Studies on the hydrolytic stability of 2′-fluoroarabinonucleic acid (2′F-ANA)

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The stability of 2′-deoxy-2′-fluoroarabinonucleic acid (2′F-ANA) to hydrolysis under acidic and basic conditions was compared to that of DNA, RNA and 2′F-RNA. In enzyme-free simulated gastric fluid (pH ~1.2), 2′F-ANA was found to have dramatically increased stability (virtually no cleavage observed after 2 days) with respect to both DNA ($t_{1/2}$ ~ 2 min) and RNA ($t_{1/2}$ ~ 3 h (PO) or 3 days (PS)). These results were observed for both phosphodiester and phosphorothioate backbones and with multiple mixed-base sequences. Under basic conditions, 2′F-ANA also showed good stability. In 1 M NaOH at 65 °C, 2′F-ANA had a $t_{1/2}$ of ~20 h, while RNA was entirely degraded in a few minutes. Furthermore, the nuclease cleavage of phosphorothioate 2′F-ANA and DNA by snake venom phosphodiesterase was studied in detail. One diastereomer of the PS-2′F-ANA linkage was found to be much more vulnerable to enzymatic cleavage than the other, which is parallel to the properties observed for PS-DNA. Additional studies of 2′F-ANA-containing oligonucleotides are warranted based on the excellent stability properties described here.

Introduction

Since the development of 2′-deoxy-2′-fluoroarabinonucleic acid (2′F-ANA),[1,2] its useful properties have continued to be discovered. Hybrids of 2′F-ANA and RNA are substrates of RNase H,[3] and 2′F-ANA-containing oligonucleotides have shown increased efficacy and prolonged activity in gene silencing through the antisense strategy.[4] 2′F-ANA shows increased binding to RNA and DNA[3,5,6] and improved nuclease resistance.[5,7] Two 2′F-ANA-containing phosphorothioate antisense oligonucleotides have recently received approval to begin clinical trials for the treatment of chronic obstructive pulmonary disease.[8] More recently we have shown that 2′F-ANA-modified RNA duplexes are substrates of the RISC complex; partial 2′F-ANA modification can thus improve the properties of siRNAs.[9,10] Various DNA polymerases recognize both 2′F-ANA triphosphates and a 2′F-ANA template.[11] The structure of 2′F-ANA is shown in Fig. 1.

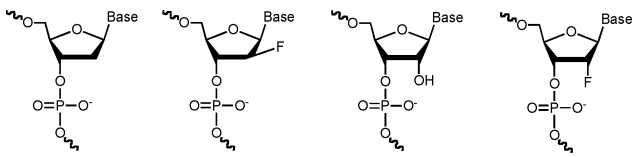

Fig. 1 Structures of DNA, 2′F-ANA, RNA and 2′F-RNA.

This paper explores the hydrolytic cleavage of 2′F-ANA in detail. We first present an investigation of the remarkable hydrolytic stability of 2′F-ANA under acidic conditions. Secondly, we examine alkaline cleavage of 2′F-ANA. Finally, we explore the stereochemistry of PS-2′F-ANA cleavage by snake venom phosphodiesterase (SVPDE), and report on the remarkable nuclease stability of a particular 2′F-ANA-DNA dinucleotide.

Results and discussion

Acid-catalyzed hydrolysis of nucleic acids

DNA oligonucleotides are vulnerable to depurination and cleavage under acidic conditions (Fig. 2).[12–14] Acid-catalyzed depyrimidination can occur as well, but several orders of magnitude more slowly.[15] The final step (β-elimination) can occur under basic conditions or in the presence of heat and salt.[14]

RNA is much less vulnerable to depurination since the electronegative 2′ oxygen renders the oxocarbonium ion intermediate less stable.[16,17] However, RNA can undergo 3′–2′-migration or cleavage under acidic conditions through attack of the 2′-hydroxyl group on the protonated phosphate group (Fig. 3).[18]

Because of the very high electronegativity of fluorine, the oxocarbonium ion intermediate is even less stable for 2′-fluorinated nucleosides, protecting them from depurination.[19] 2′-Fluorinated nucleosides have therefore been proposed as acid-stable nucleoside drugs.[19,20] One of these drugs, clofarabine, is now approved for treatment of pediatric leukemia.[21] Since enzymatic depurinations also proceed through a cation-like transition state, 2′-fluorinated nucleosides and oligonucleotides have been useful here too, for example, as inhibitors of base-excision repair enzymes.[22] 2′-Fluorinated oligonucleotide phosphoramidates have also shown increased acid-resistance relative to unmodified phosphoramidates.[23]

Because of the past success of 2′-fluorinated *nucleosides*, we predicted that 2′F-ANA *oligonucleotides* would show resistance to acid-mediated depurination, and because of the absence of a 2′-hydroxyl group, they would not be vulnerable to the phosphate attack observed for RNA. Thus, we compared the acid stability of

