Thermodynamics of the Hydrolysis Reactions of 1-Naphthyl Acetate, 4-Nitrophenyl Acetate, and 4-Nitrophenyl α-L-arabinofuranoside†

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Microcalorimetry, high-performance liquid chromatography (HPLC), and liquid chromatography−mass spectrometry (LC-MS) have been used to conduct a thermodynamic investigation of the hydrolysis reactions {1-naphthyl acetate(aq) + H_2O(l) = 1-naphthol(aq) + acetate(aq)}, {4-nitrophenyl acetate(aq) + H_2O(l) = 4-nitrophenol(aq) + acetate(aq)}, and {4-nitrophenyl α-L-arabinofuranoside(aq) + H_2O(l) = L-arabinose(aq) + 4-nitrophenol(aq)}. Calorimetrically determined enthalpies of reaction $\Delta_r H$(cal) were measured for all three reactions. However, since the positions of equilibrium for all of these reactions were found to lie very far to the right, it was only possible to set lower limits for the values of the apparent equilibrium constants K'. A chemical equilibrium model, together with pKs and standard enthalpies of reaction $\Delta_r H°$ for the H^+ binding reactions of the reactants and products, was then used to calculate the values of $\Delta_r H°$ for chemical reference reactions that correspond to the overall biochemical reactions that were studied experimentally. The values of Benson estimates of $\Delta_r H°$ for the chemical reference reactions that correspond to the first of the above two reactions were, in all cases, within 16 kJ·mol^{-1} of the results obtained in this study. Thermochemical network calculations led to $\Delta_f H° = -286.4$ kJ·mol^{-1} for 1-napthyl acetate(aq) and $\Delta_f H° = -364.9$ kJ·mol^{-1} for 4-nitrophenyl acetate(aq).

1. Introduction

Acetylxylan esterase (EC 3.1.1.72) is an enzyme that catalyzes the deacetylation of xylans and xylo-oligosaccharides. One of the simplest reactions that it catalyzes is

2-acetyl β-D-xylopyranose(aq) + H_2O(l) = D-xylose(aq) + acetate(aq) (1)

Additionally, the enzyme also catalyzes the hydrolysis reactions of 1-naphthyl acetate and 4-nitrophenyl acetate

1-naphthyl acetate(aq) + H_2O(l) = 1-naphthol(aq) + acetate(aq) (2)

4-nitrophenyl acetate(aq) + H_2O(l) = 4-nitrophenol(aq) + acetate(aq) (3)

Because efficient biomass utilization looks to breakdown acetylated xylans, the reactions that are catalyzed by acetylxylan esterase assume some practical importance. Also related to biomass utilization are the reactions catalyzed by α-*N*-arabinofuranosidase (EC 3.2.1.55) which acts on α-L-arabinofuranosides, α-L-arabinans containing (1,3) and/or (1,5)-linkages, arabinoxylans, and arabinogalactans.[1] A representative reaction that it catalyzes is

4-nitrophenyl α-L-arabinofuranoside(aq) + H_2O(l) = L-arabinose(aq) + 4-nitrophenol(aq) (4)

In this study we report calorimetrically determined enthalpy changes $\Delta_r H$(cal) for reactions 2, 3, and 4 (see Figure 1). We also attempted to measure values of the apparent equilibrium constants K' for all of the above reactions. However, since the position of equilibrium for all of these reactions lies very far to the right, we were able to only set lower limits for the values of the apparent equilibrium constants.

The results obtained in this study were treated by using a chemical equilibrium model[2,3] to obtain values of $\Delta_r H°$ for chemical reference reactions that involve specific ionic species rather than overall biochemical reactants.

2. Experimental Methods

2.1. Materials. Pertinent information on the substances used in this study is given in Table 1. (Certain commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedures adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology (NIST), nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.) The purities of those substances that are most critical for this thermodynamic investigation were checked by using HPLC (see Section 2.2). Also, the mass fractions of water in these same substances were

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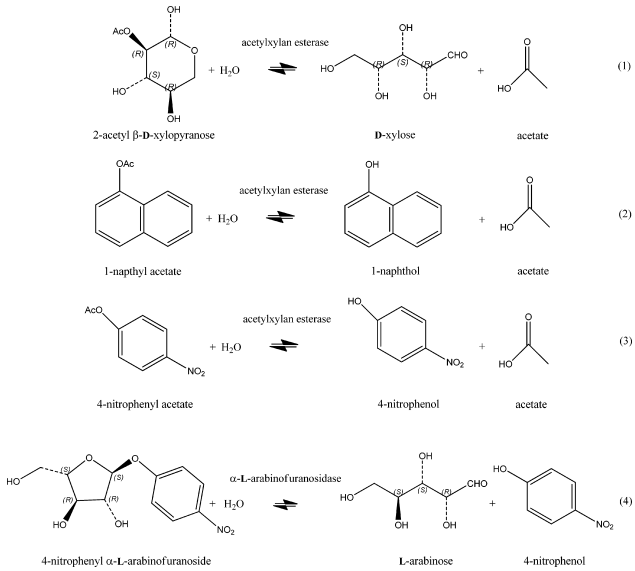

Figure 1. The structures of the substances and the reactions studied herein. The neutral forms of the aqueous species are shown.

measured by performing Karl Fischer titrations with a Metrohm 975 KFT Titrino automatic titrator. In performing these titrations, all samples were dissolved in anhydrous methanol and titrated with Hydranal composite 2 solution. Calibration of the Karl Fischer apparatus was done by using a water-saturated octanol solution.[4] Acetylxylan esterase (EC 3.1.1.72) from *Trichoderma reesei* was expressed in *Aspergillus awamori* and prepared as previously described.[5] This enzyme was stored (mass fraction $w = 0.00085$) in sodium acetate buffer (concentration $c = 0.020$ mol·dm^{-3}) containing NaCl ($c = 0.10$ mol·dm^{-3}) at pH = 5.0. Both the acetylxylan esterase and the α-*N*-arabinofuranosidase were dialyzed against citrate buffer {molality m(sodium citrate) = 0.097 mol·kg^{-1}, m(HCl) = 9.94 × 10^{-5} mol·kg^{-1}, pH = 4.80} for ∼24 h at the temperature T = 277 K and then kept frozen at $T = 253$ K until ready for use in either the extent of reaction or calorimetric experiments.

