VOLUME 122, NUMBER 4
FEBRUARY 2, 2000

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Hydride Transfer in Oxidation of Nucleic Acid Sugars: Electronic Effects of 2′-Substituents on Activation of the 1′-C−H Bond by Oxoruthenium(IV)

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Received June 9, 1999

Abstract: The effect of polar 2′-substituents on the oxidation of nucleic acids at the 1′-C−H bond was investigated. Oligonucleotides with the sequence 5′-ATI $CCC_{2'-X}$ TTI CIT AT-3′ (I = inosine, −X = −H, $-NH_2$, $-OCH_3$, −F) were synthesized and subjected to oxidation by Ru(tpy)(bpy)O^{2+} (tpy = 2,2′:6′,2″-terpyridine; bpy = 2,2′-bipyridine). High-resolution electrophoresis revealed a similar cleavage pattern for all of the sites in the oligonucleotides except for the 2′-substituted site, which varied in the extent of cleavage relative to the other sites according to H > NH_2 > OCH_3 > F. Because the cleavage was monitored following piperidine treatment, this analysis could not be applied to the 2′-hydroxy oligonucleotide. The extent of cleavage was a linear function of oxidant concentration, and the slope of this plot was used as a relative rate after normalizing to the adjacent, unsubstituted 5′-cytosine. When the relative rates were determined using other nucleotides in the sequence for normalization, the results did not change outside the quoted error limits. The log of the relative rates was a linear function of the polar Hammett parameter, *F*, and gave a slope of $\rho = -1.4 \pm 0.1$. Rate constants on 2′-H and 2′-OH monophosphates from previous studies by stopped-flow spectrophotometry fall on the line defined by the electrophoresis data, supporting the assumption that the C−H bond activation is rate-limiting. The observed Hammett correlation supports an accumulation of positive charge at the 1′-carbon, as would be expected for hydride transfer. These results provide a basis for the oxidation resistance of 2′-substituted oligonucleotides.

The activation of C−H bonds in nucleic acid sugars provides a mechanism for the chemical footprinting of nucleic acids,[1−3] the action of drugs that cleave nucleic acids,[4] and natural DNA and RNA damage.[5] In general, agents that damage nucleic acid sugars exhibit fewer cleavage sites in RNA than in DNA,[6−8] an effect that could arise in part from a chemically more demanding oxidation reaction in RNA compared to DNA, although the more diverse structures of RNA certainly play a role. For example, the complete chemical mechanism for oxidation of the 1′-C−H bond in RNA by iron bleomycin has been elucidated,[9] and the cleavage of natural RNAs by bleo-

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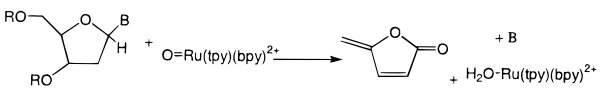
10.1021/ja991967+ CCC: $19.00
Published on Web 01/15/2000

mycin is much more selective than that of DNA.[7] Further, the cleavage of stem-loop RNAs and their DNA analogues by oxoruthenium(IV) is more extensive in the DNA analogues compared to that in the corresponding RNAs.[10−12] Finally, the absolute rate constants for oxidation of the 1′-C−H bond in mononucleotides by oxoruthenium(IV) have been measured by stopped-flow spectrophotometry, and the 2′-OH decreases the second-order rate constant by a factor of 5−10 depending on the identity of the attached base.[11] In addition to elucidating the differences between natural DNA and RNA, the effect of 2′-substituents is also of interest in the preparation of nuclease-resistant aptamers that are of utility as drugs and biological tools.[13−16]