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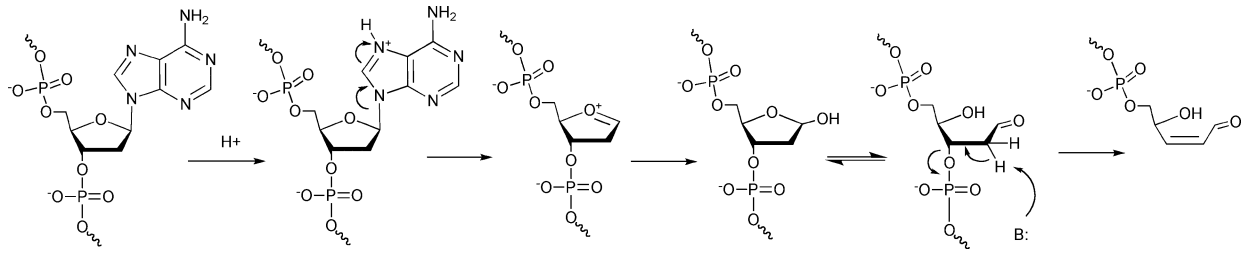

Fig. 2 Mechanism of acid-catalyzed depurination and cleavage in DNA, shown for adenine base but which can also occur with guanine.[13] Protonation at N-7 can allow breakage of the N-glycosidic bond.[16] The resulting structure is in equilibrium with an open-chain aldehyde form[14,38] and can undergo β-elimination. In the case of 2′F-ANA, the presence of fluorine at the 2′-position destabilizes the oxocarbonium ion intermediate and disfavors depurination, largely preventing this mechanism of degradation.

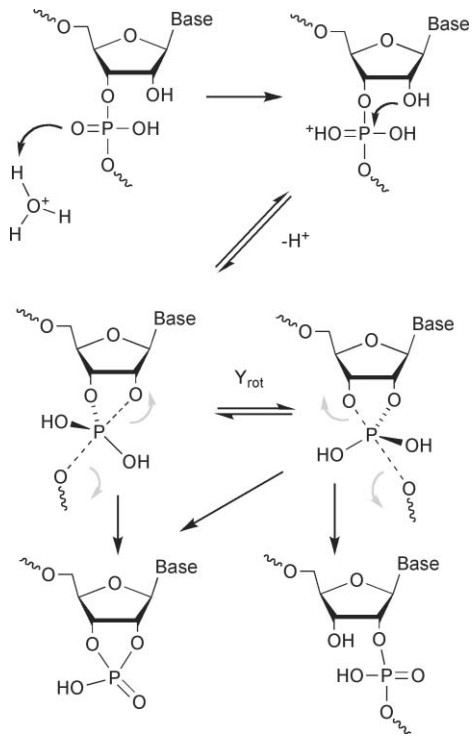

Fig. 3 Mechanism of RNA cleavage and 3′–2′-migration under strongly acidic conditions (pH < 2).[18] The grey arrows indicate alternate pathways for the breaking of the pentavalent phosphate. During phosphate hydrolysis, groups can only enter and leave through apical positions (dashed lines). Under acidic conditions, pseudorotation (Ψ_{rot}) is favorable, allowing 3′–2′ phosphate migration (rightmost structures) as well as cleavage.[39]

2′F-ANA, DNA and RNA oligonucleotides with either phosphodiester or phosphorothioate backbones.

2′F-ANA is highly resistant to acidic hydrolysis

Sequences of DNA, RNA and 2′F-ANA were prepared (Table 1) and exposed to enzyme-free simulated gastric fluid[24] for varying amounts of time. Aliquots were removed, frozen in dry ice and immediately lyophilized to dryness, then analyzed by PAGE. Results are shown in Fig. 4.

The DNA strands were degraded very rapidly, with half-lives on the order of minutes. RNA strands were longer-lasting, with half-lives on the order of hours (PO linkages) or days (PS linkages).

Table 1 Strands prepared for the acid stability study. Legend: dna, RNA, ***2′F-ANA***, **2′F-RNA**. Abbreviations: PO, phosphodiester; PS, phosphorothioate. Note that although sequences **Ac1**–**Ac6** are self-complementary, no duplex formation was observed under the acidic conditions of simulated gastric fluid

Name	Description	Sequence (5′–3′)
Ac1	PO-RNA	PO(AGCUAGCU)
Ac2	PO-2′F-ANA	PO(***AGCTAGCT***)
Ac3	PO-DNA	PO(agctagct)
Ac4	PS-RNA	PS(AGCUAGCU)
Ac5	PS-2′F-ANA	PS(***AGCTAGCT***)
Ac6	PS-DNA	PS(agctagct)
Ac7	PO-RNA	PO(AGAUUGGAGAAGGCUUGUAUU)
Ac8	PO-2′F-ANA	PO(***AGATTGGAGAAGGCTTGTATT***)
Ac9	PO-2′F-RNA	PO(**TCCTTCTC**)
Ac10	PO-2′F-ANA	PO(***TCCTTCTC***)
Ac11	PO-DNA	PO(tccttctc)
Ac12	PO-RNA	PO(UCCUUCUC)

2′F-ANA strands, however, showed minimal degradation up to 46 h.

