Supporting Information

Fluorine substituted adenosines as probes of nucleobase protonation in functional RNAs

*Ian T. Suydam and Scott A. Strobel**

Extinction Coefficients. Extinction coefficients for analogs **7c - 7e** were estimated by obtaining UV-vis spectra of solutions of known concentration of the corresponding nucleosides in 100 mM phosphate buffer, pH = 7.0 (Figure S1).

Determining Site of Analog Protonation. The first site of protonation for adenosine and 7-deaza-adenosine has been determined previously via a combination of ^{13}C and ^{15}N NMR[1-6]. Both nucleosides are protonated at N1 (purine numbering), as indicated by large upfield shifts in the N1 resonance upon protonation[3,6]. Due to the strong electron withdrawing effect of fluorine substitutions the first protonation site of the analogs used in this study may be different than their unsubstituted parent nucleosides. For this reason we determined the site of protonation for the fluoro-adenosine analogs using a combination of ^{19}F, ^{15}N and ^{13}C NMR. The results presented below confirm that 7-fluoro-7-deaza-adenosine (7F-7dA) and 2-fluoro-7-deaza-adenosine (2F-7dA) are protonated at N1, while the first protonation site of 2-fluoro-adenosine (2F-A) is N7.

^{19}F NMR Titrations. ^{19}F NMR titrations for 7F-7dA, 2F-7dA, and 2F-A were carried out as described in the experimental section. Titrations were also obtained for 3-fluoro-pyridine (3F-Pyr) and 2-fluro-pyridine (2F-Pyr) to aid in the interpretation of the ^{19}F chemical shifts. The pK_a values determined by ^{19}F NMR titrations for 3F-Pyr and 2F-Pyr (Figures S2 D,E) are within ± 0.03 pK_a units of those determined by UV absorption[7] and confirm the large shift in pK_a observed for the 2-fluoro substitution (Pyr pK_a = 5.17, 2F-Pyr pK_a = -0.43). A dramatic feature of these titrations is that although the site of protonation is unambiguous, the titration curves for 3F-Pyr and 2F-Pyr show an opposite dependence of ^{19}F frequency on protonation state (3F-Pyr $\Delta\delta$ = +9.24, 2F-Pyr $\Delta\delta$ = -11.09). The origin of the upfield shift for the ^{19}F resonance of 2F-Pyr is likely related to the well documented upfield shift observed for ^{13}C resonances at the α position of nitrogen heterocycles[8].

The ^{19}F NMR titrations of the fluorine substituted analogs of this study also show both upfield and downfield shifts upon protonation (Figures S2 A-C). While 2F-7dA shows a large upfield shift upon protonation, 7F-7dA and 2F-A show more modest downfield shifts. The simplest interpretation of these titrations is that 7F-7dA and 2F-7dA are protonated at N1 or N3, while 2F-A is protonated at N7. While the pK_a of adenosine's N7 nitrogen cannot be determined directly it has been estimated to be the second most basic site, with a pK_a between 2.5 and 3.0[9,10]. Given the dramatic effect of fluorine substitution on the pK_a of 2F-Pyr it is reasonable to expect N7 would become the most basic site in 2F-A. This expectation is confirmed by the ^{15}N and ^{13}C NMR data presented below.

^{15}N NMR Spectra. To confirm the site of protonation for 7F-7dA and 2F-A we obtained ^{15}N NMR spectra of these nucleosides in d_6-DMSO solutions after adding various equivalents of H_2SO_4 (nucleoside concentrations ranged from 70 - 100 mM). Because these compounds contain ^{15}N at natural abundance an indirect method of detection was required. We chose to employ a ^{1}H to ^{15}N heteronuclear multi-bond connectivity experiment[11] (^{1}H-^{15}N HMBC). Experimental parameters were set to filter single bond couplings with $^1J_{NH}$ near 90 Hz, and to detect multiple bond coupling with $^nJ_{NH}$ near 15 Hz. To validate the method we obtained ^{1}H-^{15}N HMBC spectra of adenosine (Figure S3 A), and used the ^{15}N dimension of these spectra to track peak positions as a function of added equivalents of H_2SO_4 (Figure S3 B). Before the addition of H_2SO_4 the N1 nitrogen is readily identified by its strong coupling to the C2 proton and weak coupling to the exchangeable protons of the exocyclic amine (Figure S3 A). Addition of H_2SO_4 leads initially to a shift in N1 frequency as well as a loss of signal due to exchange. The N1 resonance is recovered once adenosine is fully protonated, and is shifted by -78.9 ppm relative to the unprotonated state. The chemical shifts of all nitrogens before and after protonation match those reported previously reported[3].

