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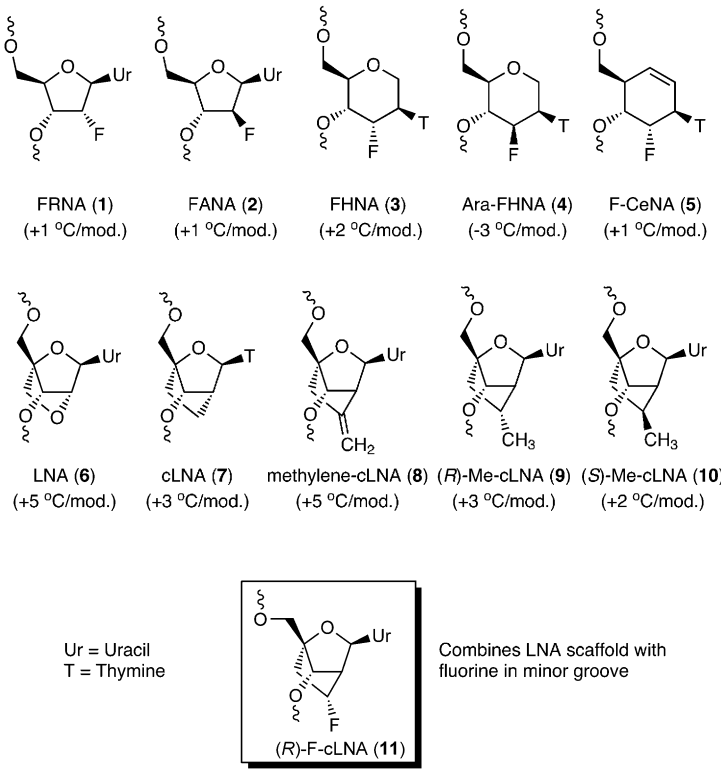
Synthesis, Duplex Stabilization and Structural Properties of a Fluorinated Carbocyclic LNA Analogue

Punit P. Seth,*[a] Pradeep S. Pallan,[b] Eric E. Swayze,[a] and Martin Egli[b]

Fluorine enjoys a privileged status in medicinal chemistry by virtue of its "polar hydrophobic" nature.[1] Fluorine has roughly the same atomic radius as hydrogen but it is highly electronegative. In the context of oligonucleotide medicinal chemistry, fluorine has been used as an isostere for the 2′-oxygen atom in RNA and also as an isostere for one of the 2′-hydrogen atoms in DNA, as exemplified by 2′-fluoro RNA (FRNA, **1**) and 2′-fluoro arabino nucleic acid (FANA, **2**) respectively (Scheme 1). DNA oligonucleotides modified with FRNA and FANA, show improved duplex thermal stability when paired with RNA complements relative to unmodified DNA. However, while FRNA increases oligonucleotide duplex thermal stability by increasing the strength of the Watson–Crick base-pairing and nucleobase-stacking interactions,[2] improved thermal stability of FANA-modified duplexes results from the formation of pseudo H···F bonds at pyrimidine–purine steps.[3]

Recent reports have unequivocally shown the beneficial effects of FRNA substitution on the biological properties of modified oligonucleotides for several antisense mechanisms.[4] We showed that introducing fluorine in an axial or equatorial orientation at the 3′-position of hexitol nucleic acid (HNA) provided analogues FHNA (**3**) and Ara-FHNA (**4**) respectively.[5] In thermal denaturation experiments, **3** had a stabilizing effect upon duplex thermostability whereas **4** was destabilizing. Using crystal structure data, we showed that the axial fluorine atom in **3** projects into the minor groove where it is well tolerated. In contrast, the equatorial fluorine in **4** experiences a steric clash with the electronegative 4′-oxygen atom of the 3′-adjacent nucleotide resulting in duplex instability. In animal experiments, FHNA-modified antisense oligonucleotides (ASOs) showed excellent activity for downregulating gene expression in mouse liver despite lower RNA affinity as compared to a benchmark locked nucleic acid (LNA, **6**) ASO control. We also showed that 2′-fluorocyclohexenyl nucleic acid (F-CeNA, **5**) behaves like a nuclease-stable mimic of **1** and exhibits significantly improved activity in animals in comparison to a sequence-matched 2′-*O*-methoxyethyl ASO with comparable RNA affinity.[6] In both cases, the improved activity with the fluorinated ASOs was attributed to improved functional uptake of the modified ASOs in liver tissue.