We confirmed the integrity of the PO-FANA 21-mer **Ac8** by neutralizing a portion of the reaction mixture at 48 h using a phosphate buffer, desalting and analyzing by ESI-MS. More than 95% of the resulting peaks corresponded to full-length product with no evidence of depurination. As a further test of the integrity of the acid-treated **Ac8**, we neutralized and desalted it, annealed it to complementary RNA, and verified the T_m of the resulting duplex, which was unchanged with respect to untreated **Ac8**.

A dramatic improvement in the acid stability of RNA was obtained upon phosphorothioation. This is consistent with the results of Lonnberg and coworkers, who explain the greater stability of PS-RNA to strong acid by noting that the stronger acidity of a P(V)SH$^+$ moiety would disfavor the monocationic cleavage mechanism adopted by PO-RNA at very low pH (Fig. 3).[18,25] The increased stability is particularly striking in light of the fact that replacing a *bridging* oxygen by sulfur leads to decreased acid stability.[26] As for DNA, thioation of the phosphodiester leads to slightly increased resistance to acid hydrolysis, but it is beyond the scope of this study to examine whether this is due to slower depurination or slower β-elimination.

We attempted to compare the stability of 2′F-ANA and its 2′-epimer, 2′F-RNA, to acid. However, only the pyrimidine monomers were available to us at the time, and pyrimidines are much more resistant to acid-catalyzed cleavage. After three months in acid at 55 °C, 2′F-ANA or 2′F-RNA pyrimidine sequences

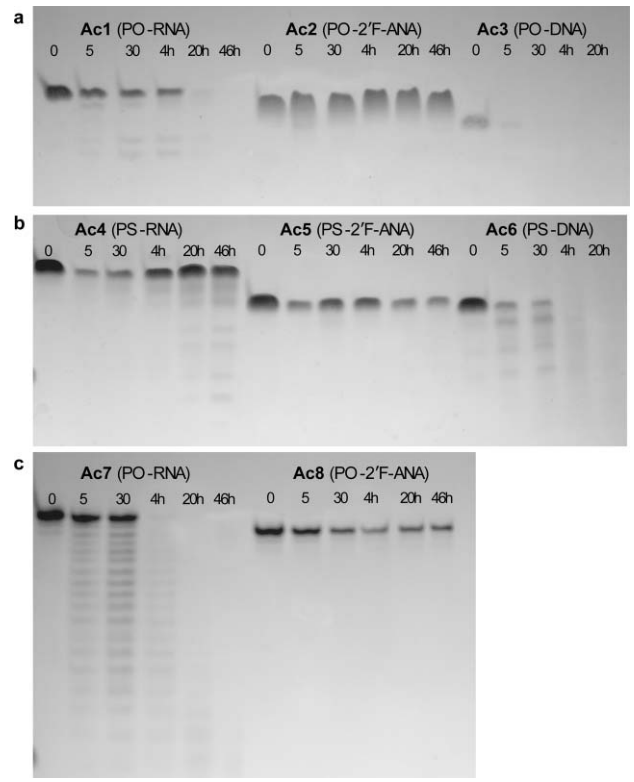

Fig. 4 24% Denaturing PAGE comparing the acid stability of DNA, RNA and 2′F-ANA to simulated gastric fluid at 37 °C. (a) PO sequences **Ac1**–**Ac3**; (b) PS sequences **Ac4**–**Ac6**; (c) 21mer PO sequences **Ac7**–**Ac8**. Gels were developed using Stains-All dye. Timepoints are in minutes unless otherwise indicated.

(**Ac9**–**Ac10**) were at least 90% intact (Fig. 5). There seems to have been slightly more degradation for 2′F-ANA than 2′F-RNA at the 97 day timepoint. An isosequential DNA, **Ac11**, had a half-life of 2–3 weeks under similar conditions. Thus both 2′-fluorinated analogues are highly stable compared with DNA.

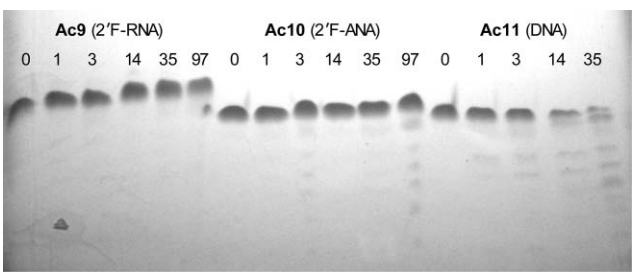

Fig. 5 24% Denaturing PAGE comparing the acid stability of 2′F-RNA, 2′F-ANA and DNA sequences **Ac9**–**Ac11** to simulated gastric fluid at 55 °C. Gels were developed using Stains-All dye. Timepoints are indicated in days.

