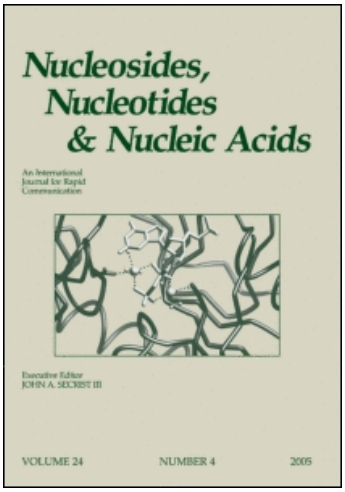

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HYDROLYSIS OF 2'-DEOXY AND 2'-FLUORONUCLEOSIDE-3'-PHOSPHODIESTERS

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HYDROLYSIS OF 2'-DEOXY AND 2'-FLUORONUCLEOSIDE-3'-PHOSPHODIESTERS

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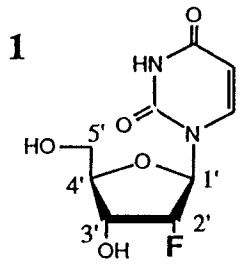
Abstract. Two nucleoside analogs were synthesized to test the ribose conformational and electronic effects on phosphate hydrolysis at the 3' position. It was found that under alkaline conditions, a 2'-fluoro-nucleoside (C3'-endo) resulted in a phosphate degradation that was ten times faster than the 2'-deoxynucleoside analog (C2'-endo). In addition to kinetic differences, product distributions will be presented.

Introduction

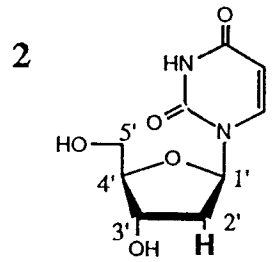
Fluorine incorporation at the 2' position of nucleic acid ribose rings has yielded interesting insights into the importance of the 2'-OH functionality for ribose[1] and oligonucleotide conformation.[2] To this end, we present results that describe how a 2'-fluorine in the ribo configuration affects phosphate hydrolytic chemistry at the neighboring 3' position. Such fundamental studies have potential bearing for pharmaceutical and RNA chemistry, for 2'-fluorinated oligonucleotides have been used as putative antisense agents[3] and as probes of RNA enzymes.[4]

The nucleoside, 1'-(2'-fluoro-β-ribofuranosyl)uracil, or 2'-deoxy-2'-fluorouridine[5] (**1**) is known to assume the C3'-endo, ribose-like, sugar conformation in comparison to pyrimidine-2'-deoxynucleosides which are known to be in the C2'-endo conformation (Figure 1).[6] Therefore, a nucleoside such as **1** can approximate a "conformational isomer" of 2'-deoxyuridine (**2**) since the substituents in the 2' position for **1** and **2** cannot form strong hydrogen bonds in aqueous solution, and are roughly the same size.[7]

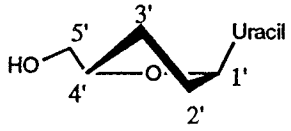
In this connection, the effects of this conformational and electronic alteration can be addressed with phosphodiesters **3** and **4** which serve to examine how the 2' substitution affects the rates of phosphate hydrolysis at the 3' site.



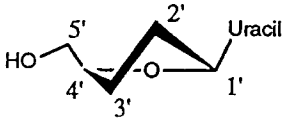
2'-fluoro-2'-deoxyuridine



2'-deoxyuridine

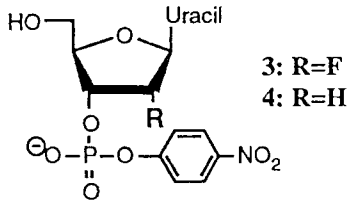


C3'-endo



C2'-endo

Figure. 1 2'-fluoro-2'-deoxyuridine and 2'-deoxyuridine with their corresponding ribose puckering mode.