Scheme 1. Structures and relative duplex stabilization properties of DNA oligonucleotides modified with various fluorinated and cLNA analogues and paired with complementary RNA.

Given the excellent antisense activity observed with ASOs containing fluorinated nucleoside building blocks, we wanted to study the impact of introducing fluorine on the [2.2.1]dioxabicyclo ring scaffold of locked nucleic acid (LNA, **6**).[7] We envisaged that ASOs modified with such building blocks could have the dual advantage of high RNA affinity afforded by the LNA scaffold combined with the beneficial effects of fluorine substitution for facilitating tissue uptake. We further anticipated that the 2′,4′-bridge in LNA was an ideal position for incorporating fluorine as this would position the appended fluorine atom in the same general orientation (into the minor groove) as the 3′-fluorine atom in **3**. However, it is not feasible to introduce a fluorine atom directly on the 2′,4′-bridging group in LNA due to the synthetic difficulties associated with assembling a bicyclic geminal fluorinated ether. To circumvent these issues, we

[a] *Dr. P. P. Seth, Dr. E. E. Swayze*
Department of Medicinal Chemistry, Isis Pharmaceuticals
2855 Gazelle Court, Carlsbad, CA 92010 (USA)
E-mail: pseth@isisph.com

[b] *Dr. P. P. S. Pallan, Prof. M. Egli*
Department of Biochemistry, Vanderbilt University School of Medicine
Nashville, TN 37232-0146 (USA)

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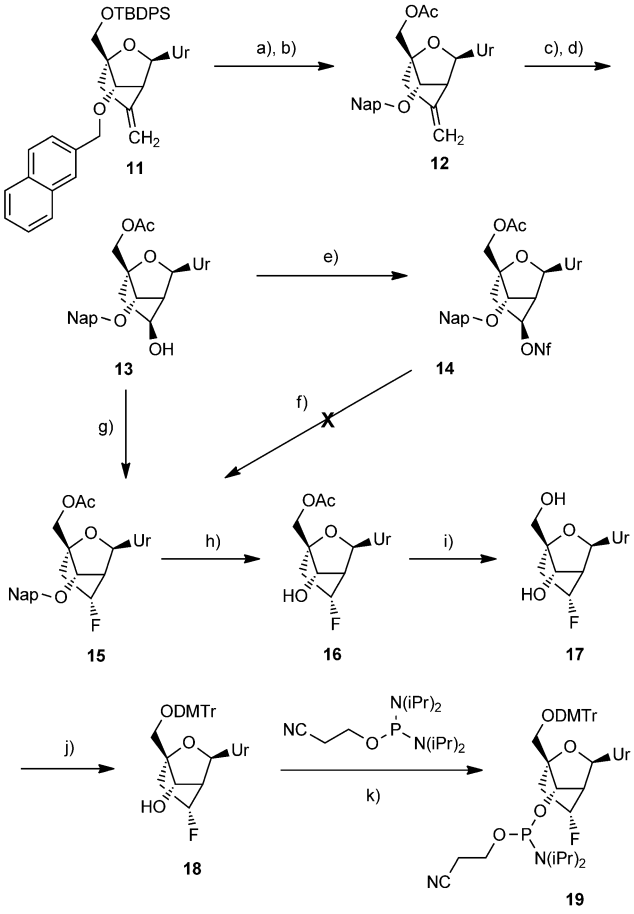
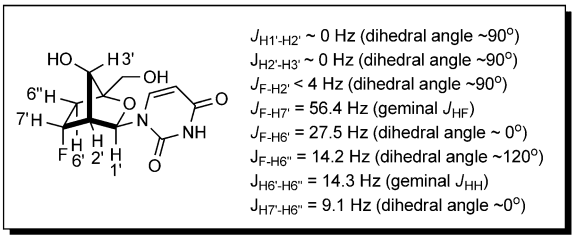
examined the feasibility of introducing fluorine on the 2′,4′-bridge of carbocyclic LNA (cLNA, **7**) where the 2′-oxygen atom in LNA is replaced with a carbon atom (Scheme 1).