Base-catalyzed hydrolysis of nucleic acids

The phosphate diester is remarkably unreactive to nucleophilic attack.[27] Indeed, the half-life of a dialkyl phosphate diester to water attack *at phosphorus* has been estimated at 30 million years at 25 °C.[28] For a simple phosphodiester anion like dimethyl phosphate, essentially all the cleavage derives from attack at carbon instead of phosphorus (Fig. 6).[28,29] DNA in neutral or basic solution is predominantly hydrolyzed by neither of these mechanisms: breakdown likely begins at the sugar or nucleobase, allowing subsequent strand cleavage at abasic or base-modified sites.[28]

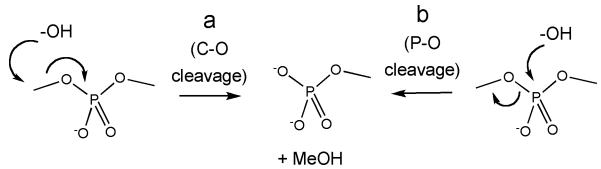

Fig. 6 C–O bond cleavage (pathway a) is the major contributor to basic phosphate cleavage of dimethyl phosphate.

The story is quite different for RNA because of its 2′-OH group. When deprotonated, this group can undergo an intramolecular attack on phosphorus to generate a 2′,3′-cyclic phosphate and cleave the chain (Fig. 7). Attack by the hydroxyl group itself (*i.e.* before deprotonation) is negligible.[30]

Because 2′F-ANA does not contain a 2′-OH group, we assumed that its stability to basic hydrolysis would be much greater than that of RNA. However, it was not clear whether the fluorine itself would make other sites in the molecule more electrophilic (and therefore susceptible to attack by base). This is important in light of the fact that nucleobase or sugar degradation is the starting point of DNA strand cleavage under alkaline conditions[28] (as discussed above).

Furthermore, we wished to compare the base stability of 2′F-ANA with that of its 2′-epimer, 2′F-RNA. Other groups have observed some breakdown of 2′F-RNA under basic conditions, with both phosphodiester and phosphoramidate backbones.[23,31] We hoped to explore the effect of stereochemistry at 2′ on the base stability of oligonucleotides.

2′F-ANA is slightly less stable than DNA under strongly alkaline conditions

Oligonucleotides **Ac9**–**Ac12** were subjected to basic conditions and aliquots were analyzed by PAGE. No degradation was observed by PAGE when DNA, 2′F-ANA or 2′F-RNA were subjected to methylamine for up to several days (data not shown). Under more rigorous conditions (1 M NaOH, 65 °C), 2′F-RNA, 2′F-ANA and DNA were still all quite stable. Some breakdown products were observed at longer timepoints, especially for 2′F-ANA and 2′F-RNA, but a significant amount of starting material was intact up to 20 h (Fig. 8). In sharp contrast, the corresponding RNA sequence was cleaved completely by the first timepoint (0.2 h).

It is possible that some alkaline degradation products co-migrate with the full-length oligo. Previous authors observed modification of 2′F-RNA without chain cleavage yielding products of similar mobility on HPLC.[23] The predominant transformation in that case was displacement of fluorine by pyrimidine O2, followed by hydroxide attack at C2 of the nucleobase to yield an arabinonucleotide. This transformation would not necessarily affect the stability of the phosphate backbone. Since fluorine is the poorest leaving group of the halogens, this reaction is only favored for 2′F-RNA where it is intramolecular and entropically favored. Consistent with this thinking, no direct displacement of fluorine was observed for 2′F-ANA (this would have transformed

