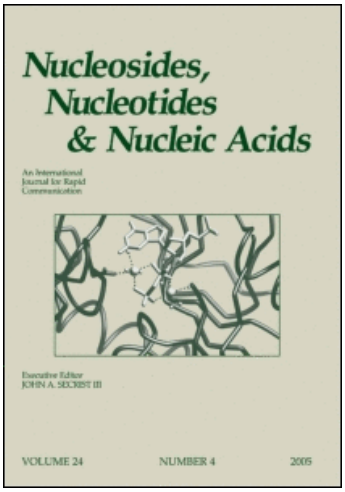

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Synthesis of 4-Amino-8-(2, 2-Difluoro-2-Deoxy-β-D-Ribo Furanosyl Amino)Pyrimido [5, 4-D]Pyrimidine (dFARPP). Stability and Cellular Cytotoxicity

T. E. Mabry[a]; C. D. Jones[a]; T. S. Chou[a]; J. M. Colacino[a]; G. B. Grindey[a]; J. F. Worzalla[a]; H. L. Pearce[a]
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NUCLEOSIDES & NUCLEOTIDES, 13(5), 1125-1133 (1994)

SYNTHESIS OF 4-AMINO-8-(2,2-DIFLUORO-2-DEOXY-β–D-RIBO FURANOSYL AMINO)PYRIMIDO[5,4-D]PYRIMIDINE (DFARPP). STABILITY AND CELLULAR CYTOTOXICITY.#, 1

T. E. Mabry*, C. D. Jones, T. S. Chou, J. M. Colacino, G. B. Grindey, J. F. Worzalla and H. L. Pearce

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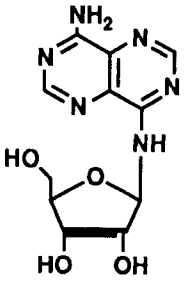
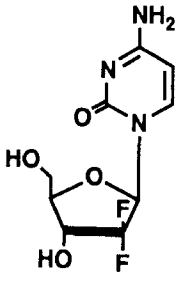
Abstract: The synthesis of β-dFARPP was accomplished by Mitsunobu coupling of 6-cyanopurine with 2-deoxy-2,2-difluororibose followed by purine ring-expansion under the action of methanolic ammonia. The title compound inhibited the growth of proliferating human leukemic CCRF-CEM cells at 6.1μg/ml but had no effect on plaque formation by a variety of DNA and RNA viruses.

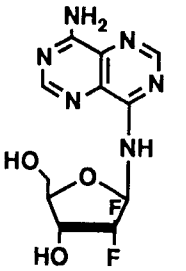
Several groups[2-6] have described the synthesis and biological evaluation of a novel class of quasi-nucleosides best represented by 4-amino-8-(β-D-ribofuranosylamino) pyrimido[5,4-d]pyrimidine (β-ARPP) **1**. The most potent of these ribosyl analogs possess significant activity against a variety of DNA and RNA viruses[3-5] as well as immunosuppressive properties and *in vivo* cytotoxic activity against L1210 cells[2,4,7]. Willis[8] and Jackson[9] have studied the basis of antitumor activity and found **1** to be a substrate of adenosine kinase.