cLNA represents an interesting class of nucleic acid analogues of which several members have been synthesized by the Chattopadhyaya group.[8] The majority of cLNA analogues reported in the literature show slight to significant decrease in binding affinity for complementary nucleic acids relative to LNA. The RNA affinity of cLNA-modified oligonucleotides can be partially restored by introducing polar functionality on the 2′,4′-bridge of these analogues.[9] The polar functionality helps to restore the water of hydration network in the minor groove which is important for maintaining the stability of oligonucleotide duplexes.[10] Interestingly, the cLNA analogue **8**, in which the 2′-oxygen atom was replaced with a exocyclic methylene group, showed LNA-like duplex stabilization properties while the corresponding saturated analogues (**9** and **10**) were less stabilizing.[11] Using crystal structure data, we showed that the exocyclic methylene group retains net negative electrostatic potential at the brim of the minor groove and participates in CH···O type interactions with acceptor molecules in the minor groove of the modified duplex. In contrast, the saturated cLNA analogues **9** and **10** were unable to extend the water of hydration network around the backbone phosphates and the nucleobases resulting in diminished duplex stabilizing properties. Given this background, we hypothesized that a fluorinated cLNA analogue such as **11** (Scheme 1) could exhibit improved duplex stabilizing properties as a consequence of introducing a highly electronegative fluorine atom in the vicinity of the 2′-carbon atom of the nucleoside furanose ring.[2a] In addition, oligonucleotides modified with such building blocks could benefit from fluorine substitution for potential therapeutic applications. In this report, we describe the synthesis, biophysical and structural evaluation of oligonucleotides modified with a fluorinated cLNA analogue **11**.

Synthesis of the fluorinated cLNA phosphoramidite **19** was initiated from the known nucleoside intermediate **11** (Scheme 2).[11] Attempts to cleave the exocyclic double bond in **11** using osmium tetroxide and sodium periodate gave poor conversions to the intermediate ketone. Presumably, the bulky *tert*-butyldiphenylsilyl (TBDPS) and 2-naphthylmethyl (Nap) protecting groups on the 5′- and the 3′-hydroxyl groups create steric crowding and limit access of the catalyst to the exocyclic methylene group in **11**. To address this, the TBDPS group was removed using tetra-*n*-butylammonium fluoride (TBAF) and the 5′-hydroxyl group was re-protected as the acetate ester to provide **12**. In the absence of the 5′-*O*-TBDPS protecting group, oxidative cleavage of the double bond was accomplished using osmium tetroxide and sodium periodate in moderate yield (~50% for two steps). Attempts to improve the yield by optimizing the reaction conditions were unsuccessful because of oxidative decomposition caused by reaction of osmium tetroxide with the C5=C6 double bond in the uracil nucleobase. The intermediate ketone was typically not isolated but directly reduced with sodium borohydride in methanol at −78 °C to yield the alcohol **13** as a single diastereoisomer. Presumably, attack of the hydride nucleophile occurs from the less hindered *re* face of the intermediate ketone away from the bulky 3′-*O*-Nap protecting group.





