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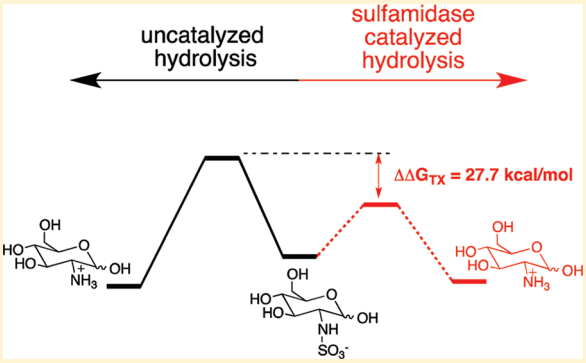
Hydrolysis of *N*-Alkyl Sulfamates and the Catalytic Efficiency of an S–N Cleaving Sulfamidase

Danielle C. Lohman, Richard Wolfenden, and David R. Edwards*

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: The final step in the degradation of heparin sulfate involves the enzymatic hydrolysis of its 2-sulfamido groups. To evaluate the power of the corresponding sulfamidases as catalysts, we examined the reaction of *N*-neopentyl sulfamate at elevated temperatures and found it to undergo specific acid catalyzed hydrolysis even at alkaline pH. A rate constant of 10^{-16} s^{-1} was calculated using the Eyring equation for water attack on the *N*-protonated species at pH 7, 25 °C. As a model for the pH neutral reaction, a rate constant for hydroxide attack on $(CH_3)_3CCH_2N^+H_2SO_3^-$ at pH 7, 25 °C was calculated to be 10^{-19} s^{-1}. The corresponding rate enhancement (k_{cat}/k_{non}) produced by the N-sulfamidase of *F. heparinum* is approximately 10^{16}-fold, which is somewhat larger than those generated by most hydrolytic enzymes but considerably smaller than those generated by S–O cleaving sulfatases.



INTRODUCTION

The roles of sulfuric acid derivatives in biological structure and signaling have drawn attention to the chemical properties of these molecules and to the enzymes responsible for their biosynthesis and degradation. Sulfate esters of lipids and polysaccharides are involved in maintaining cell structure, in regulating receptor–ligand binding affinities, and in the control of hormone levels.[1,2] Recent experiments have shown that sulfate esters are exceptionally stable to hydrolysis, and that S–O cleaving sulfatases achieve the largest rate enhancements (10^{26}-fold) known to be generated by any enzyme.[3] Sulfamate esters have received less attention, but are no less important. Heparin sulfate (HS), containing a repeating trisulfated disaccharide unit, is the most negatively charged polymer in mammalian tissues, and is joined covalently to serine residues of proteins in proteoglycans.[4] These macromolecules, in which N-acetylglucosamine residues originally present in the disaccharide units of heparin have been deacetylated and then sulfurylated,[5] affect the binding and turnover of signaling proteins at cell surfaces and in the extracellular matrix, and have been implicated in developmental changes including angiogenesis, blood coagulation and tumor metastasis. HS is degraded by certain bacteria including *Flavobacterium heparinum*, in a sequence of events that has been characterized in detail by Sasisekharan and his associates (Scheme 1).[6−9] In the final step, an *N*-sulfamidase selectively cleaves the sulfamate (sulfur–nitrogen) linkage of 2-sulfamino-2-deoxyglucose (**4**).[9] In the work described here, we set out to measure the power of this *N*-sulfamidase as a catalyst.

Rate constants for the specific acid catalyzed hydrolysis of *N*-alkyl sulfamates are known.[10−13] However, a rate constant for pH neutral hydrolysis is required to estimate the catalytic rate enhancement (k_{cat}/k_{uncat}) provided by the enzyme and the dissociation constant of the altered substrate at the transition state ($K_{TX} = k_{uncat}/(k_{cat}/K_m)$, units of M).[14] To this end, neopentyl sulfamate (**6**) was prepared and the kinetics of its hydrolysis were determined at elevated temperatures.