β-ARPP 1

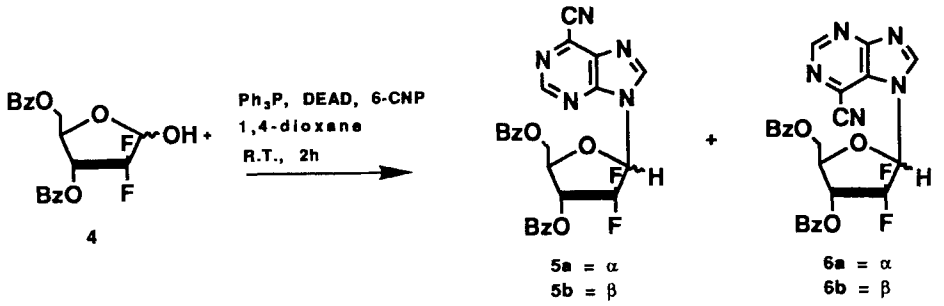


Gemcitabine 2



β-dFARPP 3

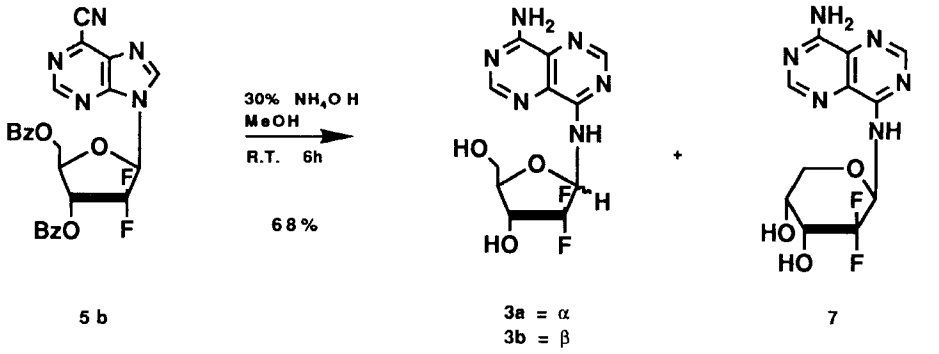
\# *This paper is dedicated to the memory of Dr. G. B. Grindey, deceased November 16, 1993.*



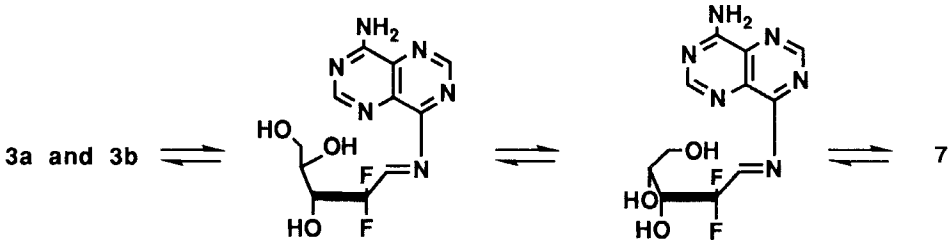
Scheme I

Subsequent cellular accumulation of β-ARPP monophosphate inhibits PRPP synthase (ribose-5-phosphate pyrophosphokinase), a critical enzyme involved in the *de novo* biosynthesis of both purine and pyrimidine nucleosides. Although **1** and its analogs have been invaluable for studying the complex regulation of PRPP synthase[10] their clinical potential has been compromised due to anomerization of the β-anomer[6,8]-the preferred substrate for adenosine kinase. Hertel[12] has reported on a series of highly active nucleoside antimetabolites, represented by Gemcitabine **2**, in which a gem-difluoromethylene occupies the 2' position. We envisioned such substitution in β-ARPP might stabilize the glycosidic bond and provide an anaolg with a longer half-life at physiological pH. Also, we were interested in determining if a fluorine atom would be a suitable bioisostere for the hydroxy group in this series of aminoglycosides. Herein, we wish to report the synthesis of 4-amino-8-(2,2-difluoro-2-deoxy-β-D-ribofuranosylamino) pyrimido[5,4-d]pyrimidine (β-dFARPP) **3b** and describe results obtained from antiviral and anticancer testing as well as anomeric stability studies at various pH.

RESULTS AND DISCUSSION: The preparation of the requisite 6-cyano-2',2'-difluoro-2'-deoxyadenosine derivative **5b** was accomplished via activation of an anomeric mixture of 3,5-di-O-benzoyl-2,2-difluoro-2-deoxyribose [13] **4a/4b** and coupling with 6-cyanopurine (commercially available from Sigma) to give the desired N-9 regioisomer **5a/5b** as a 1:1 α/β mixture in 58% isolated yield as well as 10% of the N-7 regioisomers **6a/6b** as depicted in Scheme 1. Structural assignment of the N-9 regioisomer was



Scheme II



Scheme III

based on the precedent of Szarek[14], as recently applied by Hertel[15], who report the predominant formation of the N-9 regioisomer when 6-substituted purines are ribosylated utilizing Mitsunobu conditions.

Purine to pyrimido[5,4-d]pyrimidine rearrangement of the N-9 regioisomer and deprotection was carried out by treatment of **5b** (purity >95% β after silica gel chromatography) with methanolic ammonia at room temperature to give **3a** and **3b** as 1:1 α/β mixture in 68% yield along with a small amount of the pyranose derivative **7**. Chromatography on silica gel gave β-dFARPP, **3b**, which displayed the characteristic UV absorption spectra[3] commensurate with the pyrimido[5,4-d]pyrimidine ring system. Only the N-9 regioisomer could rearrange to give this heterocycle. The pyranoside **7** could be isolated in high yield by prolonged exposure of **5b** to methanolic ammonia at elevated temperatures and may have been formed as a consequence of the reaction mechanism proposed in Scheme III.