2.2. Chromatography. Three chromatographic methods were used in this study. The first was a Hewlett-Packard Model 1100 HPLC equipped with a UV detector set at the wavelength $\lambda = 280$ nm. This method was used for the separation of the 1-naphthyl acetate, 4-nitrophenyl acetate, and 4-nitrophenyl α-L-arabinofuranoside, and their respective reaction products. A Zorbax Extend C-18 column and a Zorbax Eclipse Plus C18 guard column were used for the separations. These columns were thermostatted at $T = 303.15$ K. The mobile phases were (I) aqueous H_3PO_4 ($c = 0.01$ mol·dm^{-3}) and (II) acetonitrile. The volume fractions ϕ of these mobile phases were ϕ(I) = 0.80, ϕ(II) = 0.20 at time $t = 0$; ϕ(I) = 0.55, ϕ(II) = 0.45 at $t = 15.0$ min; and ϕ(I) = 0.55, ϕ(II) = 0.45 at $t = 40.0$ min. The flow rate was 0.0167 cm^3·s^{-1}. Typical retention times were 4-nitrophenol-α-L-arabinofuranoside, 6.9 min; 4-nitrophenol, 11.7 min; 4-nitrophenylacetate, 17.4 min; 1-naphthol, 19.3 min; and 1-naphthyl acetate, 26.9 min. By means of successive injections into the HPLC of solutions having increasingly smaller concentrations, the following approximate limits of detection were determined: 1-naphthylacetate, 6 × 10^{-9} mol·dm^{-3}; 4-nitrophenyl acetate, 8 × 10^{-12} mol·dm^{-3}; and 4-nitrophenyl α-L-arabinofuranoside, 3 × 10^{-8} mol·dm^{-3}. In the determination of these approximate limits of detection, injections of pure buffer into the HPLC were always performed in between the injections of solutions containing known concentrations of the substance(s) of interest.

The second method used a Dionex DX 500 Ion Chromatograph with an ED50 amperometric detector (cell set at $T = 303$ K; a carbohydrate quadruple waveform) and a Carbopac PA20 column with an amino trap guard column, and a borate suppressor. Both the column and the guard column were thermostatted in an LC25 chromatography oven at $T = 303$ K. The mobile phase consisted of (I) NaOH(aq), $c = 0.010$ mol·dm^{-3}; (II) NaOH(aq), $c = 0.25$ mol·dm^{-3}; and (III) H_2O(l). The following step-gradient of these mobile phases was formed: volume fraction ϕ(I) = 0.10, ϕ(II) = 0, and ϕ(III) = 0.90 at time $t = 0$; ϕ(I) = 0.10, (II) = 0, and ϕ(III) = 0.90 at $t = 12.0$ min; ϕ(I) = 1.00, ϕ(II) = 0, and ϕ(III) = 0 $t = 20.0$ min; ϕ(I) = 0.75, ϕ(II) = 0.25, and ϕ(III) = 0 at $t = 25.0$ min; ϕ(I) = 0.0, ϕ(II) = 0.80, and ϕ(III) = 0.20 at $t = 25.1$ min; ϕ(I) = 0.0, ϕ(II) = 0.80, and ϕ(III) = 0.20 at $t = 35.0$ min; ϕ(I) = 0.10, ϕ(II) = 0, and ϕ(III) = 0.90 at $t = 35.1$ min; and ϕ(I) = 0.10, ϕ(II) = 0, and ϕ(III) = 0.90 at $t = 55.0$ min. The flow rate was 0.00833 cm^3·s^{-1}. The retention time of D-xylose was 9.3 min; its approximate limit of detection was 1 × 10^{-10} mol·dm^{-3}.

Third, to study reaction 1, we used a Waters UPLC Acquity liquid chromatography−mass spectrometry (LC-MS) with an evaporative light scattering detector (ELSD) and QToF micro MS detector in parallel. This system utilized a Shodex SZ5532 column thermostatted at $T = 333$ K with a mobile phase consisting of (I) acetonitrile and (II) water. The following step-gradient program of these mobile phases was formed: ϕ(I) = 0.80 and ϕ (II) = 0.20 at time $t = 0$; ϕ(I) = 0.83 and ϕ(II) = 0.17 at $t = 9.0$ min; ϕ(I) = 0.70, and ϕ(II) = 0.30 at $t = 25.0$ min; and ϕ(I) = 0.20 and ϕ(II) = 0.80 at $t = 30.0$ min. The flow of the mobile phase (flow rate = 0.015 cm^3·s^{-1}) was split 2:1 to the respective detectors with the ELSD receiving the majority of the flow (0.010 cm^3·s^{-1}). The Waters Acquity ELSD was run at a pressure of 0.193 MPa and a drift tube temperature of 340 K. Mass spectroscopy detection was carried out with electrospray under positive mode with a cone voltage of 23 V, capillary voltage of 3000 V, desolvation temperature of 573 K, and a source temperature of 373 K. The MS was calibrated and tuned with reserpine and subsequent samples were scanned from masses of 40 to 400 Da with a scan time of 1 s. The MS effluent line was infused with a solution consisting of formic acid (volume fraction = 0.002) in acetonitrile at a ratio of 1:10 to ensure adequate ionization of the samples. To ionize the samples, direct-infusion MS was carried out under the same instrument conditions as LC-MS, except that the sample was directly injected into the MS at a flow rate of 1.67 × 10^{-4} cm^3·s^{-1} with a cone voltage of 16 V. These samples were diluted in a 1:1 ratio with a solution of formic acid (volume fraction = 0.002) in water. The data was collected for 30 to 60 s in direct-infusion mode.