The same series of ^{1}H-^{15}N HMBC spectra were obtained for the 7F-7dA and 2F-A analogs (Figures S4 and S5). For 7F-7dA the weak coupling between N1 and the C6 amine was not observed due to a broadened exocyclic amine ^{1}H resonance. However, the N1 and N3 chemical shifts of 7F-7dA are within 1 ppm of those reported for 7-deaza-adenosine, providing an assignment of these resonances (this assignment is confirmed by the ^{13}C chemical shifts provided below). The N1 resonance of 7F-7dA shifts -78.2 ppm upon protonation. For 2F-A only the N7 and N9 resonances are observable in the ^{1}H-^{15}N HMBC spectra since the C2 proton has been replaced with fluorine in this analog. These resonances are easily distinguished by the large chemical shift difference between N7 and N9 seen in a large number of purine ribonucleosides[5]. Unlike 7F-7dA, protonation of 2F-A requires more than a single equivalent of H_2SO_4 because of its very low pK_a (see above). The N7 resonance of 2F-A shifts -65.1 ppm upon protonation, confirming N7 as the most basic site of 2F-A. ^{1}H-^{15}N HMBC spectra of 2F-7dA were not acquired since the 2-fluoro substitution prevents the detection of either titratable nitrogen.

^{13}C NMR Spectra. A final confirmation of analog protonation site was obtained with ^{13}C NMR spectra taken before and after protonation. Assignment of protonation sites with this method relies on the observation that protonation of nitrogen heterocycles produces large upfield shifts at the adjacent carbons[1,8]. In the case of adenosine upfield shifts are observed at all carbons in the pyrimidine ring, with the largest of these shifts adjacent to the site of protonation[2]. ^{13}C NMR spectra of 7F-7dA, 2F-7dA and 2F-A were obtained from d_6-DMSO solutions (nucleoside concentrations ranged from 40 - 100 mM). Spectra were assigned by a combination of ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC spectra, and by the observed $^nJ_{CF}$ coupling constants (Table S1). The observed chemical shift changes for 7F-7dA confirm N1 as the site of protonation. Large upfield shifts are observed for C2 and C6, with significant downfield shifts for C8, as is the case for adenosine. For 2F-A the pattern is reversed, with the largest upfield shift occurring at C5 and the largest downfield shifts at C2 and C1', supporting the assignment of N7 as the most basic site in this analog. The modest upfield shift at C8, despite its proximity to N7, is also observed for the second protonation of adenosine at N7[2]. For 2F-7dA substantially more acidic solutions were required to accumulate a significant fraction of the protonated form. Because these solutions led to slow degradation of 2F-7dA we limited averaging times in the collection of ^{13}C spectra after the addition of H_2SO_4. The resulting spectra provided clear peak positions and assignment for all resonances except C2 (the weakest resonance due to its large ^{19}F coupling). The observed chemical shift changes are consistent with N1 protonation of 2F-7dA, with a larger upfield shift observed at C6 than at C4.