Scheme 2. Synthesis of (*R*)-F-cLNA Uracil phosphoramidite **19**. a) TBAF, THF, RT, 16 h, 63%; b) Ac_2O, pyridine, RT, 16 h, 95%; c) OsO_4, $NaIO_4$, 1,4-dioxane, H_2O, RT, 16 h; d) $NaBH_4$, MeOH, -78 °C to RT, 16 h, 46% (2 steps); e) $CF_3(CF_2)_3SO_2F$, DBU, THF; f) CsF, *t*BuOH, 50 °C, 4 h; g) DAST, CH_2Cl_2, 0 °C, 3 h, 10%; h) DDQ, CH_2Cl_2, RT, 16 h, 65%; i) NH_3, MeOH, RT, 3 h; j) DMTrCl, pyridine, RT, 16 h, 70% (2 steps); k) 2-Cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, DMF, NMI, 1*H*-tetrazole, RT, 8 h, 73%.

Fluorination of the secondary hydroxyl group in **13** was very problematic. Use of nonafluorobutanesulfonyl fluoride[12] provided the nonaflate intermediate **14** which was recalcitrant to further displacement with fluoride. Triflation of the secondary alcohol in **13** followed by reaction with cesium fluoride in *tert*-butanol[13] at elevated temperatures did not yield the desired fluorinated nucleoside and only decomposition prevailed. Successful fluorination was finally achieved by reacting **13** with diethylaminosulfur trifluoride (DAST) in dichloromethane at 0 °C to provide **15** as a single diastereoisomer, presumably through

an S_N2 displacement of the secondary hydroxyl group in **13**. However, attempts to scale up this reaction on a gram scale lead to significant decomposition and only trace amounts of the fluorinated nucleoside **15** could be isolated after purification by silica gel chromatography. The Nap group was next removed using 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in dichloromethane[14] to provide nucleoside **16**. Further removal of the 5′-acetyl group to provide **17**, followed by selective protection of the 5′-hydroxyl group with 4,4′-dimethoxytrityl chloride (DMTrCl) provided nucleoside **18**. A phosphitylation reaction provided the desired phosphoramidite **19**.

The precise orientation of the fluorine atom in **17** was determined by examination of the fluorine–proton coupling constants (Scheme 2). The H1′ and H3′ protons in **17** appear as singlets, which is consistent with the nucleoside furanose ring being locked in the C3′-endo conformation (dihedral angles ~90°). H2′ shows a small coupling (H2′–F dihedral angle ~90°) to the neighboring fluorine atom and appears as a narrow doublet (J = 4.0 Hz). In contrast, fluorine shows a large geminal coupling to H7′ (J = 56.4 Hz), a large coupling to H6′ (J = 27.5 Hz, dihedral angle ~0°) and a smaller coupling to H6″ (J = 14.2 Hz, dihedral angle ~120°) which is consistent with an axial orientation for the fluorine atom.

Synthesis of the modified oligonucleotides was carried out on polystyrene resin using standard conditions for incorporation of the DNA monomers and an extended coupling time for introducing the modified nucleoside phosphoramidite **18** under conditions described previously.[14b] After the synthesis was complete, the cyanoethyl protecting groups on the backbone phosphodiesters were removed by using triethylamine. Removal of the heterocyclic protecting groups and cleavage from the solid support was accomplished by heating with aqueous ammonia at 55 °C. Purification of the oligonucleotide using ion-exchange chromatography followed by desalting on C18 cartridges provided the desired oligonucleotide for evaluation in thermal denaturation (T_m) experiments.