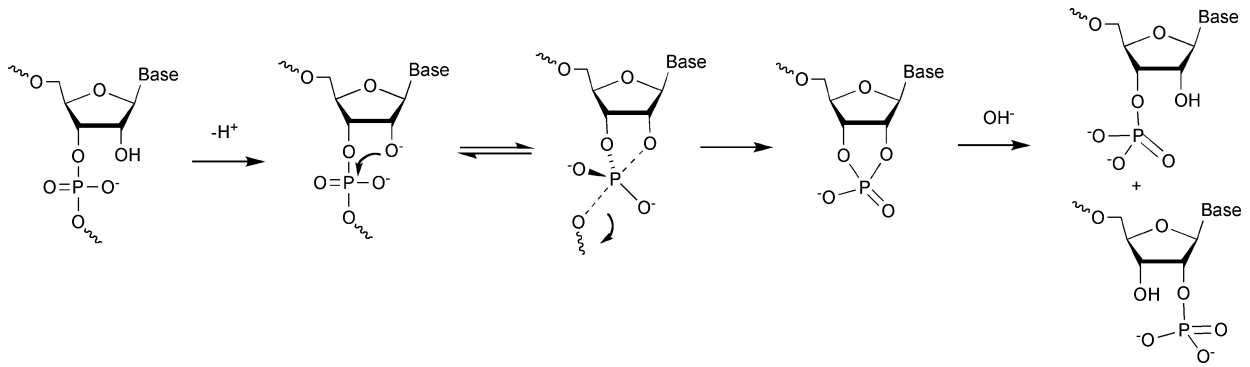

Fig. 7 Mechanism of RNA cleavage under basic conditions. As pseudorotation is not favorable under these conditions, since the negatively charged oxygens prefer to remain equatorial,[39] the cyclic phosphate is the sole product of attack by 2′-O⁻. It can subsequently be opened to a mixture of 2′ and 3′ phosphates.

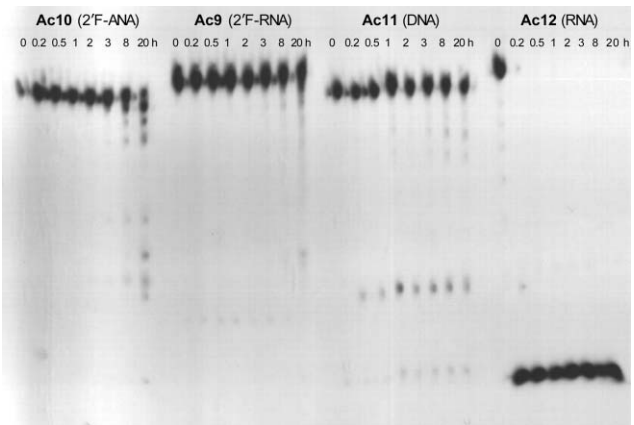

Fig. 8 Relative base stabiliy of 2′F-ANA, 2′F-RNA, DNA and RNA (sequences **Ac9**–**Ac12**). Radiolabeled oligonucleotides were treated with 1 M NaOH at 65 °C. Aliquots were removed at the indicated timepoints (in hours) and neutralized, then analyzed by 16% denaturing PAGE and visualized by autoradiography.

2′F-ANA into RNA and therefore been readily observed by the PAGE analysis.)

At this point we do not know why 2′F-ANA oligomers are slightly less stable than DNA under these strongly basic conditions. Given that DNA undergoes sugar or nucleobase breakdown under basic conditions,[28] we conclude that the inductive effect of the 2′-fluorine accelerates this process and the resultant cleavage of the oligonucleotide chain.

Nuclease stability of 2′F-ANA

The improved nuclease stability of 2′F-ANA oligonucleotides has long been appreciated. For example, the first group to work on 2′F-ANA found that $(2'F\text{-}araT)_{11}$ and $(2'F\text{-}araC)_{20}$ had a 4–8 fold increase in stability to nuclease P1, with respect to DNA.[5] Others have also observed increased stability of 2′F-ANA to snake venom phosphodiesterase (SVPDE, a highly active 3′-exonuclease) or serum.[7]

2′F-ANA can also confer nuclease resistance to duplexes and higher order structures. Dowler *et al.* showed that 2′F-ANA modification of one strand of an siRNA duplex provided significant additional serum stability to the whole duplex ($t_{1/2}$ ~5 h, *vs.* ~5 min for the unmodified duplex).[9] 2′F-ANA-modification of a G-tetrad-containing thrombin-binding aptamer resulted in up to 48-fold greater serum stability compared to the unmodified DNA tetrad.[32]

While phosphodiester (PO)-2′F-ANA does provide some advantages in terms of nuclease stability, it cannot match the dramatic increase in stabilization provided by a phosphorothioate (PS) backbone.[33] However, when the two modifications (PS and 2′F-ANA) are combined, the resulting PS-2′F-ANA oligonucleotide is highly resistant to most nucleases. Kalota *et al.* studied chimeric PS-2′F-ANA–DNA oligomers, and found that more than 70% of the oligonucleotide was still recoverable from K562 cells after 96 h, while very little PS-DNA remained after this time, leading to a lower effective dose and longer duration of action for the PS-2′F-ANA–DNA with respect to PS-DNA.[4] Another study found that two PS-2′F-ANA sequences were more stable to serum nucleases than most other sequences (with some exceptions.)[34] Again, this paralleled excellent activity (luciferase knockdown in HeLa cells) over extended assay times.[34] Thus the nuclease resistance of 2′F-ANA is key to its clinical relevance.

To better understand the nuclease resistance of 2′F-ANA, we decided to explore the stereochemistry and sequence dependence of cleavage by SVPDE.