There has been an earlier suggestion that the greater oxidative reactivity of DNA over RNA toward iron bleomycin, termed the "DNA/RNA Paradox,"[17] is attributable to destabilization of a cationic species adjacent to the 2′-hydroxy group. In this previous study, model sugars were synthesized that produced a cation radical at the 3′-position, which was less stable when there was a 2′-OMe substituent than in the 2′-deoxy case.[17] This observation suggests that the rate of cleavage at a 2′-OMe nucleotide in a polymer should be slower than that for a 2′-H nucleotide and that substituents that stabilize the adjacent cation radical to different extents should modulate the reactivity appropriately. Further, the effect should also be observed for generation of a cationic transition state at the 1′-carbon. Here we show that oxidation at the 1′-carbon by an oxidant known to generate a transition state with significant cationic character proceeds in single-stranded oligonucleotides with a rate that is dependent on the Hammett polarity of the 2′-substituent. Our observations expand and support the assertions of Crich[17] by showing that other substituents exert the same effect to differing degrees depending on the polarity of the 2′-substituent, that the effect is apparent in cleavage studies on intact oligonucleotides, and that the 2′-substituent modulates the rate of oxidation at the 1′-site in addition to the 3′-site.

The oxidant used in these studies is a complex based on oxoruthenium(IV), $Ru(tpy)(bpy)O^{2+}$ (tpy = 2,2′:6′,2″-terpyridine, bpy = 2,2′-bipyridine), which undergoes a thermal reaction with DNA to produce 5-methylenefuranone (5-MF) and free bases:[10,18] A number of other oxidants react with DNA via this



(1)

pathway to generate 5-MF.[19−21] In the oxoruthenium(IV) case, isotopic labeling shows direct incorporation of the oxo ligand of the metal complex into the 5-MF product.[10] These observations combined with the attenuation of the reaction rate constant by the 2′-OH substituent[11] strongly support a mechanism of oxidation where the rate-limiting step is activation of the 1′-C−H bond by the metal−oxo functionality. Numerous detailed studies on the mechanisms of C−H activation in small molecules by oxoruthenium(IV) suggest that the initial oxidation step exhibits two-electron character.[22−24] As shown here, the effect of a range of 2′-substituents on the nucleic acid sugar oxidation is consistent with significant accumulation of positive charge on the 1′-carbon in the transition state.

Experimental Section

Materials. The $[Ru(tpy)(bpy)O](ClO_4)_2$ complexes were prepared as previously described[18] except Cl_2 instead of Br_2 was used as the oxidant in the final step. Oligonucleotides (5′-ATI $CCC_{2'-X}$ TTI CIT AT-3′) were purchased from the Nucleic Acids Core Facility at UNC. Nucleotide phosphoramidites used in the syntheses were purchased from Glen Research. The oligonucleotides were piperidine treated (held at 90 °C in 1 M piperidine in water) and purified on a 20% denaturing gel before use. Water was house distilled and further purified by passage through a Millipore Milli-Q water purification system. Vitreous carbon working electrodes were purchased from The Electrosynthesis Co. Ag/AgCl reference electrodes were purchased from Cypress Systems.

DNA Oxidation. A stock solution of $[Ru(tpy)(bpy)O](ClO_4)_2$ was made by dissolving a small amount of the complex in 50 mM sodium phosphate buffer (pH 6.8). This solution was held at a potential of +0.85 V (vs Ag/AgCl, Pt wire counter, vitreous carbon working) to ensure the absence of lower oxidation state Ru. This solution was then diluted by a factor of 4 with water (final buffer concentration = 12.5 mM). This solution (0−10 μL) was added to a solution of 10 μL oligonucleotide (14 μM in strand) in water and 12.5 mM sodium phosphate buffer (pH 6.8); the total solution volume was 20 μL. The reaction was allowed to proceed for 10 min at room temperature when 800 μL cold (−4 °C) ethanol was added to quench the reaction. A solution of 1.5 M sodium acetate (20 μL) was added, and the mixture was placed on dry ice for 30 min to precipitate the DNA. The solutions were centrifuged and lyophilized to produce a white pellet that either was loaded onto a gel or piperidine-treated by literature methods.[18] Resulting pellets were dissolved in 10 μL gel loading buffer (80% formamide in water with 0.0025% bromophenol blue and 0.0025% xylene cyanol FF) and run on a 20% polyacrylamide denaturing gel, using a published protocol.[25]