Biophysical evaluation of the (*R*)-F-cLNA **11** modified oligonucleotide (A2, ΔT_m +2.9 °C per modification) showed that this modification exhibited similar duplex stabilizing properties as the (*R*)-Me-cLNA, **9** (A3, ΔT_m +2.9 °C/mod.) and the (*S*)-Me-cLNA **10** (A4, ΔT_m +2.0 °C/mod.) but improved properties relative to the **3**-modified oligonucleotide (**A5**, ΔT_m +0.6 °C/mod.; Table 1). The improved hybridization of **11** relative to **3** is a consequence of locking the furanose ring in the RNA-like C3′-endo sugar pucker. The hexitol ring system in **3** mimics the RNA-like C3′-endo sugar pucker but this scaffold is conformationally more dynamic as compared to the locked sugar moieties in LNA and related analogues. However, the duplex stabilizing property of analogue **11** was clearly reduced as compared to LNA **6** (A6, ΔT_m +4.5 °C/mod.) and methylene-cLNA **8** (A7, ΔT_m +4.8 °C/mod.).

We next examined the structural properties of a self-complementary DNA duplex modified with the F-cLNA analogue **11** to ascertain if the fluorine atom can participate in interactions with acceptor molecules in the minor groove. For the crystallographic studies, we chose the DNA decamer of sequence 5′-d(GCGTA**U***ACGC)-3′ with (*R*)-F-cLNA (**U***) replacing thymidine at position 6 (nucleotides in strands 1 and 2 of the duplex are numbered 1–10 and 11–20, respectively). We previously observed that this decamer adopts an A-form conformation if at least one of the 2′-deoxynucleotides is replaced with a 2′-substituted residue.[11,15] In addition, we relied on the same sequence in our studies of the conformational properties of **3** and **4**[5a] as well as of **5**.[6] The decamer was crystallized and its structure determined at 1.80 Å resolution. Final electron density is shown in Figure 1 A and selected crystal data and final refinement parameters are listed in Table S1 in the Supporting Information (PDB ID: 4HQH).

Table 1. Sequence, analytical data and duplex stabilizing properties of modified oligonucleotides evaluated in thermal denaturation experiments.

ODN	Modification	Sequence (5′→3′)[a]	T_m [°C]	ΔT_m/mod. [°C][b]
A1	DNA	d(GCGTTTTTTGCT)	45.6	control
A2	F-cLNA	d(GCGTT<u>U</u>TTTGCT)	48.5	+2.9
A3	(*R*)-Me-cLNA[c]	d(GCGTT<u>U</u>TTTGCT)	48.5	+2.9
A4	(*S*)-Me-cLNA[c]	d(GCGTT<u>U</u>TTTGCT)	47.6	+2.0
A5	FHNA	d(GCGTT<u>U</u>TTTGCT)	46.2	+0.6
A6	LNA[c]	d(GCGTT<u>U</u>TTTGCT)	50.1	+4.5
A7	methylene-cLNA[c]	d(GCGTT<u>U</u>TTTGCT)	50.4	+4.8

[a] Underlined letter indicates modified nucleotide. [b] T_m values were measured in sodium phosphate buffer (10 mM, pH 7.2) containing NaCl (100 mM) and EDTA (0.1 mM). Sequence of RNA complement: 5′-r(AGCAAAAAACGC)-3′. [c] Ref. [11].

As expected, the duplex adopts an A-form conformation with average values for helical rise and twist of 2.9 Å and 31°, respectively. All sugars except for those of residues G11 and G13 adopt a C3′-*endo* pucker; both G11 and G13 exhibit a C2′-*exo* pucker. The backbone torsion angles of both nucleotides U*6 and U*16 fall into the standard $sc^-/ap/sc^+/sc^+/ap/sc^-$ ranges for α to ζ. P···P distances of 5.64 Å (**U***6pA7 step) and 5.61 Å (**U***16pA17 step) are consistent with the A-form character of the backbone. Consistent with previous crystal structures of DNA duplexes modified with cLNA analogues **8**, **9** and **10**,[11] A5 is the only residue in the duplex that displays an extended variant of the backbone with torsion angles α and γ both in the *ap* range rather than the standard sc^- and sc^+ conformations, respectively.