One diastereomer of PS-2′F-ANA is selectively cleaved by SVPDE

Four 3′,5′-linked dinucleoside monophosphorothioates were synthesized (Table 2). The purified dimers were incubated with SVPDE and aliquots were removed and analyzed by HPLC. Data on the half-lives of their diastereomers are given in Table 2 and Fig. 9. (For **AT** and ^{f}AT, the phosphorothioate diastereomers proved very hard to resolve, even after trying several systems on both anion exchange and reverse-phase columns. However, the half-lives of their diastereomers were estimated based on the degradation curves of the sum of their two diastereomers, which easily fit the assumption that one of the diastereomers was essentially untouched by the enzyme during our assay.)

It is clear from the results on ^{f}A^{f}T that SVPDE prefers one isomer of PS-2′F-ANA as it does for PS-DNA, where the R_p isomer is cleaved several orders of magnitude more quickly than the S_p isomer.[35] The preference has been associated with the mechanism of cleavage of PS-DNA by SVPDE,[33] which thus does not appear to be affected by the switch to PS-2′F-ANA.

Table 2 Dinucleoside monophosphorothioates prepared for this study. Legend: dna, ***2′F-ANA***. Abbreviations: PS, phosphorothioate; $t_{1/2}$ (d1), the half-life for SVPDE degradation of the shorter-lived diastereomer. The $t_{1/2}$ of the longer-lived diastereomer was too long to measure using our techniques

Name	Sequence (5′–3′)	$t_{1/2}$ (d1)
AT	PS(at)	~40 h
A^{f}T	PS(a***T***)	~250 h
^{f}AT	PS(***A***t)	~2–8 h
^{f}A^{f}T	PS(***AT***)	~10 h

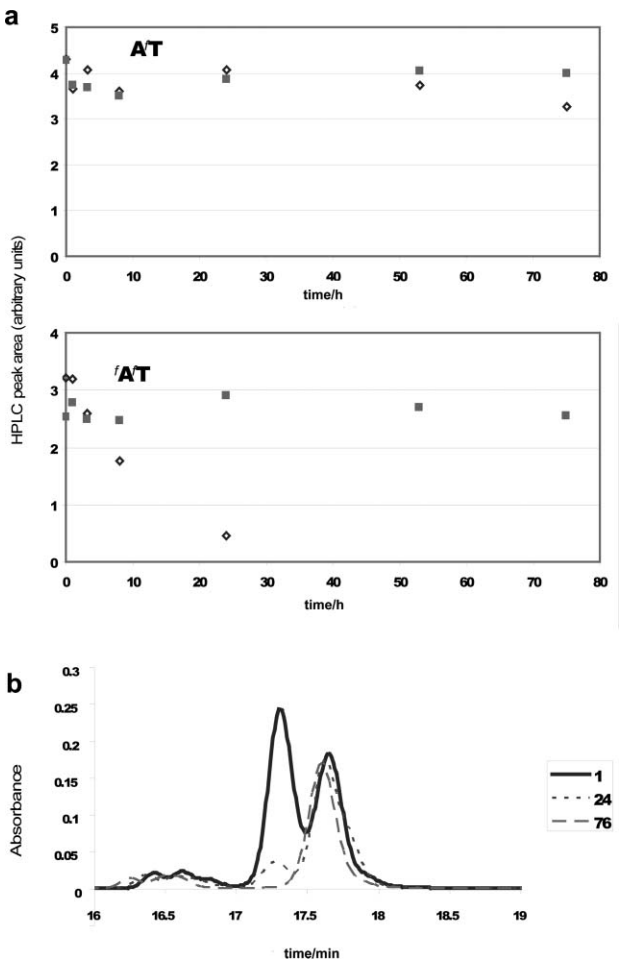

Fig. 9 SVPDE selectively cleaves one diastereomer of PS-2′F-ANA. (a) Peak areas from HPLC analysis of SVPDE degradation of **A^{f}T** and ^{f}A^{f}T. The shorter-lived diastereomer is indicated with hollow diamonds and the longer-lived diastereomer with filled squares. (b) The PS-dimer diastereomer region of HPLC traces from SVPDE degradation of ^{f}A^{f}T after 1 h, 24 h and 76 h. The x axis of the 24h trace was shifted by nine seconds to make the superposition clearer. The small broad peak at 16.5 min is unidentified.