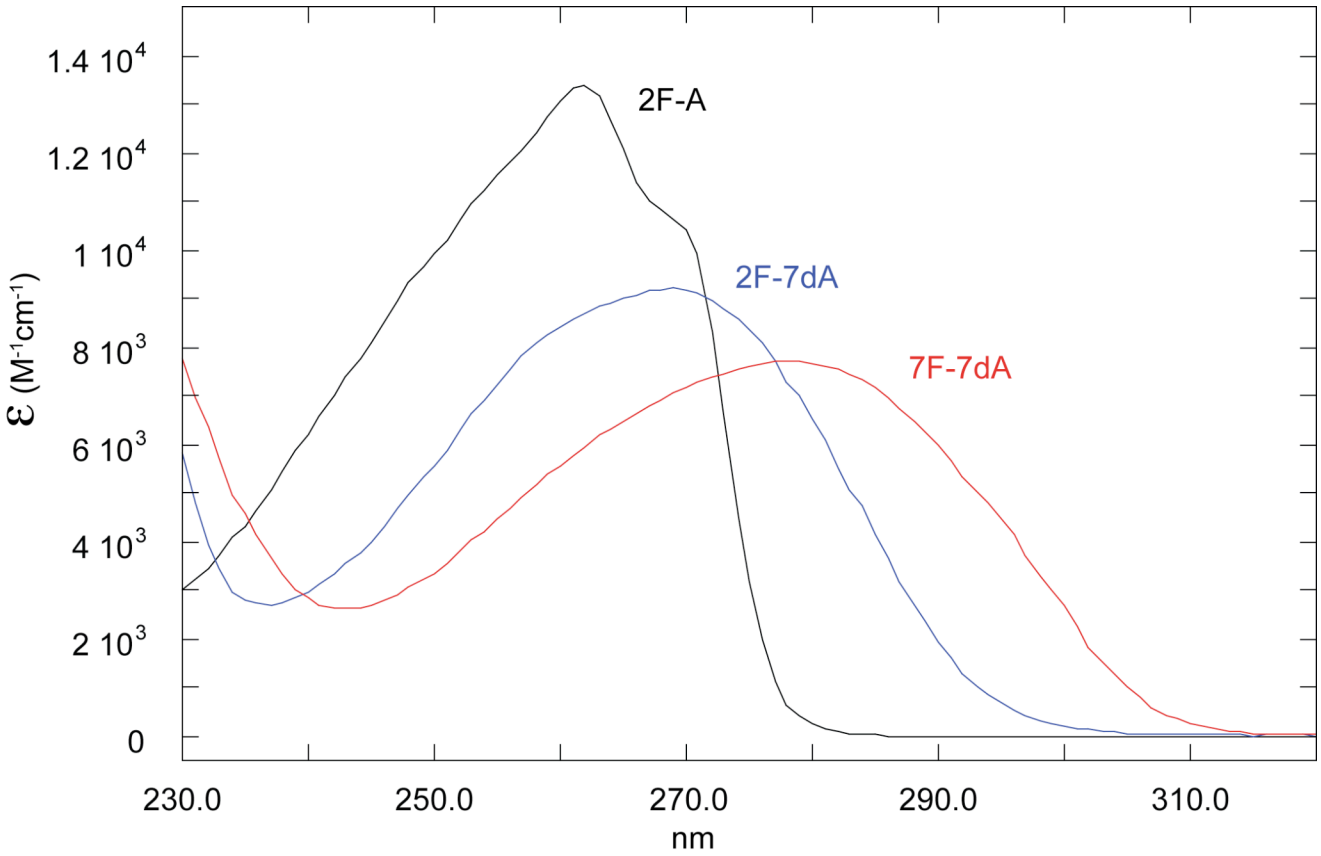


Figure S1: UV-vis spectra of fluorine substituted adenosines in 100 mM phosphate buffer (pH = 7.0).