Fluorine atoms jut into the minor groove but do not engage in lattice interactions with atoms from neighboring duplexes (Figure 1 B). Water molecules in the minor groove are hydrogen bonded to acceptors from the base and sugar moieties whereas distances between water and fluorine are inconsistent with hydrogen bond formation (Figure 1 B). This observation is consistent with the conclusion based on crystal structures of FRNA duplexes that showed fluorine to be a poor hydrogen-bond acceptor in the minor groove.[2b] Superimposition of the modified base-pair steps from the decamer containing LNA-Ts[16] (Figure 1 C) and the corresponding decamer with (*R*)-F-cLNA-Us indicates nearly identical conformations (Figure 1 D).

In conclusion, we report the synthesis, biophysical and structural evaluation of oligonucleotides modified with a fluorinated cLNA analogue. We show that fluorine substitution does not

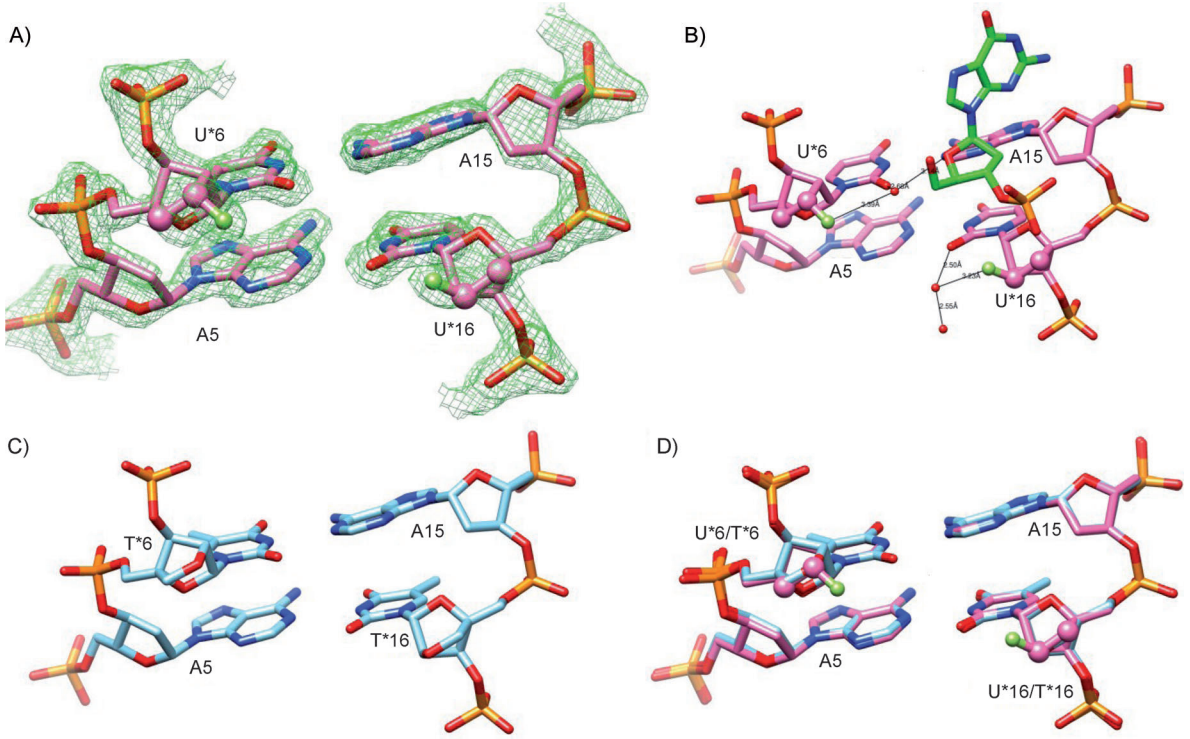


Figure 1. A) The central A5pU*6:A15pU*16 base-pair step (U* = (*R*)-F-cLNA-U) viewed into the minor groove. The green meshwork represents Fourier $2F_o$-F_c sum electron density drawn at the 1.2 σ level. Carbon, oxygen, nitrogen and phosphorus atoms are colored in pink, red, blue and orange, respectively, and C6′, C7′ and F7′ in the modified uridines are highlighted as spheres. B) Solvent environment of fluorine atoms in the minor groove. Hydrogen bonds between water molecules (small red spheres) and minor-groove acceptors are indicated with thin lines. Residue G1 from an adjacent duplex is shown with carbon atoms colored in green. Relatively long distances of > 3.3 Å between fluorine and water are inconsistent with hydrogen bond formation. C) The central A5pT*6:A15pT*16 (T* = LNA-T) in the crystal structure of the LNA-modified decamer viewed into the minor groove (PDB ID: 1I5W).[16] Carbon, oxygen, nitrogen and phosphorus atoms are colored in light blue, red, blue and orange, respectively. D) Superimposition of the (*R*)-F-cLNA-modified A5pU*6:A15pU*16 and LNA-modified A5pT*6:A15pT*16 base-pair steps. The root-mean-square deviation between the two fragments amounts to 0.63 Å.