Gratifyingly, stability studies[16] indicated that β-dFARPP, **3b**, was essentially unchanged after exposure to pH 4.3 buffer and 37° C for 100 hours (data not shown). Unfortunately, exposure of **3b** to conditions at or above physiological pH led to anomerization with the formation of the pyranose analog as shown in Figures 1 and 2. The half-life of β-dFARRP was determined to exceed 100 hours at pH 7.4 while a pH of 10.0 eliminates 50% of β-dFARPP in just 2 hours. It is suggested that an intermediate, open-chain trihydroxyimine is formed. This compound cyclizes through two routes: closure by the secondary C-4' hydroxyl from either face yields and α/β mixture of ribofuranosylpyrimido[5,4-d]pyrimidines; or addition of the primary C-5' hydroxyl to the imine yielding a single pyranose analog **7**. Therefore, it appears that incorporation of the gem-difluoromethylene at C-2' stabilizes the glycosidic bond in acidic media presumably by reducing the basicity of the exocyclic nitrogen. However, at high pH increasing the acidity of the 4-NH proton by the intense electron withdrawing capacity of the geminal fluorine substitiuion facilitates the furanose to pyranose ring-expansion.

ANTIVIRAL TESTING: Compounds **3a**, **3b** and **7** were evaluated *in vitro* against Herpes simplex type I (Mayo strain) and type II (G Strain) in BSC-1 cells using a plaque reduction assay[17] with acyclovir as postive control and found to be devoid of measurable activity (IC_{50} >25 μg/mL). These three anaolgs were also found to be devoid of antiviral activity against influenza A/Ann Arbor and influenza B/Great Lakes in Madin-Darby canine kidney cells as determined by plaque reduction assays[18] with amantadine and ribavirin as positive controls, respectively. Srivastava[3] prepared the 2'-deoxy analog (without fluorine) but failed to mention results of antiviral testing. The corresponding arabino derivative was devoid of activity when tested against RNA and DNA viruses in cell culture. Compound **3b** inhibited the growth of CCRF-CEM (human T-cell) by 50% after 72h incubation at a concentration of 6.1μg/mL. The α anomer **3a** and the pyranose derivative **7** failed to inhibit growth at concentrations less than 20.0 μg/mL. β-dFARPP failed to inhibit tumor growth of mice inoculated with murine solid tumors X5563[19] (plasma cell myeloma) or C3H[20] (mammary adenocarcinoma) at 15.0 or 30.0 mg/kg (i.p. dailyX10).

Currently, we are investigating how the 2',2'-difluoromethylene influences the conformation of the sugar and what effect this has on the orientation of various heterocyclic bases.