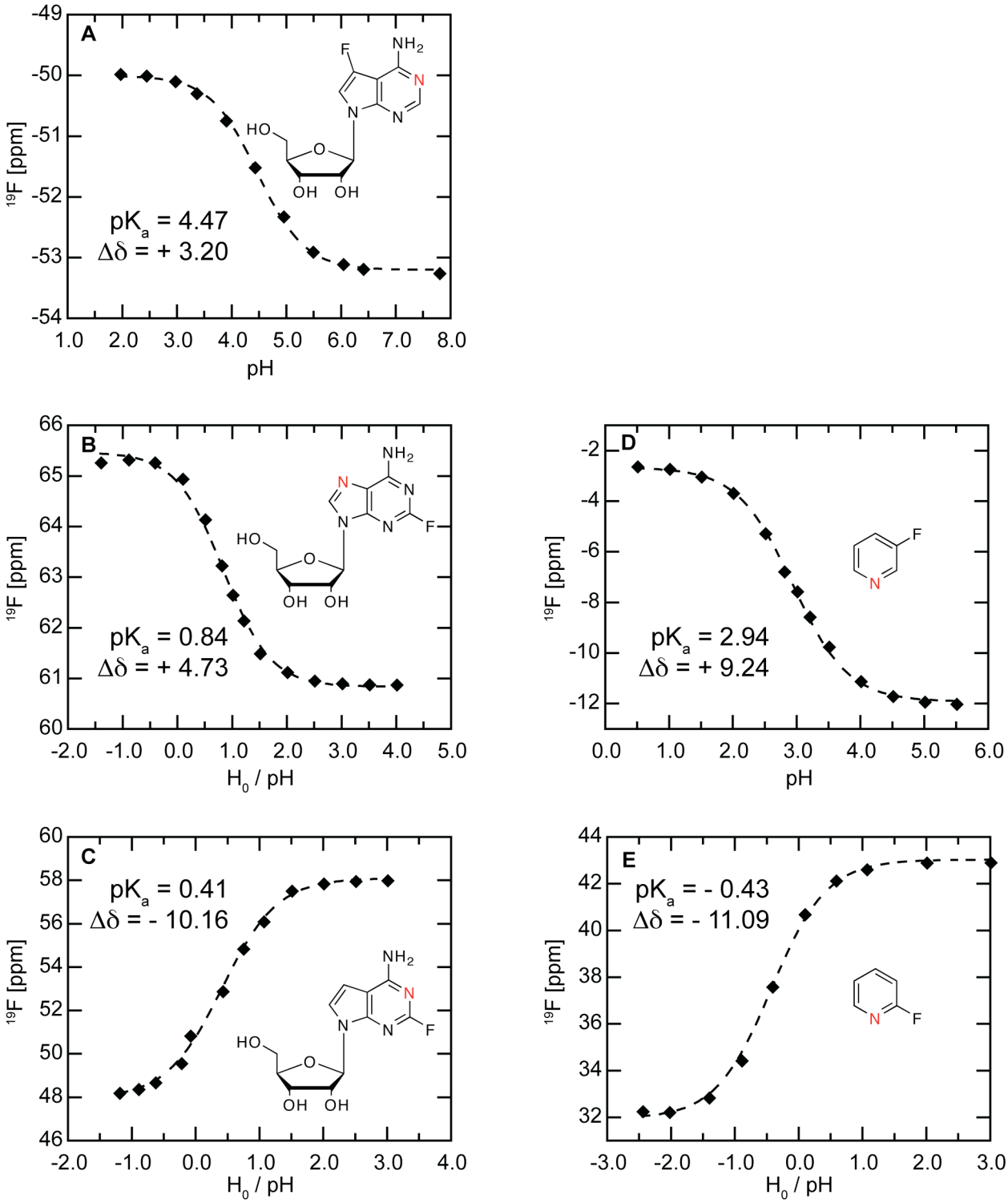


Figure S2: ^{19}F NMR titrations of (A) 7-fluoro-7-deaza-adenosine, (B) 2-fluoro-adenosine, (C) 2-fluoro-7-deaza-adenosine, (D) 3-fluoropyridine, and (E) 2-fluoropyridine. ^{19}F frequencies are reported relative to an internal fluorobenzene standard. pH values above 2.0 were adjusted with appropriate buffer systems. For pH values below 2.0 the Hammett acidity function (H_0) was used and adjusted by addition of H_2SO_4.