2.3. Extent of Reaction Measurements. Attempts were made to measure values for the apparent equilibrium constants[6] K' of reactions 1, 2, 3, and 4. These studies used the HPLC/MS methods described in Section 2.2. In each case, a citrate buffer {m($Na_2C_6H_5Na_3O_7$) = 0.097 mol·kg^{-1} + m(HCl) = 9.94 × 10^{-5} mol·kg^{-1}, pH = 4.80} was used. In the absence of a sample of 2-acetyl β-D-xylopyranose, it was not possible to study reaction 1 from the forward direction of reaction. Nevertheless, the reaction mixture used for the reverse direction of reaction consisted of {D-xylose ($m = 0.0022$ mol·kg^{-1}) + sodium acetate ($m = 0.012$ mol·kg^{-1}) in the citrate buffer. The final pH of the reaction mixture was 4.77. The mass fraction of acetylxylan esterase in the respective reaction mixtures was ∼3 × 10^{-5}. The reaction mixture was allowed to proceed with gentle lateral

TABLE 1: Principal Substances Used in This Study with Their Chemical Abstracts Service (CAS) Registry Numbers, Empirical Formulae, Relative Molecular Masses M_r, Mass Fraction Moisture Contents w Determined by Karl Fischer Analysis, Supplier (S = Sigma-Aldrich, F = Fluka, M = Megazyme), Approximate Mole Fraction Purities x, and the Method(s) Used by the Vendors

substance	CAS No.	formula[a]	M_r[a]	$10^2 \cdot w$	supplier	x[b]	methods of analysis
acetylxylan esterase[c]	188959-24-2		2.5×10^4		[d]		
2-acetyl β-D-xylopranose	133391-16-9	$C_7H_{12}O_6$	192.167				
α-*N*-Arabinofuranosidase[e]	9067-74-7				M		
L-arabinose	5328-37-0	$C_5H_{10}O_5$	150.130		S	≥0.995	HPLC; trace analysis
dimethylsulfoxide	67-68-5	C_2H_6OS	78.134		S	≥0.999	UV; titration; residue on evaporation
1-naphthol	90-15-3	$C_{10}H_8O$	144.170		S	≥0.99	GC; trace analysis
1-naphthyl acetate	830-81-9	$C_{12}H_{10}O_2$	186.207	0.308	S	≥0.98	TLC; elemental analysis
4-nitrophenol	100-02-7	$C_6H_5NO_3$	139.109		F	≥0.995	HPLC; elemental analysis; trace analysis
4-nitrophenyl acetate	830-03-5	$C_8H_7NO_4$	181.145	0.152	S	≥0.98	GC; elemental analysis
4-nitrophenyl α-L-arabinofuranoside	6892-58-6	$C_{11}H_{13}NO_7$	271.223	0.426	S	≥0.99	TLC
sodium acetate	127-09-3	$C_2H_3NaO_2$	82.034		S	≥0.990	trace analysis; titration
sodium citrate tribasic dihydrate	6132-04-3	$C_6H_5Na_3O_7 \cdot 2H_2O$	294.10		S	≥0.995	trace analysis; titration
D-xylose	58-86-6	$C_5H_{10}O_5$	150.130		S	≥0.99	GC; trace analysis

[a] Except for sodium citrate tribasic dihydrate, the empirical formulas and the relative molecular masses refers to the anhydrous substances. [b] The estimated mole fraction purities are those reported by the vendor(s) and are exclusive of the amount of water in the samples. By using the chromatographic method described in Section 2.2, approximate mole fraction purities x were found to be 0.994 for 1-naphthyl acetate, 0.997 for 4-nitrophenyl acetate, and 0.986 for 4-nitrophenyl α-L-arabinofuranoside. [c] Acetylxylan esterase (EC 3.1.1.72), was from *A. niger*. The recombinant acetylxylan esterase is *T. reese*i enzyme expressed in *A. awamori*. The enzyme was purified by ion exchange and size-exclusion chromatography from the commercial product. Its mass concentration was 0.72 mg cm^{-3} in the buffer {$NaC_2H_3O_2$ (c = 0.020 mol·dm^{-3}) + NaCl (c = 0.10 mol·dm^{-3}), pH = 5.0}. [d] Prepared for this study (see section 2.1). [e] α-*N*-Arabinofuranosidase (EC 3.2.1.55), also called α-L-arabinosidase and α-L-arabinofuranoside arabinofuranohydrolase, was from *A. nidulans* expressed in *A. awamori*. Its mass concentration was 1.74 mg cm^{-3} in the buffer {$NaC_2H_3O_2$ (c = 0.020 mol·dm^{-3}) + NaCl (c = 0.10 mol·dm^{-3}), pH = 4.8}.

shaking (~100 shakes min^{-1}) at T = 298.15 K for ~24 h. The chromatogram of the reaction mixture prior to addition of the enzyme and the chromatogram of the enzyme in citrate buffer were compared with the resulting chromatogram of the final reaction mixture. These comparisons showed no new peaks. Thus, there was no evidence for the formation of 2-acetyl β-D-xylopyranose. If one assumes that this substance would not be degraded by 0.01 mol·dm^{-3} NaOH, and thus would produce a peak on the chromatogram, and that its approximate limit of detection would be approximately the same as D-xylose (see Section 2.2), one can conclude that, for reaction 1, $K' \geq 3 \times 10^5$.

The LC-MS was also used to study reaction 1. In this case reactions were carried out using D-xylose (c = 0.133 mol·dm^{-3}) and sodium acetate (c = 0.133 mol·dm^{-3}) stock solutions in ratios of 1:1, 1:3, and 3:1. The mass fraction of acetylxylan esterase in the respective reaction mixtures was ~3×10^{-6}. The pH of the reaction mixture(s) was 5.0 (no additional reagents were added to the aforementioned reaction mixtures other than the acetylxylan esterase). After an equilibration time of 2 h at T = 313.15 K, the samples were frozen until the time at which the LC-MS and direct-infusion MS experiments were performed. Control experiments that involved only the enzyme, D-xylose, and sodium acetate in deionized water were performed. Although xylose and acetate were readily detected by both LC-MS and direct-infusion MS, in no case was acetylated xylose detected. By using the approximate limit of detection for D-xylose (1×10^{-7} mol·dm^{-3}) and by assuming that it is the same as 2-acetyl β-D-xylopyranose, one can conclude that, for reaction 1, $K' \geq 2 \times 10^5$.

For reaction 2, the reaction mixture used for the forward direction of reaction consisted of 1-naphthyl acetate ($m = 5.3 \times 10^{-4}$ mol·kg^{-1}) in the citrate buffer. The reaction mixture used for the reverse direction of reaction consisted of {1-naphthol (m = 0.0017 mol·kg^{-1}) + sodium acetate (m = 0.015 mol·kg^{-1}) in the citrate buffer}. The final pHs of the reaction mixtures were 4.70 for the forward and 4.80 for the reverse directions of reaction. The mass fraction of acetylxylan esterase in the respective reaction mixtures was ~2×10^{-4}. The forward and reverse reaction mixtures were allowed to proceed with gentle lateral shaking (~100 shakes min^{-1}) at T = 298.15 K for ~48 h. There was no evidence for the formation of 1-naphthyl acetate via the reverse direction of reaction. Additionally, all of the 1-naphthyl acetate from the forward reaction mixture had reacted to form (acetate +1-naphthol). On the basis of the approximate limit of detection of 1-napthyl acetate (see Section 2.2), one can conclude that, for reaction 2, $K' \geq 47$ (based on the forward reaction) and $K' \geq 4 \times 10^4$ (based on the reverse reaction).