RESULTS AND DISCUSSION

First order rate constants, k_{obs}, for the hydrolysis of **6** (0.02 M) in water were determined at T = 200 °C, 0 < pH < 7. ^{1}H NMR (500 MHz, D_2O) analysis of the reaction mixture shows clean conversion of **6** to neopentylamine (**7**) without detectable formation of byproduct (Figure S1, Supporting Information). Shown in Figure 1 is a plot of log(k_{obs}) versus pH and a nonlinear least-squares fit of these data to eq 1 yields to k_{max} = 0.3 ± 0.1 s^{-1} corresponding to reaction along the low pH plateau and a kinetic pK_a = 1.0 ± 0.2. The kinetic pK_a value determined at elevated temperature is similar to that determined by spectrophotometric titration at 25 °C where pK_a = 0.92 ± 0.08 (Figure S2, Supp. Info). The observation that the pK_a of **6** does not change significantly from 25 to 200 °C implies a very small heat of ionization consistent with the ΔH of ~0.2 kcal/mol reported for sulfamic acid.[15]

$$\log(k_{obs}) = \log\left(\frac{k_{max}10^{-pH}}{10^{-pH} + 10^{-pKa}}\right) \quad (1)$$

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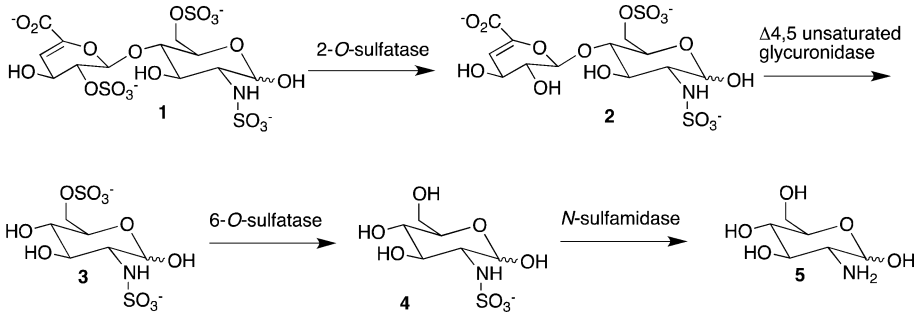
Scheme 1. Final Four Hydrolytic Steps in the Heparin Degradation Pathway of *F. heparinum*



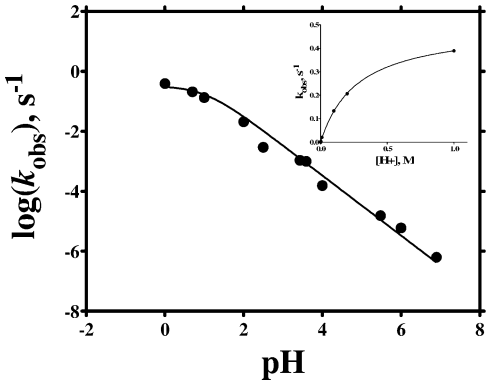


Figure 1. pH rate profile for the hydrolysis of **6** at 200 °C. The kinetic parameters k_{max} = 0.3 ± 0.1 s^{-1} and pK_a = 1.0 ± 0.2 were determined from a nonlinear least-squares fit of the data to eq 1 (r^2 = 0.9831, 18 data). (Inset) Plot of k_{obs} versus [H^+]. Note that kinetic data above pH 7 are not experimentally accessible, see ref 18.

Rate constants for the hydrolysis of **6** at pH 5.6 were measured as a function of temperature between 150 and 250 °C and second order rate constants were determined as $k_2 = k_{obs}/[H^+]$. The activation parameters were determined to be $\Delta H^{‡}$ = 34.2 ± 1.1 kcal/mol and $\Delta S^{‡}$ = +17 ± 2 cal/mol/K from an Eyring plot of the data (Figure 2). Extrapolation of the rate data in Figure 2 indicates that $k_2 = 3 \times 10^{-9}$ $M^{-1}s^{-1}$ at 25 °C.