This preference of SVPDE for one isomer of 2′F-ANA (as well as DNA) carries over to the oligonucleotide level. As seen in Fig. 10b, for both DNA and 2′F-ANA, the amount of full-length strand never drops below about 50% of the original amount. At longer timepoints, ~25% of the n-1 species is visible, ~12% of the n-2, and so on. Therefore, when the full-length strand of either DNA or 2′F-ANA was cut, the SVPDE enzyme was only able to excise a nucleotide or two before, apparently, reaching a linkage that it was

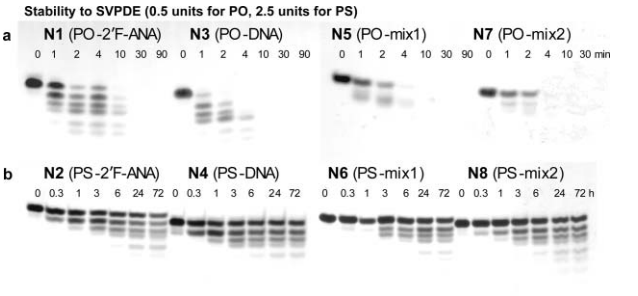

Fig. 10 24% Denaturing PAGE showing SVPDE stability of 2′F-ANA and DNA sequences **N1**–**N8** (visualized by Stains-All). (a) 5 nmol of each PO sequence was incubated at 37 °C with 0.5 units SVPDE in buffer, aliquots were removed at the times indicated, in minutes. (b) 5 nmol of each PS sequence was incubated with 2.5 units SVPDE in buffer, aliquots were removed at the times indicated, in hours.

unable to cleave. This resulted in very long-lived, nearly full-length species, all containing a 3′-terminal S_p phosphorothioate linkage if the same isomer is preferred for both DNA and 2′F-ANA.

The nuclease stability of 2′F-ANA is sequence-dependent

Another very interesting result from this study is that opposite chemistries were preferred at either end of the dimer. 2′F-araT incorporation at the 3′-end of the dimer (**A^{f}T**) led to improved stability as compared with dT (*i.e.*, **AT**). A larger and opposite preference observed at the 5′-end—dA in the 5′-position led to much slower cleavage rates than did 2′F-araA. Thus, the faster-cleaved diastereomer of **A^{f}T** is cleaved ~25 times more slowly than that of ^{f}A^{f}T. The "doubly favored" **A^{f}T** is about 100 times more stable than the "doubly disfavored" ^{f}AT. Overall, the fully-2′F-ANA-modified dimer ^{f}A^{f}T was about four times less stable than the DNA **AT**, a surprising result since 2′F-ANA oligonucleotides usually have greater nuclease resistance, as noted above.

It is striking that the 5′-nucleotide of the dimer has such a significant effect on the rate of cleavage by SVPDE, in spite of the fact that SVPDE is a 3′-exonuclease. This may be due to conformational issues. It is clear that **A^{f}T** is a poorer substrate for cleavage and/or an inhibitor of the enzyme, preventing other substrates from being cleaved.

We also sought to know whether the **A^{f}T** motif provided additional stability to oligonucleotides as well as dimers, and to PO backbones as well as PS. Therefore oligonucleotides were synthesized containing **AT**, ^{f}A^{f}T, or **A^{f}T** structures at their 3′-termini (Table 3). Upon treatment of these oligonucleodies with SVPDE or FBS, no significant stabilization was observed by introducing the **A^{f}T** motif into oligonucleotides (Fig. 10). Thus

Table 3 Further strands prepared for the nuclease stability study. Legend: dna, ***2′F-ANA***. Abbreviations: PO, phosphodiester; PS, phosphorothioate

Name	Description	Sequence (5′–3′)
N1	PO-2′F-ANA	PO(***CGACCTGTGCAT***)
N2	PS-2′F-ANA	PS(***CGACCTGTGCAT***)
N3	PO-DNA	PO(cgacctgtgcat)
N4	PS-DNA	PS(cgacctgtgcat)
N5	PO-mix1	PO(***C***g***ACCTGTGC***a***T***)
N6	PS-mix1	PS(***C***g***ACCTGTGC***a***T***)
N7	PO-mix2	PO(***C***gacctgtgca***T***)
N8	PS-mix2	PS(***C***gacctgtgca***T***)

the dramatic stability of the $\mathbf{A}^{f}\mathbf{T}$ dimer appears to be specific to the dimer itself.

Conclusions

It has long been known that 2′-fluorinated nucleosides have excellent resistance to acid-catalyzed depurination.[19,20] As this study has now demonstrated, that property enables 2′F-ANA oligonucleotides to show excellent resistance to acid-catalyzed degradation as well.

2′F-ANA is also quite stable to basic conditions. Thus, while DNA and RNA have a relatively limited functional pH range, 2′F-ANA (and 2′F-RNA) are stable to a much wider range of conditions.

The striking stability of 2′F-ANA oligonucleotides to acidic and basic conditions suggests many possible applications. These include oral absorption of oligonucleotides[36] and intracellular delivery (oligonucleotides must pass through an increasingly acidic endosome to be released into the cell).[37] Furthermore, 2′F-ANA could provide improved durability in currently used diagnostic applications: for example, 2′F-ANA-modified photoaptamers could be washed with acid or base after covalent linking to proteins. Or again, oligonucleotide microarrays made of 2′F-ANA could be treated with acid or base to wash away all of the analyte by disruption of Watson–Crick base pairing without affecting the integrity of the 2′F-ANA probes of the array, extending the lifetime of the array itself.