3: R=F
4: R=H

Experimental

All chemicals were purchased from Aldrich Chemical Co. and Tokyo Kasei, and used without purification. Dioxane (dried over Na /benzophenone), pyridine (dried over BaO), diisopropylethylamine (dried over CaH_2) and CH_2Cl_2 (dried over P_2O_5) were distilled prior to use, otherwise all other solvents were used without further purification. All ^{31}P (121.44 MHz) and ^{1}H (300.17 MHz) spectra were taken on a QE-300 NMR spectrometer and referenced to 85% H_3PO_4, and $(Me)_4Si$, respectively. ^{19}F NMR spectra were taken on a QE-300 with a dedicated fluorine probe and tank filter, and referenced to 2,2,2-trifluroethanol. Liquid chromatorgraphy was done with a Vydac C-18 column (0.5 x 30 cm) at ambient temperature (23°C) on a HP 1090 LC interfaced to a diode array detector. The mobile phase (1.5 mL/min) was a 0-8% acetonitrile gradient in a 20 mM diisopropylethylamine acetate buffer (pH 7.8).

Kinetic measurements of phosphate hydrolysis were done by monitoring p-nitrophenol production which has a λmax at 400 nm. Spectrophotometric determination was done with a HP 8452 spectrophotometer equipped with a Fisher Scientific thermostat.

The title compound (**1**) 1-(2'-fluoro-β-D-ribofuranosyl)uracil (2'-fluoro-2'-deoxyuridine)[5] was prepared by the reported procedure except that 70% hydrogen fluoride-pyridine was used in place of anhydrous hydrogen fluoride at 100 °C. Anhydrouridine (2.00 g, 8.84 mmole) was reacted with HF-pyridine to give 2'-fluoro-2'-deoxyuridine (0.840 g, 42%). The purity of the title compound was confirmed by mixed melting point (148-150 °C) and TLC analysis (Rf = 0.5 in 9:1 EtOAc:MeOH) with commercial (USB) and donated 2'-fluoro-2'-deoxyuridine (ISIS pharmaceuticals). In addition, ^{1}H NMR spectra of authentic and synthesized 2'-fluoro-2'-deoxyuridine were identical. ^{1}H NMR (300.17 MHz, D_2O): 7.75 (d, 1H, H6, $J_{5,6}$=8.0 Hz), 5.93 (dd, 1H, H1', $J_{1',F}$=18.0 Hz, $J_{1'2'}$=1.3 Hz), 5.70 (d, 1H, H5, $J_{5,6}$=8.0 Hz), 5.15 (dd, 1H, H2', $J_{2',F}$=52.0 Hz, $J_{2',3'}$=4.8 Hz), 4.30 (ddd, 1H, H3' $J_{3',F}$ = 18 Hz, $J_{3',4'}$=8.6 Hz, $J_{3',2'}$=4.7 Hz).

The first intermediate towards the synthesis of **3** and **4** is the 5'-O-(4,4'-dimethoxytrityl) nucleoside. This was accomplished through the treatment of **1** or **2** (250 mg, 1mmole) with 4,4'-dimethoxytrityl chloride (1.5 equivalents) and dimethylaminopyridine catalyst (0.25 equivalents) in pyridine. Upon completion of the transformation as judged by TLC (9:1 CH_2Cl_2:MeOH), the reaction was quenched with H_2O and extracted in Et_2O. After removal of the Et_2O by evaporation, the residual oil was dissolved in a minimum amount of CH_2Cl_2 and then precipitated out as a clean white powder by dropwise addition to cold hexane (yield 85%).

The p-nitrophenyl phosphate group was attached to the 3'-hydroxyl position of 5'-O-(4,4'-dimethoxytrityl)-2'-deoxy-2'-fluorouridine (500 mg, 2.00 mmole) according to the procedure of Turner[8]. These compounds were further purified with HPLC using the aforementioned gradient and column, and analysis of the phosphate substrates revealed at least 99% purity. Typical yields for **4** and **3** were 40% and15%, respectively.

For **3**: ^{1}H NMR (300.17 MHz, D_2O): 8.34 (d, 2H, H3 of p-nitrophenol, $^3J_{HH}$=7.7 Hz), 7.80 (d, 1H, H6, $J_{5,6}$=8.0 Hz), 7.35 (d, 2H, H2 of p-nitrophenol, $^3J_{HH}$=7.7 Hz), 6.0 (dd, 1H, H1', $J_{1',F}$=18.0 Hz, $J_{1'2'}$=1.2 Hz), 5.82 (d, 1H, H5, $J_{5,6}$=8.0 Hz), 5.45 (ddd, 1H, H2', $J_{2',F}$=52.0 Hz, $J_{2',3'}$=4.8 Hz, $J_{2',1'}$=1.2 Hz), 4.30 (ddd, 1H, H3' $J_{3',F}$ = 19 Hz, $J_{3',4'}$=8.5 Hz, $J_{3',2'}$= 4.8 Hz). ^{19}F (282.2 MHz, D_2O): 18.4 (ddd, 2'F, $J_{F,2'}$=52.0 Hz, $J_{F,1'}$=18.0 Hz, $J_{F,H3'}$=19.0 Hz). ^{31}P (121.7 MHz, D_2O): -5.9 ppm.