First order rate constants for the attack of water on a series of zwitterionic sulfamates at 25 °C are shown as a Brønsted plot in Figure 3 (●, solid line). The data used in the plot along with

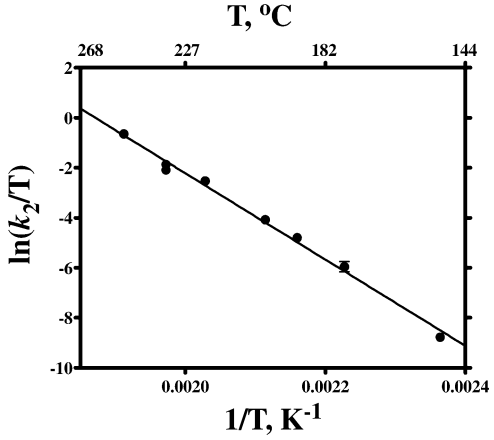


Figure 2. Eyring plot for the hydrolysis of **6** at pH 5.6. The activation parameters are determined to be $\Delta H^{‡}$ = 34.2 ± 1.1 kcal/mol and $\Delta S^{‡}$ = +17 ± 2 cal/mol/K, (r^2 = 0.9912, 17 data).

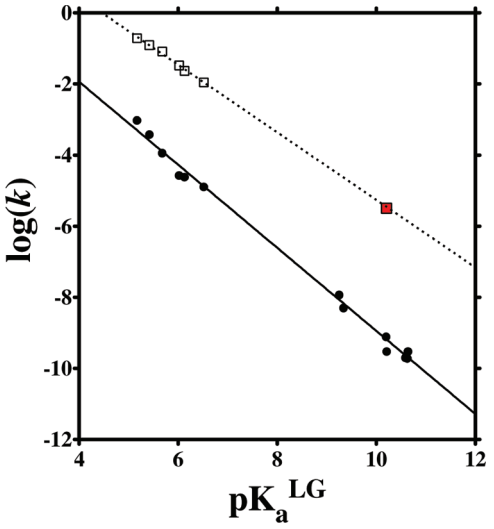


Figure 3. Brønsted plots of log(k) at 25 °C versus pK_a^{LG} for the attack of a) water on zwitterionic sulfamates (●, solid line) where the best fit line through the data adheres to log(k, s^{-1}) = (−1.16 ± 0.03)pK_a^{LG} + (2.7 ± 0.2), (r^2 = 0.9929, 13 data); and hydroxide attack on substituted pyridinium sulfamates (□, dashed line) where the best fit line adheres to log(k, $M^{-1}s^{-1}$) = (−0.95 ± 0.04)pK_a^{LG} + (4.2 ± 0.2). The filled red square corresponds to the extrapolated value for hydroxide attack on zwitterionic **6**. Rate constants and literature sources are compiled in Table S1 of the Supporting Information.

their literature sources are compiled in Table S1 of the Supporting Information. A single linear regression encompassing all the data is fit to the equation log(k_{25}) = (−1.16 ± 0.03) pK_a^{LG} + (2.7 ± 0.2), where pK_a^{LG} refers to the conjugate acid of the amine leaving groups. The Brønsted plot includes as substrates sulfamic acid ($H_3N^+-SO_3^-$), six substituted pyridinium sulfamates ($XC_5H_4N^+-SO_3^-$) and six primary *N*-alkyl sulfamates ($RH_2N^+-SO_3^-$) including **6**.[10−13,16] Williams previously presented a similar plot that did not include primary *N*-alkyl sulfamates.[16] The excellent correlation observed in Figure 3 suggests that a single mechanism describes the hydrolysis of all the zwitterionic sulfamates despite the gross structural differences of the amine leaving groups. For comparison, rate data for the base promoted hydrolysis of substituted pyridinium sulfamates are also shown in Figure 3 (□, dashed line).

N-Neopentyl sulfamate (**6**) undergoes specific acid catalyzed hydrolysis as shown in Scheme 2. The autoprotolysis constant at 200 °C can be estimated to be $K^w = 4 \times 10^{-11}$ M^2 from which it follows that neutral pH is ∼5 and that [$^-$OH] = $4 \times 10^{-11}/10^{-7} = 4 \times 10^{-4}$ M at pH 7.[17] The specific acid catalyzed mechanism for hydrolysis of **6** effectively obtains for the entire

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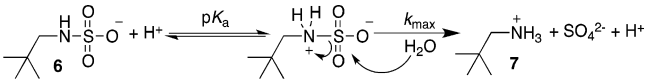
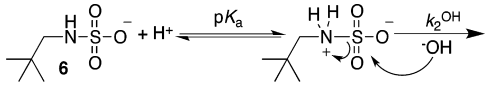
Scheme 2. Acid Catalyzed Hydrolysis of 6



accessible pH range at 200 °C and notably there is no evidence in Figure 1 for the onset of a pH neutral reaction *even in the alkaline region of the plot.*[18] The activation parameters for the acid catalyzed reaction and the pK_a of **6** at 25 °C allows us to estimate a rate constant of 10^{-16} s^{-1} at 25 °C, pH 7.