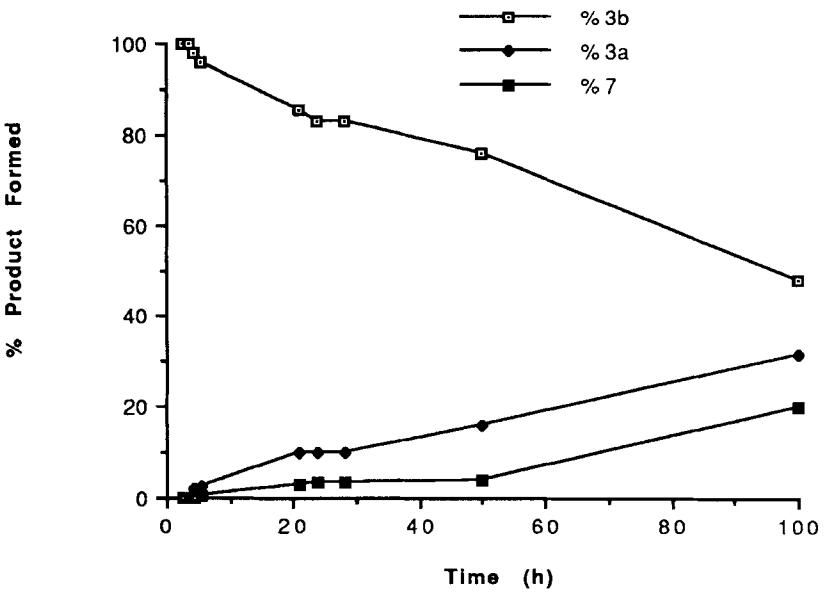


FIG. 1 Anomerization/ring-expansion of 0.16 mmol dFARPP (3b) incubated at 37 C at pH=7.4. Conditions: C18 Nova-pak; 10:90, AcCN/0.5% NH4H2P04; Det 254 nm. Beta (3b) elutes in 4.8 min, alpha (3a) elutes in 4.15 min, and 7 elutes in 3.8 min.

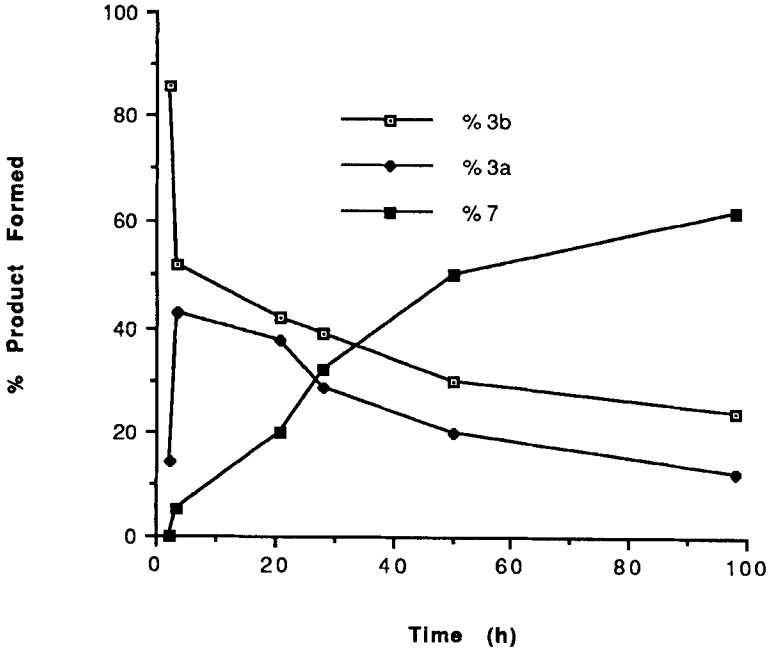


FIG. 2. Anomerization/ring-expansion of 0.16 mmol dFARPP (3b) incubated at 37 C at pH=10. Conditions: C18 Nova-pak; 10:90 AcCN/0.5% NH4H2PO4; Det 254 nm. Beta (3b) elutes in 4.8 min, alpha (3a) elutes in 4.15 min, and 7 elutes in 3.8 min.

EXPERIMENTAL SECTION

General Methods: Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were determined at 300.135 MHz with a General Electric QE300 instrument. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard. Ultraviolet spectra were recorded on a Beckman DU-50 spectrometer. Elemental analyses were performed by the Physical Chemistry Dept., Lilly Research Laboratories, Indianapolis IN. Thin-layer chromatography (TLC) was run on silica gel 60 F-254 plates (EM Reagents). Flash chromatography was run on E. Merck silica gel (230-400 mesh). Reported yields reflect the amount of purified material isolated and are unoptimized. High Performance Liquid Chromatography (HPLC) was performed on a Hitachi instrument using a Waters 3.9 X 150mm C18 Nova-Pak with a mobile phase of acetonitrile/ 0.5% ammonium dihydrogen phosphate buffer (ratio given for each example), a flow rate of 1.0 ml/min and UV detection at 254nm.