For **4**: ^{1}H NMR (300.17 MHz, D_2O): 8.05(d, 2H, H3 of p-nitrophenol, $^3J_{HH}$=7.7 Hz), 7.85 (d, 1H, H6, $J_{5,6}$=8.0 Hz), 7.15 (d, 2H, H2 of p-nitrophenol, $^3J_{HH}$=7.7 Hz), 6.05 (dd, 1H, H1', $J_{1',2'}$=6.0 Hz,), 5.62 (d, 1H, H5, $J_{5,6}$=8.0 Hz), 5.45 (dd, 1H, H2', $J_{2',F}$=52.0 Hz). ^{31}P (121.7 MHz, D_2O): -5.8 ppm.

The concentrations of compounds **3** and **4** were determined by spectrophotometric quantitation (ε=17,000 M^{-1}at pH 7.0) of the released p-nitrophenolate anion upon alkaline degradation (1.0 M NaOH, 60 °C, 48 hours). Kinetic measurements of the alkaline hydrolysis were done in 1M NaOH at 40°C with 8 μM of **3** or **4**. The initial rates of p-nitrophenolate release were measured by monitoring the rate of increase at 400 nm which was linear for at least 2 hours. Spleen phosphodiesterase II, purchased from SIGMA Chemical Co., was used only for comparative purposes for the phosphate hydrolysis of **3** and **4** and was therefore used as is (pH 5.5, 10 mM NaOAc, and 37 °C) according to literature procedures[9]. The identification of the hydrolysis products was done with HPLC techniques wherein **3** and **4** (6.5 mM) were incubated in 1M NaOH (40 °C) for up to three days. Identification of eluted samples was done with coinjection of the authentic compounds, 2'-deoxyuridine, 2'-deoxyuridine-3'-phosphate, 2'-deoxy-2'-fluorouridine, thymidine-3'-phosphate, and thymidine-3',5'-cyclic phosphate.

Results and Discussion

Geminal and vicinal ^{1}H-^{19}F coupling constants (Figure 2) for **1** and **3** (53.7 and 20.0 Hz, respectively) are in close agreement with that of 3', 5'-di-O-acetyl-2'-fluoro-2'-deoxyuridine which has been crystallographically characterized to have the C3'-endo ribose puckering mode.[10]

The close ^{1}H-^{1}H coupling constants for **2** and **4** (Table 1) implies that this C3'-endo conformation was preserved. This is further underscored by a pseudorotation analysis[11]($J_{1',2'}/J_{3',4'}$ = C2'-endo/C3'-endo) that shows the C2'endo-C3'endo equilibrium lies predominantly (~85%) on the C3'-endo side.

This suggests that the attachment of a phenyl phosphate group at the 3' position has little effect on the ribose conformation. Indeed we have found that even flanking 2'-fluoro-2'-deoxyuridine with 2'-deoxyuridine had little effect on the ^{1}H-^{19}F coupling constants[12] which suggests a rather rigid C3'-endo ribose conformation for these small molecules.

A comparison of the k_{cat}/ Km ratio ([substrate] = 5 μM-40 μM) for the enzymatic hydrolysis of **3** and **4** by spleen phosphodiesterase II, which has 3'-exonuclease activity,[9] shows little difference between the two substrates (Table 2). It has been shown that thymidine-3'-p-nitrophenyl phosphate is hydrolyzed by phosphodiesterase II to yield thymidine-3'-phosphate and p-nitrophenol.[8] When **3** and