The lack of an observable pH neutral process for hydrolysis of **6** complicates efforts to directly estimate the catalytic rate acceleration of sulfamidase enzymes and therefore an alternative strategy was devised. Hydroxide attack on protonated **6** (Scheme 3) satisfies the criteria of a pH neutral

Scheme 3. Model for pH Neutral Hydrolysis of 6



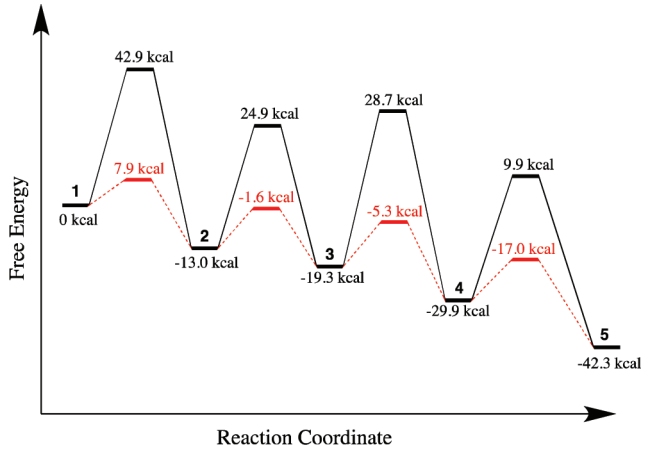
reaction (provided that pH ≫ pK_a) and the rate of this reaction can be estimated in the following manner. The second order rate constant, k_2^{OH}, for attack of hydroxide on the zwitterion of **6** (neopentyl-$N^+H_2SO_3^-$) at 25 °C can be extrapolated from the Brønsted plot in Figure 3 constructed by A. Williams for the base promoted hydrolysis of substituted pyridinium sulfamates.[16] From this we calculate $k_2^{OH} = 3 \times 10^{-6}$ $M^{-1}s^{-1}$ using a pK_a^{LG} of 10.2 corresponding to the neopentylamine leaving group of **6** (filled red square, dashed line, Figure 3). A first order rate constant, accounting for hydroxide concentration and the position of the acid−base equilibrium shown in Scheme 3, can then be determined as $k_N = k_2^{OH}(10^{(pKa-pH)}) \times [^-OH]$, which at pH 7 amounts to $(3 \times 10^{-6})(10^{(0.92-7)}) \times 10^{-7} = 3 \times 10^{-19}$ s^{-1}.[19] This value is only ∼1000 fold slower than our estimated rate constant of 10^{-16} s^{-1} for the specific acid catalyzed hydrolysis of **6** under identical conditions of temperature and pH. If we make the assumption that the attack of hydroxide on protonated sulfamates provides a suitable mechanism for their pH neutral hydrolysis, then rate constants for any primary *N*-alkyl sulfamate can be estimated by this method.[20] The spontaneous attack of water on an anionic **6** might be considerably slower than our modeled pH neutral reaction and accordingly the estimated rate constant for the process shown in Scheme 3 can be considered an upper limit for the rate constant corresponding to uncatalyzed hydrolysis of an alkyl sulfamate ester.

An N-sulfamidase from *F. heparinum* catalyzes the hydrolysis of *N*-sulfated glucoseamines as part of the degradation pathway for sulfated glycosaminoglycans.[9] The kinetic parameters for this enzyme acting on 4-methylumbelliferyl 2-sulfamino-alpha-D-glucopyranoside are $k_{cat} = 0.4$ s^{-1} and $K_m = 1.8 \times 10^{-4}$ M. We estimate a rate constant of 10^{-17} s^{-1} for the hydrolysis of **4** in the absence of enzyme using the general method described for **6** (details of this calculation are provided in the Supporting Information). Accordingly, this enzyme provides a catalytic rate enhancement of $k_{cat}/k_{uncat} = 10^{16}$ and a K_{TX} of 10^{-21} M.