For reaction 3, the reaction mixture used for the forward direction of reaction consisted of 4-nitrophenyl acetate ($m = 1.3 \times 10^{-3}$ mol·kg^{-1}) in the citrate buffer. The reaction mixture used for the reverse direction of reaction consisted of {4-nitrophenol (m = 0.0068 mol·kg^{-1}) + sodium acetate (m = 0.016 mol·kg-^{1}) in the citrate buffer}. The final pHs of the reaction mixtures were 4.70 for the forward and 4.80 for the reverse directions of reaction. The mass fraction of acetylxylan esterase in the respective reaction mixtures was ~2×10^{-4}. The forward and reverse reaction mixtures were allowed to proceed with gentle lateral shaking (~100 shakes min^{-1}) at T = 298.15 K for ~48 h. There was no evidence for the formation of 4-nitrophenyl acetate via the reverse direction of reaction. Additionally, all of the 4-nitrophenyl acetate from the forward reaction mixture had reacted to form (acetate +4-nitrophenol). On the basis of the approximate limit of detection of 4-nitrophenyl acetate (see Section 2.2), one can conclude that, for reaction 3, $K' \geq 2 \times 10^5$ (based on the forward reaction) and $K' \geq 1 \times 10^7$ (based on the reverse reaction).

For reaction 4, the reaction mixture used for the forward direction of reaction consisted of 4-nitrophenyl α-L-arabinofuranoside ($m = 5.5 \times 10^{-3}$ mol·kg^{-1}) in the citrate buffer. The reaction mixture used for the reverse direction of reaction consisted of {4-nitrophenol (m = 0.0082 mol·kg^{-1}) + L-arabinose (m = 0.029 mol·kg^{-1}) in the citrate buffer}. The final pHs of the reaction mixtures were 4.69 for the forward and 4.71 for the reverse directions of reaction. The mass fraction of α-*N*-arabinofuranosidase in the respective reaction mixtures was ~2×10^{-4}. The forward and reverse reaction mixtures were allowed

to proceed with gentle lateral shaking (~100 shakes min^{-1}) at $T = 298.15$ K for ~24 h. There was no evidence for the formation of nitrophenyl α-L-arabinofuranoside via the reverse direction of reaction. Additionally, all of the nitrophenyl α-L-arabinofuranoside from the forward reaction mixture had reacted to form (4-nitrophenol + L-arabinose). On the basis of the approximate limit of detection of 4-nitrophenyl α-L-arabinofuranoside (see Section 2.2), one can conclude that, for reaction 4, $K' \geq 1 \times 10^3$ (based on the forward reaction) and $K' \geq 8 \times 10^3$ (based on the reverse reaction).

2.4. Calorimetry. Descriptions of the microcalorimeters used in this study and their performance characteristics, the calibration and data-acquisition systems, and the computer programs used to treat the results have been given by Steckler et al.[7,8] These calorimeters were calibrated electrically by using a high-stability dc power supply, calibrated digital voltmeter, standard resistor, and time-interval counter. The electric potential differences U of the thermopiles in the microcalorimeters are measured with Agilent model 34420A Nanovolt Meters. The values of U are then recorded on a microcomputer and the areas of the thermograms are calculated by numerical integration. The calorimetric sample vessels were fabricated from high-density polyethylene. Each vessel had two compartments that held, respectively, ~0.55 and ~0.40 cm^3 of solution. The substrate solutions were placed in the 0.55 cm^3 compartment and the enzyme solutions were placed in the 0.40 cm^3 compartment. The enzyme solutions consisted of the dialyzed enzymes in the same citrate buffer used for the substrates.

The 4-nitrophenyl α-L-arabinofuranoside substrate solution was prepared by weighing a sample of this substance into the citrate buffer used for this study. However, the low solubilities of 1-napthyl acetate and of 4-nitrophenyl acetate combined with the slow rates at which these two substances dissolved in the citrate buffer constrained us to take an approach different than direct weighing of the sample into the buffer. For these two substances, we prepared solutions of each of them in citrate buffer in which crystals of the two respective substances were clearly visible. These solutions were stirred and shaken for several hours to dissolve as much of these substances as possible. After allowing some time for the settling of the crystals, the majority of the clear supernatant solution was removed from each of these two solutions. To be certain that no crystals remained and that the experiments were conducted safely below the saturation molality, additional citrate buffer was added to each of the clear supernatant solutions so as to dilute the molalities of the 1-napthyl acetate and 4-nitrophenyl acetate by ~20%. These final solutions served as the respective substrate solutions for the calorimetric measurements involving 1-napthyl acetate and 4-nitrophenyl acetate.

The concentrations of these two substrate solutions were measured by using the HPLC method described above (see Section 2.2). Calibration of the HPLC was done by using gravimetrically prepared response factor solutions of these two substances in dimethyl sulfoxide, a solvent in which these two substances were readily soluble. Note that since the injection of samples into the HPLC was done by means of an injection loop having a fixed volume, it was necessary to make a correction for the fact that the densities of the solutions containing the two substrates (1-napthyl acetate and 4-nitrophenyl acetate) in buffer are different than the densities of the solutions containing these two substrates in dimethyl sulfoxide. The area of the chromatographic peak corresponding to 1-napthyl acetate from the response factor solution differed by only 1.4% from the area of the chromatographic peak that corresponded to 1-napthyl acetate from the substrate solution. Similarly, the area of the chromatographic peak corresponding to 4-nitrophenyl acetate from the response factor solution differed by only 1.0% from the area of the chromatographic peak that corresponded to 4-nitrophenyl acetate from the substrate solution. This procedure permitted us to use 1-napthyl acetate and 4-nitrophenyl acetate solutions that were close to the saturation molality, that were essentially certain not to contain any undissolved crystals, and that had accurately known molalities.