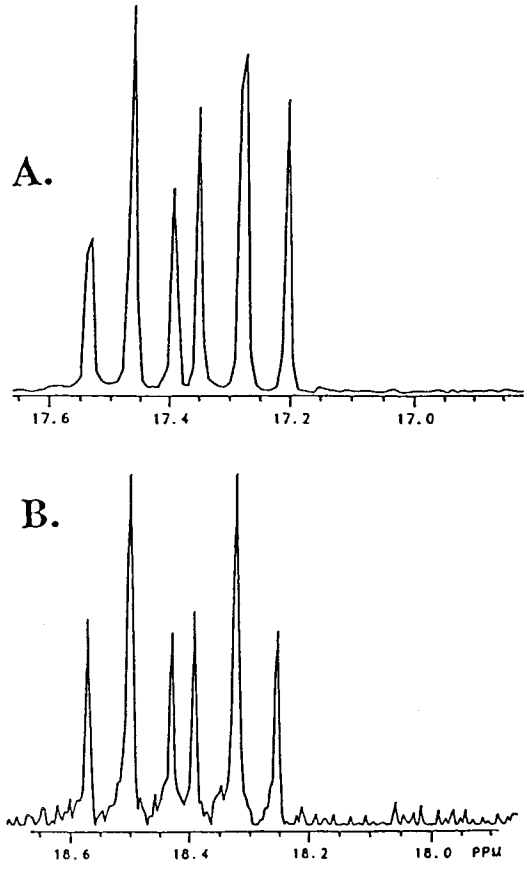


Figure 2. ^{19}F NMR spectra of (A) 2'-deoxy-2'-fluorouridine (**1**) and (B) 2'-deoxy-2'-fluorouridine-3'-p-nitrophenyl phosphate (**3**) referenced to 2,2',2"-trifluoroethanol. Geminal and vicinal ^{1}H-^{19}F coupling constants are 53.7 and 20.0 Hz, respectively.

Table 1

Ribose Coupling Constants (Hz) and Pseudorotation Analysis ($J_{1',2'}/J_{3',4}$)

	$J_{1',2'}$	$J_{2',3'}$	$J_{2',F}$	$J_{1',F}$	$J_{3',F}$	$J_{3',4'}$	$J_{1',2'}/J_{3',4}$
1	1.3	4.8	52.0	18.0	18.0	8.6	0.15
3	1.2	4.8	52.0	18.0	19.0	8.5	0.14

Table 2
Initial Rates of p-nitrophenol Production for **3** and **4**

	3	**4**
Enzymatic Digestion by phosphodiesterase II		
k_{cat}	3.3 (2) x 10^{-5} s^{-1}	1.3 (1) x 10^{-5} s^{-1}
Km	9.7 (2) x 10^{-5} M	1.5 (1)x 10^{-5} M
k_{cat}/Km	0.33 (4) s^{-1} M^{-1}	0.88 (3) s^{-1} M^{-1}
Alkaline Digestion		
rate in 1M NaOH (37°C)	3.6 (1) x 10^{-5} s^{-1}	4.9 (1) x 10^{-4} s^{-1}
E_a	17 (1) kcal mol^{-1}	14 (1) kcal mol^{-1}

4 were submitted to alkaline hydrolysis, there is a significant difference in the rates of p-nitrophenol production. At 8.0 μM concentration of **3** or **4** (1M NaOH, 40 °C), the fluorinated substrate releases p-nitrophenol ten times faster than the non-fluorinated substrate (Figure 3).

Compounds **3** and **4** have different rates of p-nitrophenol formation in 1 M NaOH as well as different hydrolyzed products. From similar work with thymidine-3'-p-nitrophenyl phosphate,[8] it is known that alkaline hydrolysis of these phosphodiesters follow two reaction pathways (Scheme 1). An intermolecular attack of the hydroxide anion yields a nucleoside-3'-phosphate product (products **5** and **6**) while an intramolecular attack of the deprotonated 5'-OH yields the 3',5'-cyclic phosphate (products **7** and **8**).

Accordingly, HPLC analysis shows that alkaline hydrolysis of **4** produces roughly equal amounts of 2'-deoxyuridine-3'-phosphate (**5**) and 2'-deoxyuridine-3',5'-cyclic phosphate (**7**) for up to two days. However, the similar degradation of **3** produces mainly the 3',5' cyclic phosphate product **8**. These result suggest that a fluorine substitution in the 2'-position of the ribose ring results in different reaction pathways as well as in different rates of phosphate hydrolysis. In agreement with the UV/Visible results, HPLC analysis of alkaline digestion also shows that **3** degrades ten times faster than **4**.