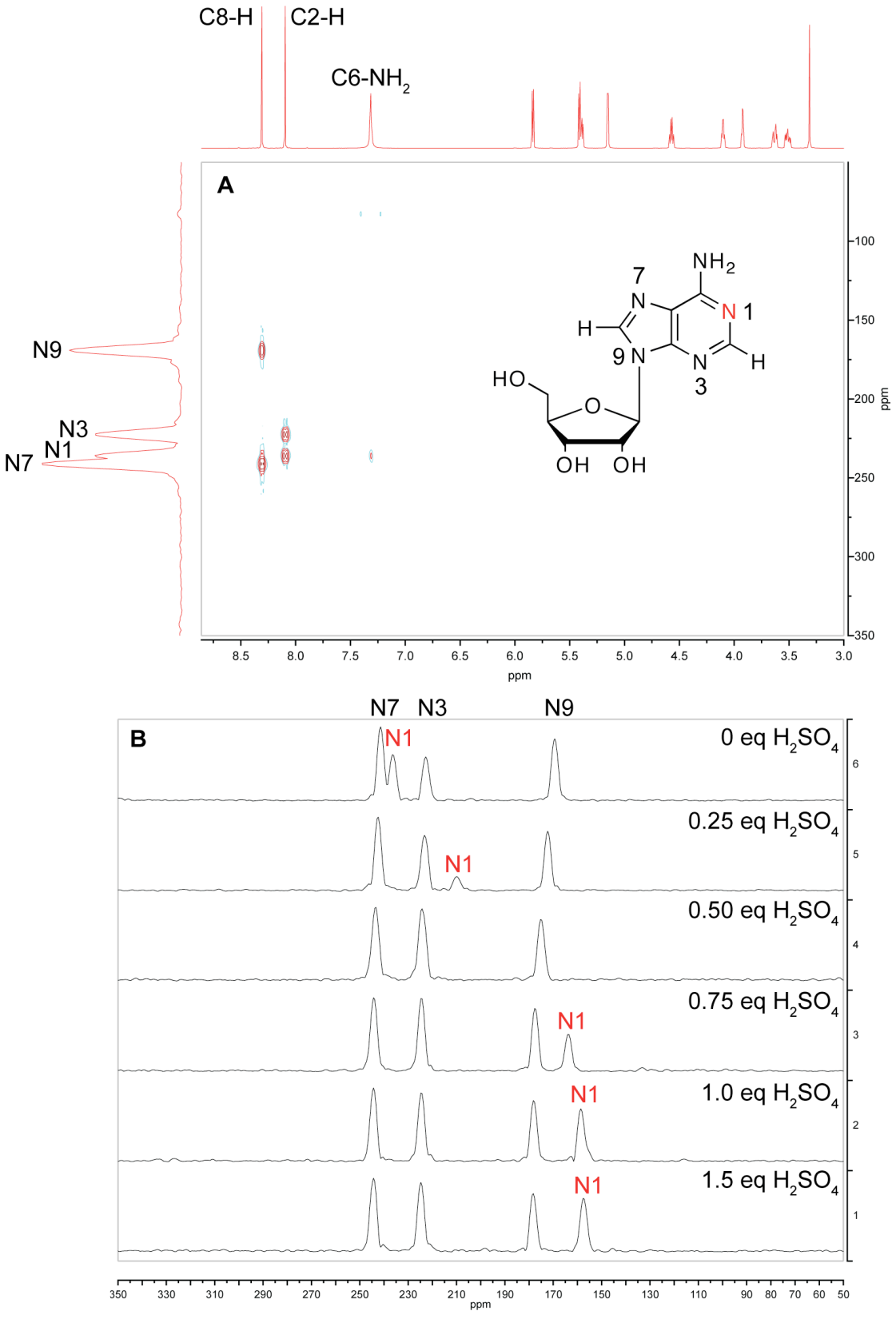


Figure S3: (A) ^{1}H-^{15}N HMBC spectra of adenosine in d_6-DMSO. (B) ^{15}N dimension of ^{1}H-^{15}N HMBC spectra after addition of H_2SO_4 (number of equivalents of H_2SO_4 noted to right of spectra). Spectra were referenced to external CH_3NO_2 and displayed on the NH_3(l) scale.