The resulting polyacrylamide gels were imaged using Molecular Dynamics phosphorimaging screens. The screens were scanned using a Molecular Dynamics Storm 840 Phosphorimager. Analysis of the resulting images was performed using ImageQuant by drawing equivalent rectangles around each of the cleavage bands and integrating the volume within the rectangle. The volumes were then plotted against $[RuO^{2+}]$ to generate the plots shown in Figure 3

Results

To assess the relative reactivities of the 2′-substituted nucleotides, we have devised a strategy that exploits the DNA cleavage reactivity of $Ru(tpy)(bpy)O^{2+}$ and the low quantities of material needed for high-resolution gel electrophoresis. As shown previously, the oxidation of nucleic acid sugars by $Ru(tpy)(bpy)O^{2+}$ is limited to activation of the 1′-C−H bond, which produces 5-MF and the appropriate termini (eq 1).[10,18]

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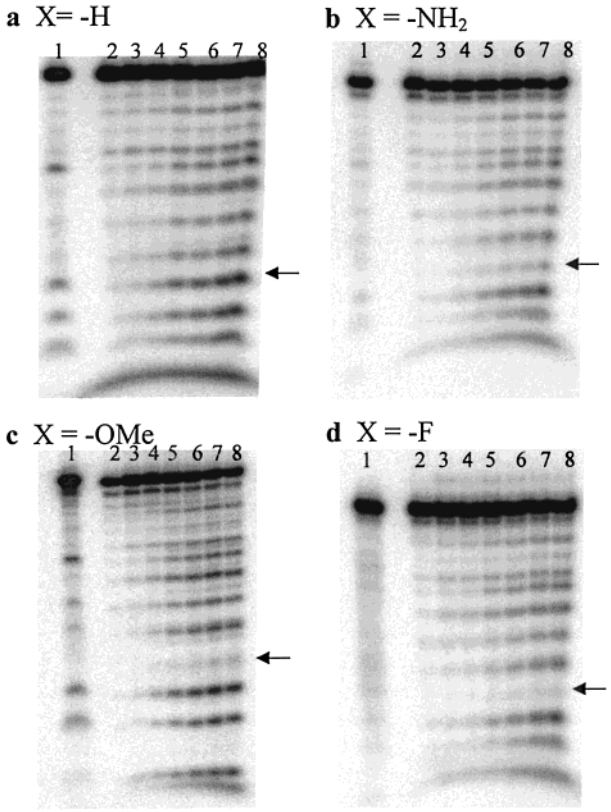

Figure 1. Representative phosphorimages showing cleavage of the oligonucleotide 5′-ATI CCC_X TTI CIT AT (14 μM) using varying concentrations of Ru(tpy)(bpy)O^{2+}. Lane 1, Maxam−Gilbert C lane; lanes 2−8, [RuO^{2+}] = 0, 1*x*, 2*x*, 4*x*, 6*x*, 8*x*, 10*x*. (A) x = 4.6 μM, −X = −H; (B) x = 4.2 μM, −X = $-NH_2$; (C) x = 3.0 μM, −X = −OMe; (D) x = 4.5 μM, −X = −F. The arrow indicates the substituted base.

Piperidine treatment leads to complete conversion of the oxidized lesions to the strand scission products. The complex does undergo a parallel reaction with guanine to produce base-labile lesions; however, this reaction was suppressed in this study by substitution of guanosine with inosine, which is oxidatively inert by comparison;[26] the redox-inertness of inosine has been similarly exploited by in studies of long-range electron transfer.[27] By comparing the reaction of Ru(tpy)(bpy)O^{2+} with a 2′-substituted nucleotide and an unsubstituted 2′-deoxy nucleotide *in the same strand*, the effect of the polar substituent on the reaction rate can be assessed on the low quantities of radiolabeled material required for high-resolution gel electrophoresis. One drawback to this strategy is that because piperidine treatment is required to realize complete conversion of the lesion to the strand scission products, analysis of 2′-hydroxy nucleotides is precluded, since these are piperidine-labile prior to oxidative damage. Fortunately, the relative reactivites of 2′-H and 2′-OH are known from oxidation kinetics with high precision, at least for the 5′-monophosphates.[11]