9-(3,5-di-O-Benzoyl-2,2-difluoro-2-deoxy-α/β-D-ribofuranosyl)purine-6-carbonitrile (5a/5b) and 7-(3,5-di-O-Benzoyl-2,2-difluoro-2-deoxy--α/β-D-ribofuranosyl)purine-6-carbonitrile (6a/6b). A 100 mL round-bottom flask was charged with triphenylphosphine (0.95 g, 3.64 mmol) and DEAD (0.46 mL, 2.92 mmol) in dioxane (40 mL) and stirred under N_2 at 23° C for 30 minutes. The yellow solution was treated with 6-cyanopurine (1.06 g, 7.3 mmol) and stirring continued for 10 minutes at which time a solution of 3,5-di-O-benzoyl-2,2-difluoro-2-deoxyribose (**4**) in dioxane (15 mL) was added dropwise over 5 minutes. After stirring for 2 hrs at 23° C the solution was evaporated to a gummy oil, diluted with EtOAc and 0.1 N HCl, separated, the organic phase washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. Chromatography (SiO_2, 10% EtOAc/toluene) gave the following nucleosides in the order isolated. Compound **5a** was isolated from the appropriate fractions and gave 230 mg (18%) as a colorless foam; ^1H NMR ($CDCl_3$) 4.78 (d, 2H, C5'H), 5.18 (m, 1H, C4'H), 5.97 (m, 1H, C3'H), 6.81 (m, 1H, C1'H), 7.7-7.5 (m, 6H, Ar), 8.0-8.2 (m, 4H, Ar), 8.6 (d, 1H, C8H), 9.15 (s, 1H, C2H); UV λ_{max} (EtOH) 283nm (ε 10400), 232nm (ε 26700); MS (FD) m/z 505; Anal. Calcd for $C_{25}H_{17}F_2N_5O_5$: C, 59.49; H, 3.39; N, 13.86. Found: C, 59.52; H, 3.46; N, 13.72. Compound **5b** was isolated from the appropriate fractions and

gave 295 mg (24%) as a colorless solid; m. p. 146-147 C; ^{1}H NMR ($CDCl_3$) 4.70-4.95 (m, 3H, $C_5'\underline{H}$ and $C_4'\underline{H}$), 5.96 (m, 1H, $C_3'\underline{H}$), 6.61 (m, 1H, $C_1'\underline{H}$), 7.7-7.5 (m, 6H, Ar), 8.0-8.2 (m, 4H, Ar), 8.51 (d, 1H, $C_8\underline{H}$), 9.10 (s, 1H, $C_2\underline{H}$); UV λ_{max} (EtOH) 283nm (ε 10400), 232nm (ε 26700); MS (FD) m/z 505; Anal. Calcd for $C_{25}H_{17}F_2N_5O_5$: C, 59.49; H, 3.39; N, 13.86. Found: C, 59.68; H, 3.56; N, 13.76.

Compound **6a/6b** was isolated as a slightly colored foam weighing 120 mg (10%); ^{1}H NMR ($CDCl_3$) of the major N-7 anomer 4.83 (m, 1H, $C_4'\underline{H}$), 4.93 (m, 2H, $C_5'\underline{H}$), 5.83 (dd, 1H, $C_3'\underline{H}$), 6.8 (dd, 1H, $C_1'\underline{H}$), 7.4-7.7 (m, 6H, Ar), 8.1 (m, 4H, Ar), 8.76 (s, 1H, $C_8\underline{H}$), 9.28 (s, 1H, $C_2\underline{H}$); UV λ_{max} (EtOH) 202 nm (ε 34270), 232nm (ε 24701), 285 nm (ε 7649); MS (FD) m/z 505.