In the case of phosphorothioate 2′F-ANA, one diastereomer is selectively cleaved by SVPDE and serum nucleases, parallel to observations on PS-DNA cleavage by these nucleases.

We have also presented data demonstrating that some sequences are protected significantly more than others upon 2′F-ANA modification. The nature of the 5′-nucleotide of a dinucleoside phosphorothioate has a significant effect on its nuclease stability. We show that the most stable combination, for the AT dimer, is dA-2′F-araT ($\mathbf{A}^{f}\mathbf{T}$). Future studies will explore the importance of base composition, the reasons for the increase in stability, and the possibility of using $\mathbf{A}^{f}\mathbf{T}$-like structures as nuclease inhibitors.

Experimental

All sequences were synthesized on an ABI 3400 DNA synthesizer. Standard conditions were used for synthesis and deprotection. Oligonucleotides were purified by preparative denaturing PAGE, followed by desalting on Nap-25 Sephadex columns. Dinucleoside monophosphorothioates were synthesized as for the oligonucleotides, desalted using Sep-Pak solid-phase extraction cartridges, and purified by HPLC. Masses of oligonucleotides were verified by ESI-MS, directly injecting 0.05–0.1 ODU of well-desalted oligonucleotides.

Pepsin-free simulated gastric fluid (SGF)[24] was made by dissolving 0.20 g NaCl in 99 mL MilliQ water and autoclaving this solution, then adding 0.70 mL HCl (final pH ~1.2). For acid stability assays on sequences **Ac1**–**Ac8**, 0.7 ODU of each sequence was dissolved in 100 μL of SGF at 37 °C. 10-μL aliquots were removed at various timepoints as indicated, frozen on dry ice, then immediately evaporated to dryness and stored in the freezer. The assay was then analyzed by 24% denaturing PAGE (7 M urea) which was developed using Stains-All dye. For acid-stability assays on pyrimidine-rich sequences **Ac9**–**Ac11**, 2 ODU of each sequence was dissolved in 100 μL of SGF at 55 °C. A microtube with a tightly sealing screw-top was used for each reaction, and the solution was topped with mineral oil to avoid loss of water and acid. The pH of the solution was verified at the end of the experiment and had not changed. 14-μL aliquots were removed at various timepoints and treated as above. The analytical gel was examined by UV shadowing since pyrimidine-only sequences do not stain well in Stains-All.

Base stability studies were carried out by 5′-labeling sequences **Ac9**–**Ac12** with γ-^{32}P-ATP and T4 polynucleotide kinase, to minimize the amount of material required for these studies since, as noted above, these pyrimidine-only sequences do not stain well in Stains-All. Labeled oligonucleotide samples were dissolved in 1 M NaOH, then the tube was submerged in water at 65 °C (to avoid condensation on the sides and top of the tube). Aliquots were removed at the indicated timepoints and neutralized by pipetting them into an equimolar amount of TBE buffer combined with an equimolar amount of HCl. The neutralized aliquots were analyzed directly by 16% denaturing PAGE and visualized by autoradiography.

Anion exchange HPLC (used to resolve the diastereomers of $^{f}\mathrm{A}^{f}\mathrm{T}$) was carried out using a Waters Protein-Pak DEAE 5PW column. A 30 min gradient from pure water to 0.20 M $LiClO_4$ was used. For reverse phase HPLC (used to resolve the diastereomers of $\mathbf{A}^{f}\mathbf{T}$), a Waters Symmetry C18 5 μm 4.6 × 150 mm column was used with a gradient of 0–25% methanol in water, containing 100 mM triethylammonium acetate.

SVPDE assays were carried out in 300-μL PCR tubes. Typically, 5 nmol of oligonucleotide was dissolved in 20 μL of SVPDE buffer (100 mM Tris-HCl, 100 mM NaCl, 14 mM $MgCl_2$, pH 8.9) and warmed to 37 °C. 2.5 μL was removed as a zero point. 17.5 μL of the same buffer containing 0.5 or 2.5 units of SVPDE (Phosphodiesterase I from *Crotalus adamanteus* venom, USB Corporation, Cleveland, Ohio) was warmed to 37 °C, then added to start the reaction. 0.5 Units of SVPDE was used in the case of PO oligonucleotides, while 2.5 units of SVPDE was used for PS oligonucleotides. Aliquots (5 μL) were removed at various timepoints as indicated, pipetted onto 5 μL formamide, heated to 95 °C for 1 minute and then stored at −20 °C until analysis by denaturing 24% PAGE. Bands were visualized using Stains-All dye. For assays longer than four hours, the reactions were topped with 10 μL mineral oil.

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