The sequential degradation of **1** → **2** → **3** → **4** → **5** (Scheme 1) during the late stages of heparin catabolism is tightly controlled by enzyme specificity.[6−9] We are now in a position to estimate the catalytic rate enhancements and transition state binding affinities for each step in the process (Scheme 1 and Table 1). Details of this analysis are presented in the Supporting Information. In the absence of enzyme, complete degradation of disaccharide **1** to **5** is very slow with an overall half-life of ∼10^{15} years that is largely dictated by the rate-limiting hydrolysis of **3** (Table 1). The four enzymes involved in promoting degradation of **1** range from being catalysts of moderate to high power with catalytic rate accelerations of 10^{16}−10^{22} and nominal dissociation constants for the altered substrates of $10^{-20} < K_{TX} < 10^{-27}$ M.[21]

Chart 1 shows an energy profile (at standard state 1 M and 25 °C) for the sequential conversion of **1** → **2** → **3** → **4** → **5**

Chart 1. Free Energy Profile for the Degradation of 1 by *F. heparinum* at Standard State 1 M, 25 °C[a]



[a]Free energies are arbitrarily referenced to **1** at 0 kcal. The solid black lines connect intermediates **1**−**5** to the uncatalyzed transition states. The dashed red lines connect intermediates to the enzyme catalyzed transition states. Enzymatic transition state energies are calculated using the k_{cat}/K_m values provided in Table 1.

where the solid black lines connect the intermediates and transition states for the uncatalyzed reactions. The free energies of the enzymatic transition states, calculated from the k_{cat}/K_m values in Table 1, are shown in Chart 1 connected to the intermediates via dashed red lines. The vertical separation

Table 1. Kinetic Constants, Enzymatic Rate Enhancements and Transition State Binding Affinities for the Hydrolytic Degradation of Heparin by *F. heparinum*[a]

	k_{uncat} (s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)[b]	catalytic rate enhancement (k_{cat}/k_{uncat})	K_{TX} ($k_{uncat}/(k_{cat}/K_m)$, M)
2-*O*-sulfatase	(4×10^{-20})[c]	814	9.4×10^6	2×10^{22}	4×10^{-27}
Δ4,5 unsaturated glycuronidase	$(\sim 10^{-15})$[d]	8.8	2.6×10^4	9×10^{15}	4×10^{-20}
6-*O*-sulfatase	(3×10^{-23})[c]	0.1	3.5×10^2	3×10^{21}	9×10^{-26}
N-sulfamidase	10^{-17}	0.4	2.1×10^3	10^{16}	10^{-21}

[a]Complete details of the calculations are provided in the Supporting Information. [b]References 6−9. [c]Reference 3. [d]Reference 22.

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between the uncatalyzed and catalyzed transition states in the diagram represents the free energy of transition state binding generated by the various enzymes. Inspection of Chart 1 reveals that each sequential step in the degradation of **1** generates a product that is lower in free energy than its immediate predecessor. Overall, the conversion of **1** to **5** releases 42 kcal/mol of free energy.

In addition to its prominent role in heparin degradation, sulfuryl transfer is also involved in regulating hormone levels, detoxification and other biological activities.[1,2] A number of sulfatases and sulfamidases have now been characterized kinetically and shown to be proficient enzymes. The catalytic rate enhancement produced by the *N*-sulfamidase estimated here is substantially lower than that produced by the O-sulfatases.[3]

EXPERIMENTAL SECTION

N-neopentyl Sulfamate (6). Into a 25 mL round-bottomed flask equipped with a magnetic stirring bar were sequentially added sulfur trioxide pyridine complex (2.0 g, 12.6 mmol), anhydrous pyridine (5 mL) and 2,2-dimethylpropylamine (3 mL, 26 mmol). The reaction flask was heated to 50 °C for 3 h, cooled to room temperature and quenched by the addition of 20 mL 1 N KOH. The aqueous solution was next pumped to dryness under reduced pressure and the resulting white solid triturated with 50 mL methanol. The methanolic solution was treated 20 mL 2-propanol to force precipitate a sticky white solid that was collected by filtration. The collected material was crystallized and then recrystallized from ethanol:water:acetone (4:1:25) to obtain **6** (260 mg, 10% yield) as fluffy colorless needles. The purity of **6** was estimated to be >90% by ^{1}H NMR comparing to a known amount of pyrazine.