An additional complication in the calorimetric measurements was caused by the spontaneous hydrolysis of 4-nitrophenyl acetate to (4-nitrophenol + acetate). The fractional rate of decomposition $\{m(\text{initial}) - m(\text{final})/m(\text{initial})\}$ of this substance was measured by using the HPLC and was found to be 8.27×10^{-7} s^{-1} at ambient temperature ($T = 295.6$ K). Corrections for this decomposition were applied in calculating the values of the calorimetrically determined molar enthalpy of reaction for reaction 3. The largest such correction that was applied was equal to $0.0183 \cdot \Delta_r H(\text{cal})$. No spontaneous hydrolysis was observed for solutions of 1-naphthyl acetate and 4-nitrophenyl α-L-arabinofuranoside after said solutions were allowed to stand at ambient temperature for 8 and 4 h, respectively. Additionally, the 4-nitrophenyl α-L-arabinofuranoside substrate solution was kept in an ice bath until ready for use in the calorimetric measurements.

The vessels and their contents were allowed to thermally equilibrate in the microcalorimeters for ~60 min before the enzyme and substrate solutions were mixed. After mixing, ~23 min was allowed for reaction 2 and ~30 min was allowed for reactions 3 and 4. Following the reaction, the vessels were removed from the microcalorimeters and the HPLC was used for the analysis of the final solutions. The fractions of unreacted substrates were found to be less than 0.0018 and 0.0004 for reactions 2 and 3. There was no evidence of any 4-nitrophenyl α-L-arabinofuranoside remaining from reaction 4. On the basis of its limit of detection (3×10^{-8} mol·dm^{-3}), the fraction of unreacted 4-nitrophenyl α-L-arabinofuranoside was $<8 \times 10^{-6}$.

"Blank" enthalpy changes $\Delta_{mix}H$ were determined in control experiments. The average of the values of $\Delta_{mix}H$ for the mixing of the enzyme solutions with the buffer was (0.52 ± 0.14) mJ and the average of the values of $\Delta_{mix}H$ for the mixing of the substrate solutions with the buffer was (0.60 ± 0.22) mJ. The uncertainties given here are equal to two estimated standard deviations of the mean. We judge the total corrections applied for the blank enthalpy changes to be uncertain by ±0.26 mJ. The measured reaction enthalpies were approximately −7, −31, and −106 mJ for reactions 2, 3, and 4, respectively. Thus, the uncertainties in the blank enthalpies lead to uncertainties of $0.037 \cdot \Delta_r H(\text{cal})$, $0.0084 \cdot \Delta_r H(\text{cal})$, and $0.0025 \cdot \Delta_r H(\text{cal})$ for reactions 2, 3, and 4, respectively. The quantity $\Delta_r H(\text{cal})$ is the calorimetrically determined enthalpy of reaction (units of kJ·mol^{-1}) pertinent to the actual experimental conditions.[9]

2.5. Measurement of pH. The pH measurements were done with a ThermoOrion Model 420 pH meter and a Radiometer combination glass microelectrode at the temperature at which experiments were performed. The pH meter was calibrated with Radiometer standard buffers that bracketed the pHs of the solutions used in this study. The pHs of the reaction mixtures were calculated by using interpolation together with the measured electric potential differences and the pHs of the standard buffers.

3. Results and Discussion

3.1. Thermodynamic Formalism. The apparent equilibrium constants for reactions 1 to 4 are

$$K' = m(\text{D-xylose})m(\text{acetate})/\{m(\text{2-acetyl }\beta\text{-D-xylopyranose})a_w m^\circ\} \quad (5)$$

$$K' = m(\text{1-naphthol})m(\text{acetate})/\{m(\text{1-naphthyl acetate})a_w m^\circ\} \quad (6)$$

$$K' = m(\text{4-nitrophenol})m(\text{acetate})/\{m(\text{4-nitrophenyl acetate})a_w m^\circ\} \quad (7)$$

$$K' = m(\text{L-arabinose})m(\text{4-nitrophenol})/\{m(\text{4-nitrophenyl }\alpha\text{-L-arabinofuranoside})a_w m^\circ\} \quad (8)$$

The molalities m in the above equations are the total molalities of the various ionic forms of the respective aqueous species. For example, m(acetic acid) = m(acetic acid^0) + m(acetate$^-$) and m(4-nitrophenol) = m(4-nitrophenol^0) + m(4-nitrophenol$^-$). The quantity m° (m° = 1 mol·kg^{-1}) has been used to make K' dimensionless in the above equations. The quantity a_w is the activity of water.

The reference reactions, which involve specific ionic species, that we have selected to correspond to the overall biochemical reactions 1 to 4 are, respectively

$$\text{2-acetyl }\beta\text{-D-xylopyranose(aq)} + H_2O(l) = \text{D-xylose(aq)} + \text{acetate}^-\text{(aq)} + H^+\text{(aq)} \quad (9)$$

$$\text{1-naphthyl acetate(aq)} + H_2O(l) = \text{1-naphthol(aq)} + \text{acetate}^-\text{(aq)} + H^+\text{(aq)} \quad (10)$$

$$\text{4-nitrophenyl acetate(aq)} + H_2O(l) = \text{4-nitrophenol(aq)} + \text{acetate}^-\text{(aq)} + H^+\text{(aq)} \quad (11)$$

$$\text{4-nitrophenyl }\alpha\text{-L-arabinofuranoside(aq)} + H_2O(l) = \text{L-arabinose(aq)} + \text{4-nitrophenol(aq)} \quad (12)$$

The equilibrium constants for these reactions are, respectively:

$$K = m(\text{D-xylose})m(\text{acetate}^-)/\{m(\text{2-acetyl }\beta\text{-D-xylopyranose})a_w m^\circ\} \quad (13)$$

$$K = m(\text{1-naphthol})m(\text{acetate}^-)/\{m(\text{1-naphthyl acetate})a_w m^\circ\} \quad (14)$$

$$K = m(\text{4-nitrophenol})m(\text{acetate}^-)/\{m(\text{4-nitrophenyl acetate})a_w m^\circ\} \quad (15)$$

$$K = m(\text{L-arabinose})m(\text{4-nitrophenol})/\{m(\text{4-nitrophenyl }\alpha\text{-L-arabinofuranoside})a_w m^\circ\} \quad (16)$$

In this study, the standard state for the solute is the hypothetical ideal solution of unit molality (m° = 1 mol·kg^{-1}) and the standard state for the solvent is the pure solvent; the standard pressure p° = 0.1 MPa.

