize intermediate **18** in 1,4-dioxane by heating under microwave conditions resulted in disappointingly low yields (17%). The presence of the bulky 5′-*O*-TBS group may hinder imidazole ring closure, resulting in poor yields. Attempts to use diethoxymethyl acetate for the cyclization yielded a complex mixture, including **19**, as shown by LC-MS (data not shown). 2′-F-NMC G was eventually synthesized from the 2-amino-6-chloropurine derivative **19** by reacting with 3-hydroxypropionitrile in the presence of NaH (Scheme 3). Standard protection of exocyclic amine using isobutyryl chloride afforded protected nucleoside **21** (95%). Desilylation using $Et_3N{\cdot}3HF$ gave diol **22** in 98% yield. Dimethoxytritylation gave DMTr-protected compound **23** (66%), which was converted to the corresponding phosphoramidite **24** in 61% yield.

To gauge the effect of modified nucleotides on RNA affinity, the 2′-F-NMC modifications were incorporated into 10-mer and 12-mer oligoribonucleotides via standard solid-phase synthesis (see Supporting Information). The 2′-F-NMC-modified oligonucleotides were mixed with complementary RNA in PBS buffer, and the melting temperatures (T_m) were determined (Table 2). Modified duplexes containing a single 2′-F-NMC nucleotide at the center showed a decrease of at most 1.3 °C in the melting temperature compared to the unmodified duplex (Table 2, Entry 1−4). In a similar context, incorporation of 2′-F-RNA-modified nucleotides resulted in

Scheme 3. Synthesis of 2′-F-NMC G Amidite



slight improvements in the thermal stability. These experiments demonstrate that the 2′-F-NMC modification is well tolerated in an RNA duplex. The slight loss in thermal stability may prove advantageous in RNAi-based applications where high binding affinities in the seed region can induce undesirable off-target effects.[43] Importantly, a 10-mer duplex with six 2′-F-NMC nucleotides in the center displayed thermal stability comparable to that of the unmodified dsRNA (entry 5) suggesting that this modification is very well accommodated in an RNA:RNA duplex.

Table 2. UV Melting Temperatures of Modified Duplexes

entry	duplex	T_m[a] (ΔT_m) (°C)[b] F-NMC	2′-F
1	5′-UACAG**U**CUAUGU 3′-AUGUCAGAUACA	53.4 (−0.2)	54.1 (0.5)
2	5′-UACAGUCUAUGU 3′-AUGUC**A**GAUACA	53.4 (−0.2)	54.2 (0.6)
3	5′-UACAGU**C**UAUGU 3′-AUGUCAGAUACA	52.3 (−1.3)	54.3 (0.7)
4	5′-UACAGUCUAUGU 3′-AUGUCA**G**AUACA	53.1 (−0.5)	54.5 (0.9)
5[c]	5′-GC**GAUCUC**AC 3′-CGCUAGAGUG	57.4 (−0.2)	65.3 (7.7)

[a] T_m values were obtained in PBS (pH 7.4) using 2.0 μM concentrations of each strand. Red letters indicate sites of modification. [b] ΔT_m is the difference in melting temperature between the duplex with the modified strand and the unmodified reference duplex (5′-UACAGUCUAUGU-3′:3′-AUGUCAGAUACA-5′, T_m = 53.6 °C). [c] Unmodified reference duplex for entry 5 is 5′-GCGAUCUCAC-3′:3′-CGCUAGAGUG-5′ with T_m = 57.6 °C.

Next, the thermal stability of duplexes carrying 2′-F-NMC base pairs was determined (Table S2). Interestingly, in this context the $U^{F\text{-}NMC}$–$A^{F\text{-}NMC}$ base pair was better accommodated (a drop of only 0.4 °C compared to unmodified RNA) than a $C^{F\text{-}NMC}$–$G^{F\text{-}NMC}$ base pair (a drop of 2.2 °C compared to unmodified RNA).

The global conformations of RNA duplexes with one or two 2′-F-NMC nucleotides were evaluated using circular dichroism (CD) spectroscopy. The CD spectra of modified duplexes were comparable to that of the unmodified RNA and featured a strong positive band at around 260 nm and a negative band

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at around 210 nm characteristic of an A-form duplex (Figure 2). This indicates that the 2′-F-NMC modification does not significantly distort the global geometry of an RNA:RNA duplex.