^{1}H NMR (500 MHz, D_2O, pD 9) δ 2.67 (2H, s, CH_2), δ 0.86 (9H, s, $C(CH_3)_3$). ^{13}C NMR (100.6 MHz, D_2O, pD 9) δ 57.6, δ 30.0 and δ 29.2 ppm. MS(-ESI) calculated for $[M\text{-}K]^-$ 166.22 and found 166.03 amu. Melting point 262 −264 °C. UV−vis (H_2O, 25 °C, 0.5 M HCl) ε^{230}=22.6 Abs $M^{-1}cm^{-1}$.

Methods. Kinetic experiments were carried out in quartz tubes sealed under vacuum containing a 0.2 mL aqueous solution of 0.02 M *N*-neopentyl sulfamate (**6**) and 0.2 M buffer (phosphate, acetate or formate). At low pH, HCl was substituted for buffer. The reaction vessels were placed in thermally equilibrated ovens equipped with ASTM thermometers for a set time period. Following reaction, the 0.2 mL reaction volume was divided into two portions. One portion was diluted 5 fold with H_2O and the pH measured. The second portion was diluted 5-fold with D_2O containing 0.3 M K_2HPO_4 for ^{1}H NMR analysis. The addition of phosphate buffer to the NMR sample allowed spectra to be recorded at the optimal pD for peak resolution. ^{1}H NMR spectra were recorded with a 30 s delay between pulses. Reaction progress was determined by comparison of the normalized integrated signal intensities corresponding to starting material (**6**) and the reaction product (**7**). Observed first order rate constants were calculated using a standard first order exponential equation and first order behavior was verified by comparison of the computed rate constants determined at different time points under otherwise identical reaction conditions. Reactions were free of byproduct and obeyed good first order behavior for >90% conversion. The general method used here for measuring hydrolytic kinetics at elevated temperature have been reviewed.[21]

ASSOCIATED CONTENT

Supporting Information

Details of the UV−visible pH titration of **6**. ^{1}H NMR spectrum of a reaction mixture containing **6** and **7**. Plots of k_{obs} versus buffer concentration. A description of the free energy calculations used to construct Chart 1. Table S1 shows kinetic data for the hydrolysis of zwitterionic sulfamates. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*dredward@email.unc.edu

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

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(17) The acidity constant, K_a, for the ionization of water ($H_2O \rightleftarrows {}^+H + {}^-OH$) is defined by the thermodynamic parameters ΔH = 13.5 kcal/mol and ΔS = −27 cal/mol/K and from this we compute $K_a = 7.3 \times 10^{-13}$ at 200 °C. It follows that $K^w = K_a \times [H_2O] = 7 \times 10^{-13} \times 55$ M $= 4 \times 10^{-11}$ under the assumption that the concentration of water is invariant with respect to temperature. Thermodynamic data obtained from: Eisenberg, D.; Kauzmann, W. *The Structure and Properties of Water*; Oxford University Press: London, 1969.
(18) Figure 1 for hydrolysis of **6** at 200 °C encompasses kinetic data determined from 1 M HCl (pH 0) to 4×10^{-4} M hydroxide (pH 7). This pH range represents the accessible limit at 200 °C. For reactions run at pH > 7 buffers are no longer appropriate for pH control and bulk hydroxide needs to be added to the reactions. To maintain pseudofirst order conditions approximately 0.2 M KOH would be required corresponding to pH 9.6 having an extrapolated rate constant of 10^{-9} s^{-1} ($t^{1/2}$ = 14 years). This is too slow to measure in a reasonable amount of time. Note that *neutral* pH at 200 °C is ∼5.
(19) The p*K*a of **6** was determined to be 0.92 by spectrophotometric titration at 25 °C (Figure S2, Supporting Information).
(20) Tertiary *N*-alkyl sulfamates, such as $Et_3N\text{-}SO_3$, do not adhere to the Brønsted correlations in Figure 3 as discussed in ref 16.
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