The effect of the 2′-substituent in intact nucleic acids was evaluated using the DNA cleavage strategy on a 14-mer oligonucleotide 5′-ATI $CCC_{2'-X}$ TTI CIT AT-3′ (I = inosine, −X = −H, $-NH_2$, − OMe, −F). The cleavage reactions were performed with varying concentrations of Ru(tpy)(bpy)O^{2+} followed by piperidine treatment, which showed a clear difference in reactivity for the substituted nucleotide, C_6 (Figure 1).

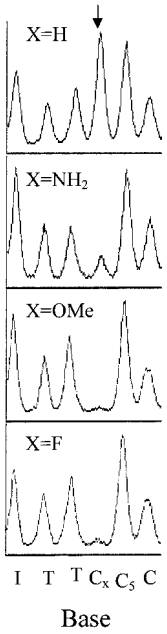

Figure 2. Histograms showing the relative cleavage intensity for oxidation of 5′-ATI CCC_X TTI CIT AT normalized at C_5. The arrow indicates the substituted nucleotide.

Importantly, the cleavage pattern at the other nucleotides was not changed significantly by addition of the 2′-substituent at C_6. This point is emphasized by inspection of quantitative traces of gel intensity (Figure 2). We chose to use the adjacent site (C_5) for normalization between oligomers because the cleavage at this site was the most intense and was highly reproducible. Further, although we took great care to ensure that no over-reaction (i.e., multiple oxidations on the same oligomer) occurred, the influence of such overreaction is minimized by choosing a normalization site adjacent to the site of interest. Suppression of the reaction at C_6 by substitution of a polar substituent apparently does not lead to increased reaction at the other sites, as evidenced by the reproducibility of the reaction pattern at the other nucleotides. A danger of choosing the adjacent site is that changes in the conformation at C_6 due to the substituent would affect the relative rate at the adjacent site; however, the consistency of reaction intensity at C_5 (and the reproducibility of the relative intensities overall) alleviates this concern. This consistency of the relative intensities for the other sites strongly suggests that the change in reactivity at the substituted nucleotide is an effect of the substituent on the individual reaction rate at that nucleotide and not a structural effect propagated through the macromolecular structure.

The relative reaction rates were quantitatively evaluated by plotting the cleavage intensity at the substituted C_6 and unsubstituted C_5 nucleotides as a function of the oxidant concentration (Figure 3). Importantly, the cleavage intensities increased linearly with Ru(tpy)(bpy)O^{2+} concentration, strongly supporting the assertion that the relative reaction rates at each site are being measured. For each gel, the slope of the linear plots for the substituted nucleotide (C_6) divided by the slope for the unsubstituted nucleotide (C_5) was used as a measure of the relative rate (Table 1). When the normalization was performed using any of the nucleotides that are sufficiently resolved on the gels (C_4, C_5, T_7, T_8, and I_9), the relative rates did not change outside the error limits quoted in Table 1. There is a strong correlation between the relative cleavage rates and the polar Hammett parameter F (Figure 4).[28] The slope of the