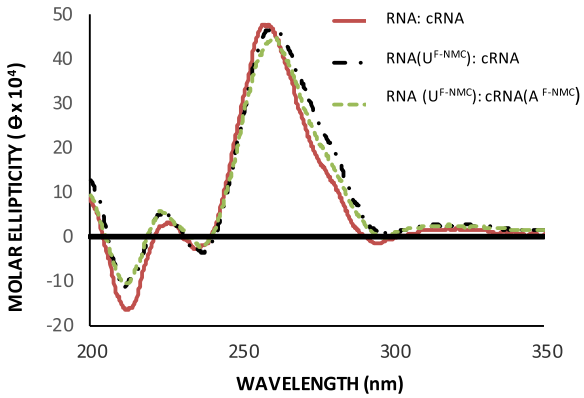

Figure 2. CD spectra of 2′-F-NMC-modified RNA and complementary RNA (cRNA) duplex at 15 °C in PBS (pH 7.4). See Table S3 for the details of the duplexes.

To determine the effect of the 2′-F-NMC modification on degradation of oligonucleotides by exonucleases, we incorporated 2′-F-NMC C ($C^{F\text{-}NMC}$) or 2′-F-RNA C (C^{F}) at the terminus or at the penultimate position of a dT oligonucleotide. The oligonucleotide modified at the 3′ end with 2′-F-NMC ($dT_{19}C^{F\text{-}NMC}$) was more resistant to degradation by snake venom phosphodiesterase (SVPD) than was the oligonucleotide modified with 2′-F-RNA (Figure 3A). The

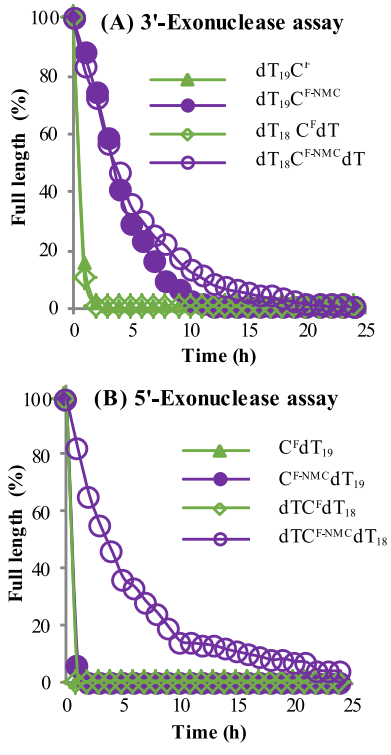

Figure 3. HPLC quantification of indicated full-length oligonucleotide after incubation with (A) SVPD and (B) PDE-II as a function of time. For $dT_{18}C^{F\text{-}NMC}dT$, the percentage of 19-mer $dT_{18}C^{F\text{-}NMC}$ remaining is plotted.

half-life of the 2′-F-NMC-modified oligonucleotide was around 90 min, whereas the half-life of $dT_{19}C^{F}$ was 15 min. The 3′-terminal dT in the oligonucleotide with the 2′-F-NMC residue at the penultimate position relative to the 3′ end ($dT_{18}C^{F\text{-}NMC}dT$) was lost within an hour; however, $dT_{18}C^{F\text{-}NMC}$ had a half-life of 170 min, whereas $dT_{18}C^{F}dT$ and $dT_{19}C^{F}$ had half-lives of around 15 min.

Oligonucleotides modified at the 5′ ends were incubated with the 5′-exonuclease phosphodiesterase II (PDE-II). Unlike the 3′-end-modified $dT_{18}C^{F\text{-}NMC}dT$, removal of the 5′-terminal dT was not observed when $dTC^{F\text{-}NMC}dT_{18}$ was incubated with PDE-II. Rather, the full-length 20-mer oligonucleotide with a penultimate $C^{F\text{-}NMC}$ was very stable with a half-life of 5 h (Figure 3B). Surprisingly, $C^{F\text{-}NMC}dT_{19}$ was degraded rapidly with a half-life of 12 min. This indicates that 2′-F-NMC modification improves stability at the penultimate position. Nevertheless, the 2′-F-NMC-modified oligonucleotides were more stable than 2′-F-RNA-modified oligonucleotides, as $C^{F}dT_{19}$ and $dTC^{F}dT_{18}$ were completely degraded by the first time point (1 h) after addition of PDE-II.

In summary, we present efficient routes for convergent synthesis of the four 2′-F-NMC ribonucleosides. 2′-F-NMC residues are well accommodated in double-stranded RNAs and do not alter the global structure of the duplex. 2′-F-NMC oligonucleotides are more resistant to nuclease degradation than are oligonucleotides modified with 2′-F-RNA. The higher lipophilicity of an oligonucleotide containing 2′-F-NMC residues compared to that with 2′-F-RNA residues (Figure S2) may result in improved cellular uptake and endosomal release of RNAs.[44] Having access to all four phosphoramidites will allow exploration of the full potential of 2′-F-NMC modification in therapeutic oligonucleotides such as siRNAs. These studies are ongoing in our laboratories.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b04153.

Experimental, compound characterization, and assays (PDF)

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Notes

The authors declare no competing financial interest.

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