3.2. Results of Experiments. While it was not possible to measure values of K' for reactions 1, 2, 3, and 4, the extent of reaction measurements (see Section 2.3) did yield lower limits for K'. Specifically, $K' > 2 \times 10^5$ for reaction 1, $K' > 4 \times 10^4$ for reaction 2, $K' > 1 \times 10^7$ for reaction 3, and $K' > 8 \times 10^3$ for reaction 4. The pHs varied from 4.69 to 4.77 (see Section 2.3). In all cases the temperature was 298.15 K.

The results of the calorimetric measurements are given in Table 2. We judge that reasonable estimates of possible systematic error in the values of $\Delta_r H$(cal) are {$\pm 0.01 \cdot \Delta_r H$(cal) to $\pm 0.02 \cdot \Delta_r H$(cal)} due to impurities (including water) in the samples; $\pm 0.0002 \cdot \Delta_r H$(cal) due to possible errors in the extents of reaction; $\pm 0.005 \cdot \Delta_r H$(cal) due to possible errors in the measurement of the concentration of the 1-naphthyl acetate solution used for the study of reaction 2; $\pm 0.007 \cdot \Delta_r H$(cal) due to possible errors in the measurement of the concentration of the 4-nitrophenyl acetate solution used for the study of reaction 3; $\pm 0.002 \cdot \Delta_r H$(cal) due to possible errors in the correction made for the concentration 4-nitrophenyl acetate due to its spontaneous hydrolysis (see Section 2.3); and {$0.003 \cdot \Delta_r H$(cal) to $0.02 \cdot \Delta_r H$(cal)} due to possible errors in the calorimetric measurements including the "blank" enthalpies. These estimates of possible systematic error are combined in quadrature together with the statistical uncertainty in the measured value of $\Delta_r H$(cal), expressed as one estimated standard deviation of the mean, to obtain combined standard uncertainties[10] for each result. These combined standard uncertainties are then multiplied by two to arrive at the final results (T = 298.15 K and citrate buffer at pH = 4.70 to 4.71) from our experiments, that is, $\Delta_r H$(cal) = $-(14.9 \pm 1.1)$ kJ·mol^{-1} for reaction 2; $\Delta_r H$(cal) = $-(27.2 \pm 1.4)$ kJ·mol^{-1} for reaction 3; and $\Delta_r H$(cal) = $-(25.2 \pm 0.8)$ kJ·mol^{-1} for reaction 4.

3.3. Equilibrium Modeling Calculations. The pK values and standard enthalpy changes $\Delta_r H^\circ$ for the proton dissociation reactions of the reactants and of the buffer are needed to relate the experimental results obtained in this study to standard thermodynamic quantities for the reference reactions (see Section 3.1). These pK and $\Delta_r H^\circ$ values are given in Table 3 together with the basis for these values. The equilibrium model used for the calculation of the equilibrium constants K and standard enthalpies $\Delta_r H^\circ$ for the reference reactions from the measured values of K' and $\Delta_r H$(cal) has been described.[2,3] The calculations include corrections for nonideality and are made self-consistent in regard to the ionic strength. The nonideality corrections are based on the extended Debye−Hückel equation in which the "ion-size" parameter has been set at 1.6 kg$^{1/2}$·mol$^{-1/2}$. Use of the equilibrium model with the experimental results for $\Delta_r H$(cal) and with the thermodynamic quantities given in Table 3, leads to values of $\Delta_r H^\circ$ for the chemical reference reactions at T = 298.15 K and ionic strength I = 0. These values are $\Delta_r H^\circ = -(13.4 \pm 1.1)$ kJ·mol^{-1} for reaction 10, $\Delta_r H^\circ = -(25.8 \pm 0.8)$ kJ·mol^{-1} for reaction 11, and $\Delta_r H^\circ = -(25.26 \pm 0.08)$ kJ·mol^{-1} for reaction 12. The uncertainties given here are based entirely on the experimental uncertainties. The calculated values of $\Delta_r N(H^+)$, the change in binding of H^+(aq) accompanying the overall biochemical reactions under the conditions (T, pH, and I) the experiments were performed in the calorimetric measurements, are −0.550, −0.555, and −0.0050 for reactions 2, 3, and 4, respectively. These calculated values were used in performing the buffer protonation corrections.[9] The equilibrium model was also used to calculate the ionic strength values.

The uncertainties discussed thus far are based on the uncertainties in the experimentally determined values of K' and $\Delta_r H$(cal). However, there is also a component of uncertainty due to uncertainties in the parameters used in the equilibrium model. This latter component of uncertainty was examined by perturbing each of the pertinent quantities in the model. These

TABLE 2: Results of Calorimetric Measurements at $T = 298.15$ K[a]

Reaction 2: 1-Naphthyl Acetate(aq) + H_2O(l) = 1-Naphthol(aq) + Acetic Acid(aq)

experiment no.	pH	m(citrate) / mol·kg^{-1}	$10^5 \cdot m$(HCl) / mol·kg^{-1}	$10^3 \cdot m$(1-naphthyl acetate) / mol·kg^{-1}	I_m / mol·kg^{-1}	$\Delta_r H$(cal) / kJ·mol^{-1}
1	4.70	0.0969	9.94	0.4852	0.26	−14.25
2	4.70	0.0969	9.94	0.4340	0.26	−15.92
3	4.70	0.0969	9.94	0.5031	0.26	−15.01
4	4.70	0.0969	9.94	0.4943	0.26	−14.52
5	4.70	0.0969	9.94	0.5212	0.26	−14.61

$\langle\Delta_r H(\text{cal})\rangle = -(14.86 \pm 0.58)$ kJ · mol^{-1}

Reaction 3: 4-Nitrophenyl Acetate(aq) + H_2O(l) = 4-Nitrophenol(aq) + Acetic Acid(aq)

experiment no.	pH	m(citrate) / mol·kg^{-1}	$10^5 \cdot m$(HCl) / mol·kg^{-1}	$10^3 \cdot m$(4-nitrophenyl acetate) / mol·kg^{-1}	I_m / mol·kg^{-1}	$\Delta_r H$(cal) / kJ·mol^{-1}
1	4.70	0.0970	9.94	1.308	0.26	−28.01
2	4.70	0.0970	9.94	1.241	0.26	−26.77
3	4.70	0.0970	9.94	1.272	0.26	−27.69
4	4.70	0.0970	9.94	1.230	0.26	−27.70
5	4.70	0.0970	9.94	1.207	0.26	−26.62
6	4.70	0.0970	9.94	1.237	0.26	−26.66