4-Amino-8-(2,2-difluoro-2-deoxy-β-D-ribofuranosylamino)pyrimido[5,4-d]pyrimidine (3b) and 4-Amino-8-(2,2-difluoro-2-deoxy-α-D-ribofuranosyl-amino)pyrimido[5,4-d]pyrimidine (3a). An equimolar mixture of **5a** and **5b** (0.390 g, 0.772 mmol) was dissolved in MeOH (5.0 mL) and treated with conc. NH_4OH (5.0 mL) and stirred at 23°C in a sealed tube for 17 h. The solvent was removed under reduced pressure, the residue diluted with H_2O (35 mL), extracted with several portions of pentane (3 X 25 mL) and the aqueous layer taken to hard dryness under reduced pressure. The residue was first dissolved in a mimimum amount of H_2O (10 mL) and the products isolated by extraction from warm EtOAc (3 X 50mL) to give 198 mg of a light colored solid. A 100 mg sample was taken-up in a 2.5 ml solution of $CHCl_3$/MeOH (97:3, v/v), a small amount of insoluble material was filtered and the filtrate chromatographed over SiO_2 to gave two main fractions in the order isolated. Compound **3a** was isolated as 42 mg (17%) of a colorless powder; ^{1}H NMR ($DMSO_{d-6}$) 3.53 (m, 2H, $C_5'\underline{H}$), 4.05 (m, 1H, $C_4'\underline{H}$), 4.22 (m, 1H, $C_3'\underline{H}$), 5.0 (t, 1H exchangeable, $C_5'O\underline{H}$), 6.26 (m, 1H, $C_1'\underline{H}$), 6.39 (d, 1H exchangeable, $C_3'O\underline{H}$) 7.92 (bs, 1H exchangeable, N$\underline{H}$), 8.08 (d, 1H exchangeable, N$\underline{H}$), 8.12 (bs, 1H exchangeable, N$\underline{H}$), 8.41 (d, 1H, $C_6\underline{H}$), 8.59 (s, 1H, $C_2\underline{H}$); UV λ_{max} (EtOH) 210 nm (ε 16400), 290 nm (ε 13300), 318 nm (ε 10200) 332 nm (ε 7400); MS (FD) m/z 315; Compound **3b** was isolated as 37 mg (15%) of a colorless powder; ^{1}H NMR ($DMSO_{d-6}$) 3.59 (m, 2H, $C_5'\underline{H}$), 3.78 (m, 1H, $C_4'\underline{H}$), 4.32 (m, 1H, $C_3'\underline{H}$), 5.18 (t, 1H exchangeable, $C_5'O\underline{H}$), 6.11 (m, 2H, $C_1'\underline{H}$ and $C_3'O\underline{H}$) 7.92 (bs, 1H exchangeable, N$\underline{H}$), 8.08 (bs, 1H exchangeable, N$\underline{H}$), 8.26 (d, 1H exchangeable, N$\underline{H}$), 8.4 (d, 1H, $C_6\underline{H}$), 8.65 (s, 1H, $C_2\underline{H}$); UV λ_{max} (EtOH) 210 nm (ε 16400), 290 nm (ε 13300), 318 nm (ε 10200) 332 nm (ε 7400); MS (FD) m/z 315;

4-Amino-8-(2,2-difluoro-2-deoxy-β-D-ribopyranosylamino)pyrimido[5,4-d]pyrimidine (7).

An equimolar mixture of **5a** and **5b** (1.3 g, 0.257 mmol) was treated with a saturated solution of methanolic ammonia and stirred at 50 °C for 24 hours in a sealed tube. The reaction was allowed to cool to 23 °C, evaporated and chromatographed (SiO_2, $CHCl_3$/MeOH) to give 410 mg of a foam. HPLC (10:90) analysis indicated the following composition: 45% pyranose, 34% **3a** and 17% **3b**. Compound **7** was crystallized by dissolving in EtOAc/H_2O/n-propanol (10:0.5:0.25 v/v), a small amount of insoluble material was filtered, the solution was concentrated to about half-volume and cooled to 5 °C. The solid was filtered, washed with cold EtOAc to give 62 mg (7%) as a colorless powder; ^{1}H NMR ($DMSO_{d-6}$) 3.66 (m, 2H, C5'H), 3.78 (m, 1H, C4'H), 4.08 (m, 1H, C3'H), 5.21 (d, 1H, C4'OH, exchangeable), 5.92 (m, 1H, C1'H, collapses to a doublet upon addition of D_2O), 6.21 (d, 1H, C3'OH, exchangable), 7.47 (d, 1H, 4-NH, exchangeable), 7.95 (bs, 1H, 8-NH, exchangeable), 8.12 (bs, 1H, 8-NH, exchangeable), 8.40 (s, 1H, C6H), 8.60 (s, 1H, C2H); UV λ_{max} (EtOH) 211 nm (ε 17600) 289 (ε 14100) 317 (ε 10400) 331 (ε 7510); MS (FD) m/z 315; Anal. Cald. for $C_{11}H_{12}F_2N_6O_3$: C, 42.04; H, 3.85; N, 26.74. Found: C, 41.80; H, 3.71; N, 26.70.

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16. Aqueous stability of anomerically pure (>95%) **3a** and **3b** was determined by exposing the individual compounds to buffered solutions at pH 4.0, 7.4 and 10.0 and 37 °C. Aliquots were removed and analyzed by HPLC. Compound **3a** reacted in similar fashion as **3b** and at essentially the same rate. Thus, the graphs were omitted for clarity.
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