In addition to the more rapid p-nitrophenol release, HPLC studies showed that alkaline hydrolysis of the fluorinated substrate also produces 2'-deoxy-2'-fluorouridine and p-nitrophenyl phosphate. Under similar alkaline conditions, release of 2'-deoxyuridine from the non-fluorinated molecule **4** was not evident. In terms of the leaving group stability, these results suggests that the 2'-fluoro-2'-deoxy-3'-

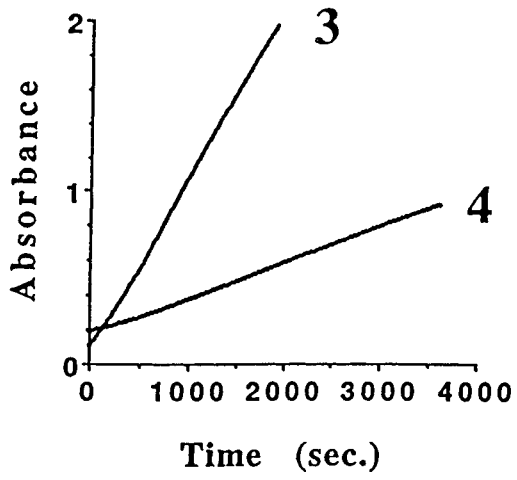
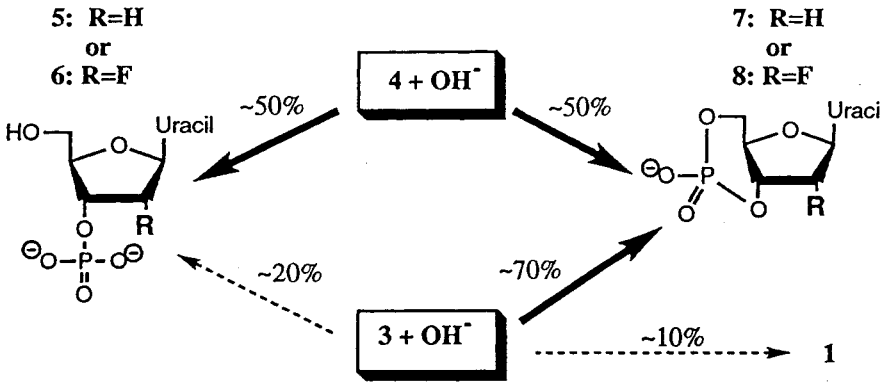


Figure 3. Alkaline degradation (1.0 M NaOH at 40°C) of 3 and 4 (8 μM) as followed by UV/Visible spectroscopy at 400 nm. See table 2 for initial rates of alkaline degradation.



Scheme 1

oxoanion is more stable than the 2'-deoxy-3'-oxoanion. This feature was also observed when the ribozyme derived from *Tetrahymena thermophila* was used to hydrolyze phosphate esters of various oligodeoxynucleotide analogs where the 2'-deoxyribose 3'-oxoanion was the leaving group.[2] It was found that the 2'-fluorine analogs accelerated rates of phosphate hydrolysis 24-fold relative to the nonfluorinated oligodeoxynucleotides due to the stabilization of the departing 3'-oxoanion by the neighboring 2'-fluorine.

We have shown that an alteration at the 2' position of the ribose ring affects phosphate hydrolytic chemistry at the 3' position. While these effects can be argued on the basis of a ribose conformational change, electronic factors cannot be ruled out. In addition to an increase in phosphate hydrolytic rates at the 3' position, the 2'-fluorinated nucleoside also produces an additional 3'-oxoanion product resulting from stabilization by the 2'-fluorine substituent. Analogous phosphate hydrolytic studies are being carried out on fluorinated oligoribonucleotide analogs, and computational techniques will be used to address the conformational basis behind the different hydrolytic reaction manifolds. The results of these fundamental studies have implications for new oligonucleotide analogs that possess a fluorine substitution in the ribose ring as they may shed more light on the stability of 2'-fluorinated nucleic acid analogs as well as reveal interesting conformational aspects of phosphate hydrolytic reactions.

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12 The trimer 5'-d(UU$_f$U)-3', where U$_f$ is 2'-deoxy-2'-fluorouridine, was made[3] to test how neighboring nucleotides affect the conformation of the central 2'-deoxy-2'-fluorouridine. No change was seen in the ^{1}H-^{19}F coupling constants upon incorporation of the 2'-deoxy-2'-fluorouridine inside a trimer.

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