$\langle\Delta_r H(\text{cal})\rangle = -(27.24 \pm 0.51)$ kJ · mol^{-1}

Reaction 4: 4-Nitrophenyl α-L-Arabinofuranoside(aq) + H_2O(l) = L-Arabinose(aq) + 4-Nitrophenol(aq)

experiment no.	pH	m(citrate) / mol·kg^{-1}	$10^5 \cdot m$(HCl) / mol·kg^{-1}	$10^3 \cdot m$(4-nitrophenyl α-L-arabinofuranoside) / mol·kg^{-1}	I_m / mol·kg^{-1}	$\Delta_r H$(cal) / kJ·mol^{-1}
1	4.71	0.0969	9.94	4.387	0.26	−25.19
2	4.71	0.0969	9.94	4.227	0.26	−25.11
3	4.71	0.0969	9.94	4.336	0.26	−25.14
4	4.71	0.0969	9.94	4.378	0.26	−25.14
5	4.71	0.0969	9.94	4.284	0.26	−25.34

$\langle\Delta_r H(\text{cal})\rangle = -(25.18 \pm 0.08)$ kJ · mol^{-1}

[a] The molalities m of the buffer components and of the reactants are given in columns three to five. These molalities are those obtained after mixing of the enzyme and substrate solutions and prior to any reaction. The approximate mass fractions of the acetylxylan esterase (reactions 2 and 3) and of the α-*N*-arabinofuranosidase (reaction 4) in the final reaction mixtures were, respectively, 1.1×10^{-4} and 1.5×10^{-4}. The values of the ionic strength I_m are calculated. The uncertainties in the average values of the calorimetrically determined standard enthalpy changes $\Delta_r H$(cal) are equal to two estimated standard deviations of the mean.

TABLE 3: The pKs and Standard Enthalpy Changes $\Delta_r H°$ at $T = 298.15$ K and at the Ionic Strength $I = 0$ for the Aqueous H^+ Dissociation Reactions of Substances Pertinent to This Study[a,b]

reaction		pK	$\Delta_r H°$/(kJ·mol^{-1})	Reference
H acetate^0 = H^+ + acetate$^-$	(17)	4.756	−0.41	11
H_3 citrate^0 = H^+ + H_2citrate$^-$	(18)	3.128	4.07	11
H_2 citrate$^-$ = H^+ + Hcitrate^{2-}	(19)	4.761	2.23	11
H citrate^{2-} = H^+ + citrate^{3-}	(20)	6.396	−3.38	11
1-naphthol^0 = H^+ + 1-naphthol$^-$	(21)	9.42	20.0	12
4-nitrophenol^0 = H^+ + 4-nitrophenol$^-$	(22)	7.15	19.4	12

[a] The standard state for the solutes is the hypothetical ideal solution of unit molality. Citrate^{3-} is $C_6H_5O_7^{3-}$ and acetate$^-$ is $C_2H_3O_2^-$. [b] For the purpose of assessing possible errors attributable to the equilibrium model (see Section 3.3), the values of pK and $\Delta_r H°$/(kJ·mol^{-1}) are, respectively, estimated to be uncertain by the following: reaction 17, ±0.003 and ±0.2; reaction 18, ±0.003 and ±0.2; reaction 19, ±0.003 and ±0.2; reaction 20, ±0.001 and ±0.2; reaction 21, ±0.01 and ±2; and reaction 22, ±0.01 and ±2.

perturbations are summarized in Table 3 (see footnote b). The "ion-size" parameter used in the activity coefficient model was also perturbed by ±0.3 kg$^{1/2}$·mol$^{-1/2}$. The effects of these perturbations on the calculated values of $\Delta_r H°$ for the reference reactions was negligible (<0.007 kJ·mol^{-1}) in all cases. Thus, the final results for the values of $\Delta_r H°$ for the chemical reference reactions are: $\Delta_r H° = -(13.4 \pm 1.1)$ kJ·mol^{-1} for reaction 10, $\Delta_r H° = -(25.8 \pm 0.8)$ kJ·mol^{-1} for reaction 11, and $\Delta_r H° = -(25.26 \pm 0.08)$ kJ·mol^{-1} for reaction 12.

The lower limits of the values of K' given above (see section 3.2), together with our equilibrium model, lead to the following lower limits of K for the respective reference reactions at $T =$ 298.15 K and $I = 0$: $K > 1$ for reaction 9; $K > 0.3$ for reaction 10; $K > 80$ for reaction 11; and $K > 8 \times 10^3$ for reaction 12. The equilibrium model[2,3] was also used to calculate the ionic strengths of the following reaction mixtures: $I = 0.28$ mol·dm^{-3} for reaction 1 studied with the HPLC, $I = 0.092$ mol·dm^{-3} for reaction 1 studied with the LC-MS, and $I = 0.26$ mol·dm^{-3} for reactions 2, 3, and 4.

3.4. Comparisons with Results in the Literature. Sirotkin et al.[13] measured the chymotrypsin-catalyzed hydrolysis of 4-nitrophenyl acetate in aqueous solutions that contained acetonitrile at volume fractions that ranged from 0.016 to 0.100. They[13] obtained values of $\Delta_r H(\text{cal}) = \{-100 \pm 8, -106 \pm 5,$

and $-102 \pm 5\}$ kJ·mol^{-1} at the respective acetonitrile volume fractions of 0.016, 0.040, and 0.10 and at $T = 298.15$ K. The buffer used in their study was tris(2-amino-2-hydroxymethyl-propane-1,3 diol) at $c = 0.033$ mol·dm^{-3} and at pH = 8.0. The concentration of the 4-nitrophenyl acetate was 0.0015 mol·dm^{-3}. By using our equilibrium model (see Section 3.3) together with the values of the pK and $\Delta_r H°$ for the ionization of Tris buffer[11] and their[13] result for $\Delta_r H$(cal) at the lowest volume fraction of acetonitrile, we calculate $\Delta_r H° = -(28.2 \pm 8)$ kJ·mol^{-1} for the reference reaction 11. The calculated ionic strength of their[13] reaction mixture was 0.019 mol·dm^{-3}. From our study, the result was $\Delta_r H° = -(25.8 \pm 0.8)$ kJ·mol^{-1} for this reaction. If we assume that a small volume fraction of acetonitrile has only a negligible effect on the value of $\Delta_r H$(cal), the aforementioned values are in agreement.