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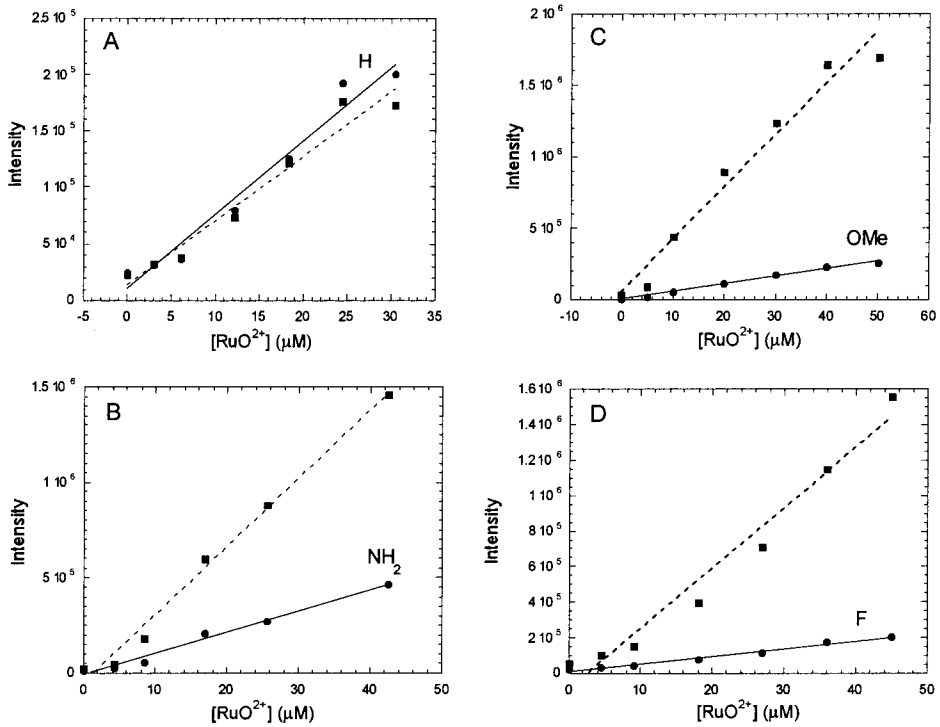


Figure 3. Intensity vs [RuO^{2+}] plots for the phosphorimages shown in Figure 2. Substituted nucleotides are shown as circles with the linear fit shown as a solid line; data for the normalizing C_5 are shown as squares with the linear fit shown as a broken line. Relative rates given in Table 1 are ratio of the slopes for C_X and C_5. (A) $-X = -H$, (B) $-X = -NH_2$, (C) $-X = -OMe$, (D) $-X = -F$.

Table 1. Relative Rates of Oxidation at 2′-Substituted Nucleotides

X	sequence	F^a	relative rate[b]
-H	5′-ATI CCC_H TTI CIT AT	0	1.13 ± 0.14
$-NH_2$	5′-ATI CCC_{NH2} TTI CIT AT	0.38	0.31 ± 0.03
-OH	−	0.46	0.17[c]
-OMe	5′-ATI CCC_{OMe} TTI CIT AT	0.54	0.15 ± 0.02
-F	5′-ATI CCC_F TTI CIT AT	0.74	0.13 ± 0.02

[a] Hammett parameters taken from reference 28. [b] Relative rates were determined by integrating the cleavage band at the substituted nucleotide (C_6) and the normalization nucleotide, C_5. These raw data were then plotted as a function of the concentration of Ru(tpy)(bpy)O^{2+} (Figure 3), and the slopes of these concentrations were divided to give the relative rates. [c] Data taken from reference 11 for oxidation rate constants from stopped-flow spectrophotometry on reactions of Ru(tpy)(bpy)O^{2+} with cytosine 5′-monophosphate (CMP) and 2′-deoxycytosine 5′-monophosphate (dCMP). The relative rate given in the table is the ratio of the second-order rate constants obtained from the kinetics data that follow the reduction of the oxoruthenium(IV) complex.

Hammett plot ($\rho = -1.4 \pm 0.1$) is indicative of significant positive charge accumulation on the 1′-carbon during the reaction. The polar Hammett parameter is the only Hammett parameter that provided a satisfactory correlation, indicating that the contribution of the 2′-substituent to the transition state is mostly through polar effects, not steric or resonant effects.[28] It could be argued that there is a change in mechanism for the −F compound, since this point lies somewhat above the line; however, exclusion of this point would only increase the magnitude of ρ, which still supports a significantly cationic transition state. Also, it would be desirable to have oligonucleotides with electron-releasing substituents (such as $-CH_3$) at the 2′-position, but to our knowledge, these nucleotides have not been synthesized. Nonetheless, it is clear simply from the relative rates for −H, $-NH_2$, and −OMe that a polar Hammett correlation is present with a negative slope.