confer added duplex stabilization beyond that imparted by locking the conformation of the furanose ring in the C3′-endo conformation using the 2′,4′-carbocyclic bridging group. A recent study of FRNA-modified duplexes using NMR and thermodynamic data showed that the axial fluorine atom at the 2′-position polarizes the nucleobase and increases RNA affinity by favorably affecting Watson–Crick base-pairing and stacking interactions.[2a] Taken together with our results, these observations collectively suggest that increased electronegativity in proximity to the 2′-position on the furanose ring alone is not sufficient for polarization of the nucleobase and underscore the importance of the anti-periplanar orientation of the nucleobase and the 2′-electron-withdrawing-group for optimal duplex stabilization, even for nucleic acid analogues with conformationally locked furanose rings (Figure 2).

In the context of cLNA analogues, replacing the electron withdrawing 2′-oxygen atom of LNA with a saturated carbon atom provides analogues that are less stabilizing than LNA. This suggests that the slight electron donating nature of the saturated carbon substituent at the 2′-position doubly handicaps these analogues as they lack the nucleobase polarization imparted by the 2′-oxygen atom and also suffer from reduced duplex hydration in the minor groove.[11] Nonetheless, fluorinated cLNA analogues such as **11**, present the opportunity to investigate the effect of combining fluorine in the minor groove of LNA duplexes with increased duplex stability (relative to FHNA and F-CeNA) on the antisense properties of modified oligonucleotides. However, a more efficient synthesis of the fluorinated nucleoside phosphoramidite is required before a complete investigation of the biological properties of fluorinated cLNA analogues can be completed. These efforts are ongoing and results will be reported in due course.

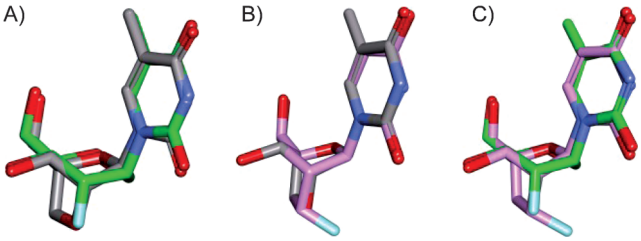


Figure 2. Overlay of A) FHNA (green carbons) with LNA (grey carbons) B) F-cLNA (pink carbons) and LNA (grey carbons) C) FHNA (green carbons) and F-cLNA (pink carbons) showing that the fluorine atom in F-cLNA, unlike the 3′-F atom in FHNA or the 2′-oxygen atom in LNA, is not anti-periplanar to the nucleobase.

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Keywords: carbocyclic LNA · duplex stability · fluorinated nucleosides · nucleic acids · X-ray crystallography

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