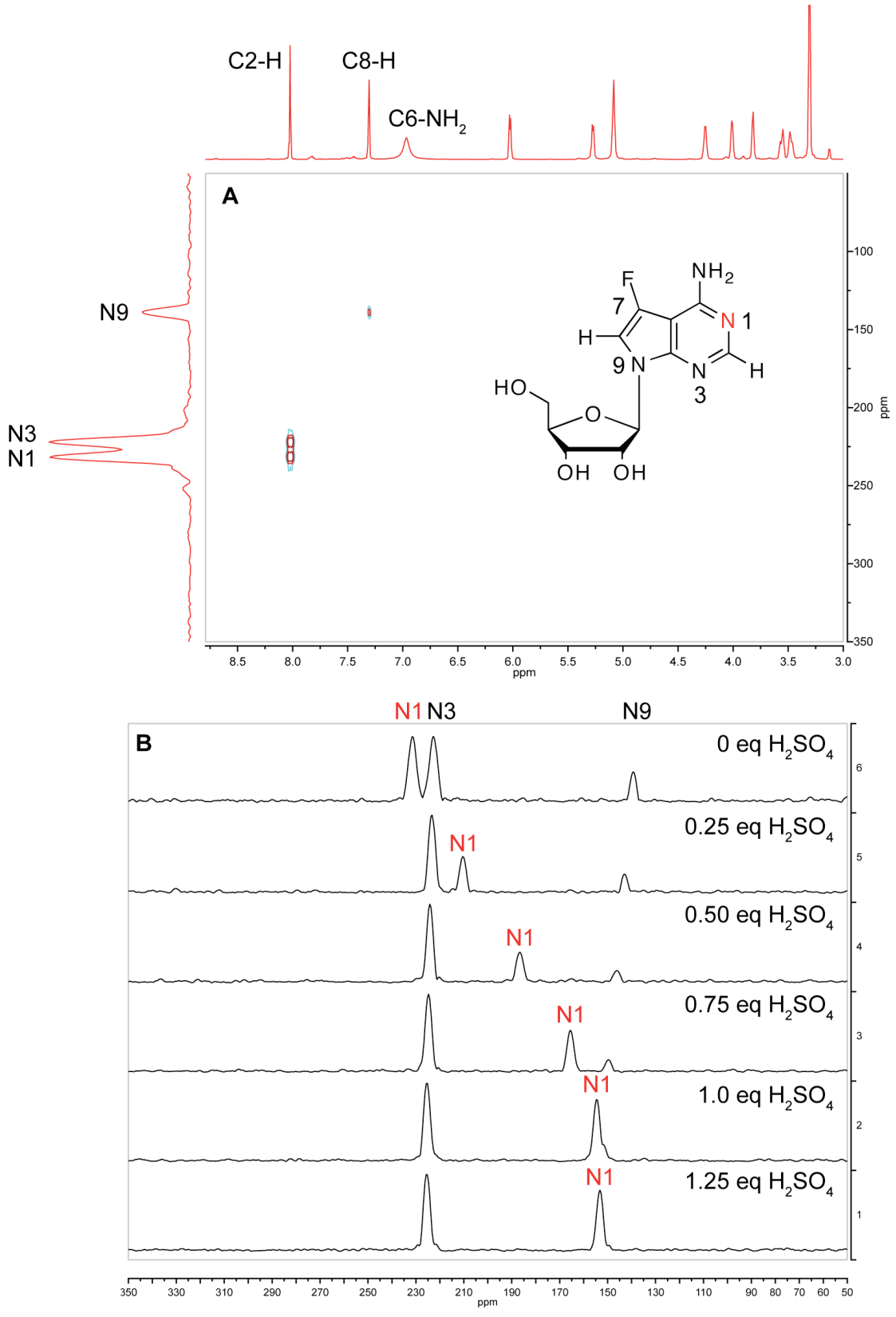


Figure S4: (A) ^{1}H-^{15}N HMBC spectra of 7-fluoro-7-deaza-adenosine in d_6-DMSO. (B) ^{15}N dimension of ^{1}H-^{15}N HMBC spectra after addition of H_2SO_4 (number of equivalents of H_2SO_4 noted to right of spectra). Spectra were referenced to external CH_3NO_2 and displayed on the NH_3(l) scale.