In addition to the relative extents of cleavage for the substituted nucleotides, also included in Figure 4 is the ratio of rates from stopped-flow spectrophotometry for oxidation of

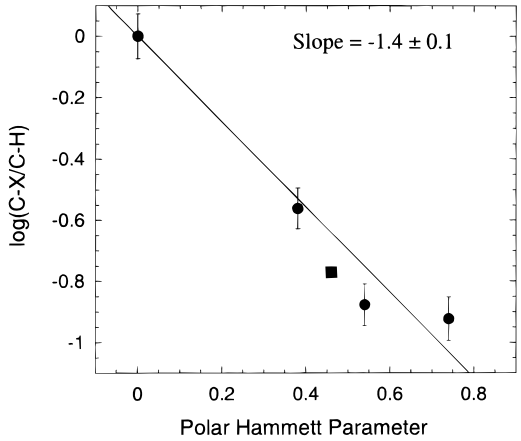


Figure 4. Plot of log of the relative rates from Table 1 versus the Hammett polar parameter *F*. The solid line represents the best linear fit to the data. Error bars are standard deviations from multiple measurements. Circles are the data from gel electrophoresis with error bars taken from standard deviations of 20 experiments. The square is the ratio of rate constants obtained from stopped-flow spectrophotometry in reference 11.

dCMP and CMP by Ru(tpy)(bpy)O^{2+}.[11] The ratio of the second-order rate constants k(CMP)/k(dCMP) falls near the line defined by the cleavage data from gel electrophoresis; the *k*(CMP)/*k*(dCMP) ratio is known with relatively high precision because sufficient quantities of the natural mononucleotides were available. The similarity between the polar effect observed in the real-time kinetics and that on the gels supports the assertion that the rate-limiting step is the C−H bond activation in both cases. As stated earlier, the relative rate for $X = -OH$ cannot be evaluated using the electrophoresis technique because this site would be piperidine-labile prior to oxidation by Ru(tpy)(bpy)O^{2+} and the cleavage yield would therefore not reflect the oxidation reactivity.

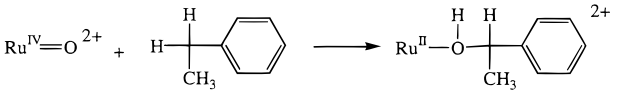
Discussion

Mechanisms of Oxoruthenium(IV) Reactions. In addition to the issues concerning nucleic acid oxidation and the DNA/RNA paradox,[17] the results reported here provide insight into the action of oxoruthenium(IV) on organic substrates. Early studies by Meyer et al. following oxidation of alcohols and alkylaromatics by these complexes using single-wavelength spectroscopy were initially interpreted in terms of a simple, two-electron hydride transfer to form a carbocation and hydroxoruthenium(II):[23,24]

$$Ru{=}O^{2+} + H{-}C(OH)R_2 \rightarrow RuOH^{+} + {}^{+}C(OH)R_2 \quad (2)$$

$$RuOH^{+} + {}^{+}C(OH)R_2 \rightarrow RuOH_2{}^{2+} + O{=}CR_2 \quad (3)$$

The carbocation can then simply lose a proton to form the ketone product, and the hydroxoruthenium(II) complex is protonated to form the stable aquaruthenium(II) product (eq 3). Later studies on ethylbenzene oxidation involving multiple wavelength spectroscopy provided evidence for insertion of the oxo ligand into the C−H bond to produce a coordinated intermediate:[29]



(4)

The Ru(II)−alcohol complex is then oxidized by an additional equivalent of $Ru(IV)O^{2+}$ to generate a Ru(III)−alkoxide complex and $Ru(III){-}OH^{2+}$.