We have used the Benson group parameters given by Domalski and Hearing[14] together with the standard enthalpies of formation $\Delta_f H°$ of acetate$^-$(aq) and H_2O(l) from the NBS Tables[15] to estimate the value of $\Delta_r H°$ for reactions 10 and 11. Note that the use of the Benson method will give the same value of $\Delta_r H°$ for both of these reactions. Use of this estimation method led to the following values: $\Delta_r H° = -14.0$ kJ·mol^{-1} by use of the parameters for the gas phase, $\Delta_r H° = -29.5$ kJ·mol^{-1} by use of the parameters for the liquid phase, and $\Delta_r H° = -29.1$ kJ·mol^{-1} by use of the parameters for the solid phase. These Benson estimates, which completely neglect the enthalpies of solution of all of the reactants excepting H_2O(l) and acetate$^-$(aq), are not far from the values $\Delta_r H° = -(13.4 \pm 1.1)$ kJ·mol^{-1} for reaction 10 and $\Delta_r H° = -(25.8 \pm 0.8)$ kJ·mol^{-1} for reaction 11 that were obtained in this study. In the absence of Benson parameters for the O$-$(C$_B$)(CO) group, it was not possible to estimate $\Delta_r S°$ for reactions 10 and 11. Because of the uncertainty in the conformational changes accompanying reaction 12, no Benson estimates are made for this reaction.

3.5. The Standard Enthalpies of Formation of 1-Naphthyl Acetate(aq) and of 4-Nitrophenyl Acetate(aq). The results obtained in this study, together with thermodynamic property values from the literature, enable the calculation of the standard enthalpies of formation $\Delta_f H°$ of 1-naphthyl acetate(aq) and of 4-nitrophenyl acetate(aq). The pertinent data from the literature are summarized in Table 4. In all cases we have carried more than the number of significant figures in the calculation to avoid rounding errors. First, we use the standard enthalpies of combustion of 1-naphthol(s) from Colomina et al.[18] and of 4-nitrophenol from Finch et al.[22] to calculate $\Delta_f H° = -121.05$ kJ·mol^{-1} for 1-napthol(s) and $\Delta_f H° = -212.43$ kJ·mol^{-1} for 4-nitrophenol(s). In performing this calculation, we used the standard enthalpies of formation of CO_2(g) and of H_2O(l) from the CODATA Tables.[31] We then use these $\Delta_f H°$ values together with the standard enthalpies of solution of 1-naphthol(s) from Hunter et al.[30] and of 4-nitrophenol(s) from Finch et al.[22] to calculate $\Delta_f H° = -99.65$ kJ·mol^{-1} for 1-napthol(s) and $\Delta_f H° = -190.53$ kJ·mol^{-1} for 4-nitrophenol(aq). These values, together with the standard enthalpy changes obtained for reactions 10 and 11 in this study and with $\Delta_f H° = -486.01$ kJ·mol^{-1} for acetate$^-$(aq) from the NBS Tables,[15] gives $\Delta_f H° = -286.4$ kJ·mol^{-1} for 1-napthyl acetate(aq) and $\Delta_f H° = -364.9$ kJ·mol^{-1} for 4-nitrophenyl acetate(aq).

It would be desirable to have quantum chemical calculations for the thermodynamic properties reported in this study. The fact that these reactions occur in aqueous media makes this calculation particularly challenging, particularly for the computation of the entropy changes for these reactions.

TABLE 4: Enthalpies of Combustion $\Delta_c H°$ and Enthalpies of Solution $\Delta_{sol} H°$ That Are Pertinent to This Study[a,b]

$C_{10}H_8O$(s) + 11.5 O_2(g) = 10 CO_2(g) + 4 H_2O(l)	
reference	$\Delta_c H°$/(kJ·mol^{-1})
Leman and Lepoutre[16]	-4966.2 ± 15
Pushina[17]	-4980.1
Colomina et al.[18]	-4957.37 ± 0.89
$C_6H_5NO_3$(s) + 5.75 O_2(g) = 6 CO_2(aq) + 2.5 H_2O(g) + 0.5 N_2(g)	
Reference	$\Delta_c H°$/(kJ·mol^{-1})
Swarts[19]	-2880.3
Garner and Abernethy[20]	-2874.4
Rinkenbach[21]	-2872.3
Finch et al.[22]	-2863.21 ± 0.54
Sabbah and Gouali[23]	-2868.5 ± 1.0[c]
4-nitrophenol(s) = 4-nitrophenol(aq)	
reference	$\Delta_{sol} H°$/(kJ·mol^{-1})
Arnett et al.[24]	23.7 ± 0.4
Fernandez and Hepler[25]	22.6
Parsons et al.[26,27]	23.3
Larsen and Magid[28]	21.6 ± 1.1
Liotta et al.[29]	23.2 ± 0.05
Parsons and Rochester[27]	23.3
Finch et al.[22]	21.91 ± 0.05[d]
1-napthol(s) = 1-napthol(aq)	
reference	$\Delta_{sol} H°$/(kJ·mol^{-1})
Hunter et al.[30]	21.4 ± 0.6[e]

[a] $C_{10}H_8O$(s) is 1-naphthol and $C_6H_5NO_3$(s) is 4-nitrophenol(s). [b] The uncertainties given in this table are those that were reported in the references. The only exception is the statistically based uncertainty (equal to two estimated standard deviation of the mean) given to the value of $\Delta_{sol} H°$ calculated from the temperature dependence of the solubilities reported by Hunter et al.[30] [c] Sabbah and Gouali[23] used only a few milligrams of 4-nitrophenol(s) in their combustion measurements. For this reason, we prefer the value of $\Delta_c H°$ obtained by Finch et al.[22] [d] Finch et al.[22] also report $\Delta_{sol} H° = (21.84 \pm 0.06)$ kJ·mol^{-1} for the metastable white form of 4-nitrophenol(s). [e] The value of $\Delta_{sol} H°$ given here was calculated from solubilities measured by Hunter et al.[30] at temperatures from 288.15 to 301.65 K. Note that the value of $\Delta_{sol} H°$ calculated by Hunter et al.[30] is in error.

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