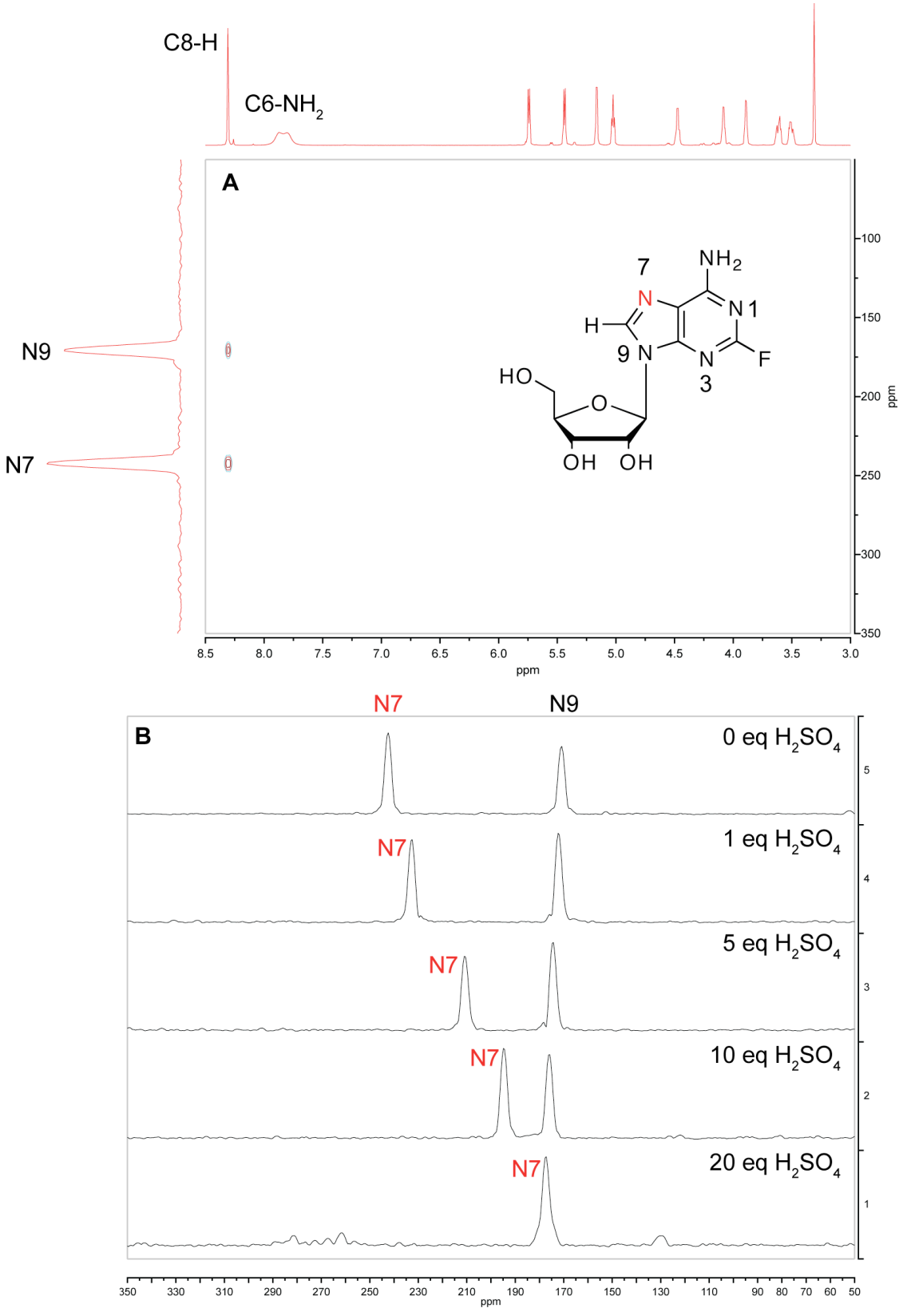
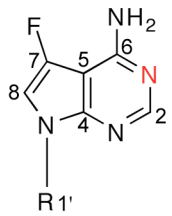
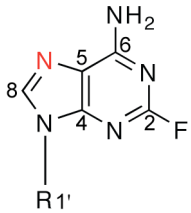
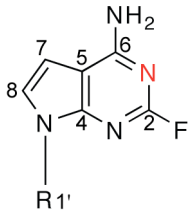


Figure S5: (A) ^{1}H-^{15}N HMBC spectra of 2-fluoro-adenosine in d_6-DMSO. (B) ^{15}N dimension of ^{1}H-^{15}N HMBC spectra after addition of H_2SO_4 (number of equivalents of H_2SO_4 noted to right of spectra). Spectra were referenced to external CH_3NO_2 and displayed on the NH_3(l) scale.

Table S1: ^{13}C chemical shifts of analogs before and after addition of H_2SO_4.

Analog	Equiv. H_2SO_4		C2	C4	C5	C6	C7	C8	C1'
	0	δ (ppm)	152.74	146.25	92.50	155.80	142.57	104.34	86.48
		J_{CF} (Hz)			15		245	27	
	1.25	δ (ppm)	143.82	142.89	91.46	149.53	143.50	107.62	86.81
		J_{CF} (Hz)			14		250	25	
		Δδ (ppm)	-8.92	-3.36	-1.04	-6.27	0.93	3.28	0.33
	0	δ (ppm)	158.54	150.47	117.51	157.65		139.93	87.42
		J_{CF} (Hz)	202	19		20			
	20	δ (ppm)	160.08	149.87	110.86	155.81		139.89	90.41
		J_{CF} (Hz)	210	22		22			
		Δδ (ppm)	1.54	-0.60	-6.65	-1.84		-0.04	2.99
	0	δ (ppm)	159.17	151.15	100.70	159.33	100.31	121.86	86.81
		J_{CF} (Hz)	201	19		21			
	60	δ (ppm)	NA	149.2	100.41	156.34	101.7	122.92	86.95
		J_{CF} (Hz)	NA	NA		NA			
		Δδ (ppm)	NA	-1.95	-0.29	-2.99	1.39	1.06	0.14

Complete References.

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Supplemental References.

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