The oxidation of the 1′-position in DNA sugars by Ru(tpy)(bpy)O^{2+} proceeds with formation of a ketone product where the oxo ligand is added to the organic substrate (i.e., eq 1),[18] so that the reaction likely proceeds via a coordinated intermediate similar to that shown in eq 4 rather than simple hydride transfer. In either case, considerable accumulation of positive charge at the oxidized carbon is anticipated, since both are two-electron oxidations. For either of these mechanisms then, a polar effect such as that deduced from the data in Figure 4 would be expected. These results therefore provide further evidence that the $Ru{=}O^{2+}$ functionality acts as a two-electron oxidant, as observed with oxo transfer to alkylaromatics,[23,29] alcohol oxidation,[24] and reaction of olefins by both allylic oxidation[29] and epoxidation.[22]

Nucleic Acid Oxidation Mechanisms. The findings described here raise a number of interesting points concerning the effects of both natural and synthetic 2′-substituents in nucleic acids. The first is that RNA sugars are less chemically reactive toward oxidation than DNA sugars, a point that is counter to the hydrolytic reactivity, where RNA is considerably more labile.[30] The combination of oxidation resistance and hydrolytic lability may be advantageous for RNA, which requires a finite lifetime in the cell but is also exposed to many more potential oxidants than DNA, which is protected in the nucleus from reactive species present in the cytoplasm.[5] Thus, RNA can be hydrolytically degraded when desirable while avoiding inadvertent deleterious oxidation.

The origin of the DNA/RNA paradox has been previously ascribed to a polar effect of the 2′-substituent on an adjacent cationic intermediate.[11,17] In particular, earlier studies have shown that 3′ oxidation products are destabilized by 2′-OMe in model sugars.[17] In our own group, we have shown previously that the rate of CMP oxidation by Ru(tpy)(bpy)O^{2+} is much slower than that for dCMP, a phenomenon we also ascribed to a polar effect of the 2′-OH.[11] Here we extend the observations further to show that different electron-withdrawing substituents modulate the reaction rate to different extents according to the polar Hammett parameter, which indicates in particular that 2′-NH_2 sugars are intermediate in reactivity compared to 2′-H and 2′-OH sugars, a point that is clear in Table 1. The appropriateness of 2′-substituted nucleic acids as drugs is now readily apparent, because with polar substituents other than −OH, these molecules are both *oxidatively AND hydrolytically more stable* than their natural counterparts.[13−16]

Finally, it is appropriate to comment on the relevance of these effects to sugar oxidation by the wide range of transition metal oxidants under investigation.[31] As noted by Crich and Mo,[17] the effect of the polar substituent is expected to be more pronounced for reactions involving carbocations than for radical reactions resulting from hydrogen atom transfer. In terms of metal complex oxidants, this implies that species with more two-electron character will be most susceptible to the effect of the polar substituents. An advantage of oxoruthenium(IV) in these studies is the well-characterized two-electron nature of the oxidant,[22,29] strongly indicating that the correlation in Figure 4 is due to the polar effect on a two-electron carbocation-type reaction rather than a smaller effect on a radical-type reaction. In addition, oxomanganese porphyrins[32] and high-valent chromium[21] are both known to activate the 1′-C−H bond and are oxidants of significant two-electron character. These reactions should also be highly sensitive to the electronic properties of the sugar. An interesting question is whether metal−oxygen nucleases, such as Cu−phenanthroline and Fe−bleomycin, will exhibit similar effects since the reactive metal−oxygen species in these cases can be viewed as either one- or two-electron oxidants, depending on the reaction sequence.[4,7,19,33−35] The observation of large polar effects in these systems would imply significant two-electron character. Indeed, the high selectivity for RNA oxidation by Fe-bleomycin may result in part from the formation of a reactive two-electron oxidant.[7] Another interesting case is that of activation of the 3′-C−H bond by rhodium(III) photooxidants where the 2′-substituent would again be in the β position.[36] The proposed photooxidation mechanism likely generates a radical,[36] so in this case, selectivity of RNA oxidation may arise solely from structural effects.[37] In each of these cases, quantitation of the effects of 2′-substituents as in Figure 4 may provide a means for resolving mechanisms of selective RNA oxidation.

Acknowledgment. We thank the National Science Foundation for support of this research. B.T.F. thanks the Department of Education for a GAANN Fellowship.

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