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Reactive Thioglucoside Substrates for β -Glucosidase

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Abstract

A new, very efficient, class of thioglycoside substrates has been found for β -glucosidase. While thioglycosides are usually resistant to hydrolysis, even in the presence of acids or most glycohydrolases, the β -D-glucopyranosides of 2- mercaptobenzimidazole (GlcSBiz) and 2- mercaptobenzoxazole (GlcSBox) have been found to be excellent substrates for β -glucosidase from both sweet almond (a family 1 glycohydrolase) and *Aspergillus niger* (a family 3 glycohydrolase), reacting nearly as well as *p*-nitrophenyl β -D-glucoside. The enzyme-catalyzed hydrolysis of GlcSBiz proceeds with retention of configuration. As with the (1000-fold slower) hydrolysis of phenyl thioglucosides catalyzed by the almond enzyme, the pL (pH/pD)-independent k_{cat}/K_M does not show a detectable solvent deuterium kinetic isotope effect (SKIE), but unlike the hydrolysis of phenyl thioglucosides, a modest SKIE is seen on k_{cat} [$^{D_2O}k_{\text{cat}} = 1.28 (\pm 0.06)$] at the pL optimum (5.5 pL 6.6). A solvent isotope effect is also seen on the K_M for the N-methyl analog of GlcSBiz. These results suggest that the mechanism for the hydrolysis of the β -thioglucoside of 2-mercaptobenzimidazole and of 2- mercaptobenzoxazole involves remote site protonation (at the ring nitrogen) followed by cleavage of the thioglucosidic bond resulting in the thione product.

Keywords

β -glucosidase; 2-mercaptobenzimidazolyl β -thioglucopyranoside; solvent kinetic isotope effect

Introduction

Activated glycosyl donors are essential for synthetic carbohydrate chemistry. Thioglycosides, which can be readily activated by oxidation or by Lewis acids (e.g., Ag^+ , Br^+ , Hg^{+2}) have been used for a long time [1]. In 1980 Hanessian *et al.* [2] introduced the concept of “remote activation” where a thioglycoside such as pyridin-2-yl 1-thio- β -glucoside becomes activated *via* electrophilic addition to the ring nitrogen. More recently, other heteroaryl thioglycosides have been shown to be useful as glucosyl donors [3]:

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⁷ Based on the data for 14 amines, collected by Laughton and Robertson [22] the following correlation is obtained: $\Delta pK_a = .033pK_a + 0.31$, $r^2=0.76$

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GlcSBox X=O

GlcSBiz X = NH

These include protected 2-benzoxazolyl 1-thio β -*D*-glucopyranoside (GlcSBox) which can be easily activated under mild conditions [3]. While it would seem likely that the high reactivity of these compounds might be due to remote activation (e.g., by methylation of the β -nitrogen) a mechanistic investigation [4] suggested that this may not be the case. For example, activation by methyl triflate of glucoside formation with the tetraacetate of GlcSBox in 2-propanol yields 2-methylthiol/benzoxazole and β -2-propyl tetraacetyl glucoside as the sole products. This indicates that activation may involve methylation (or protonation) on the sulfur. Thioglucosides of some 2-mercaptopyridines have been shown to be substrates for sweet almond β -glucosidase [5]. Niemiec-Cyganek and Szeja [5] demonstrated transglucosidase activity of the enzyme with β -thioglucosides of 2-mercaptopyridine and 2-mercapto-5-nitropyridine. These compounds were shown to be good substrates for the enzyme, however no mechanistic studies were carried out. The enzyme has much poorer activity with other thioglucosides (e.g., phenyl β -thioglucosides) [6]. For example, while the enzyme catalyzes the hydrolysis of *p*-nitrophenyl β -thioglucoside, it does so with rate constants (k_{cat} or $k_{\text{cat}}/K_{\text{m}}$) about 1000-fold lower than that of the corresponding oxygen glucoside. Not surprisingly, there is no solvent deuterium isotope effect in the β -glucosidase-catalyzed hydrolysis of *p*-nitrophenyl β -thioglucoside, consistent with the known insensitivity to general acid catalysis of the hydrolysis of thioglycosides. In order to probe the possible mechanisms for the enhanced reactivity of aryl thioglucosides with leaving groups containing a nitrogen *ortho* to the thiol, we investigated the β -glucosidase-catalyzed hydrolysis of GlcSBox and GlcSBiz. A solvent kinetic isotope effect on the hydrolysis would provide evidence of remote site activation in the reaction.

Materials and methods

GlcSBox [4] and 2-thiazolyl 1-thio- β -*D*-glucopyranoside (GlcSTaz) [7] were prepared as previously described. Synthetic details and product characterization of GlcSBiz and its N-methyl derivative, 1'-methylbenzimidazol-2'-yl 1-thio- β -*D*-glucopyranoside are given in the supplementary material (Sup.1). The tetraacetate of GlcSBiz was prepared as described by El Ashry *et al.* [8] and the N-methyl derivative (using N-methyl-2-mercaptobenzimidazole) was prepared in a similar fashion. Deacetylation was carried out under basic conditions (NaOCH_3 in methanol).

p-Nitrophenyl β -*D*-glucopyranoside (pNPG) and the buffers and other reagents were obtained from Sigma-Aldrich. D_2O (99.9%) was obtained from Cambridge Isotope Laboratories. The chromatographically purified almond β -glucosidase (MW = 65 kD on SDS PAGE), specific activity ≈ 25 units/mg (salicin, 37°, pH=5.0), was obtained from Sigma-Aldrich Chemical Co., St. Louis, MO. Purified enzyme from *Aspergillus niger* (MW = 120 kD, SDS PAGE [9]), specific activity = 52 units/mg (pNPG, 40°, pH=4.0), was obtained from Megazyme International, Wicklow, Ireland. Stock solutions of the thioglucosides were prepared in DMSO.

Kinetics

The reactions were monitored on a Hewlett-Packard model 8452A diode array spectrophotometer, with a 290 nm cutoff filter, equipped with a circulating water bath ($T = 25.0 \pm 0.1^\circ\text{C}$). Concentrated enzyme solutions ($\sim 10 \text{ mg/mL} \approx 80\text{--}150 \mu\text{M}$ subunits) were prepared in 0.01 M MES¹ buffer containing 0.01 M NaCl at pH 6.3, and the reactions were initiated by addition of enzyme (typically to a final concentration of $\sim 25 \mu\text{g/mL} \approx 1 \text{ unit/mL}$ for first-order conditions or $\sim 2 \mu\text{g/mL}$ under initial velocity conditions). With pNPG¹ as substrate, product production was monitored spectrophotometrically by measuring the absorbance at 400 nm. With GlcSBiz¹, its N-methyl derivative, or with GlcSBox as substrate, under first-order conditions ($[S] \ll K_M$) the reaction was monitored in a continuous assay by the change in absorbance (300–312 nm) to follow the production of the thione product. Under initial velocity conditions, the reaction was monitored in a stopped assay by the following rate of glucose production. This was determined by removal of an aliquot of the reaction mixture and then measuring the glucose concentration using a coupled enzyme assay of hexokinase (yeast) and glucose-6-phosphate dehydrogenase (*L. mesenteroides*), containing 10 mM ATP, 5 mM MgSO₄, 1 mM NAD⁺, pH=7.5, and measuring the resulting absorbance at 340 nm due to the formation of NADH ($\epsilon = 6.32 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ [10]). (This method was also used in an attempt to detect hydrolysis of GlcSTaz¹.) The $V_{\max}/K_M$ values were obtained under pseudo first-order conditions ($[S] \ll K_M$), following the reaction to >90% completion. The $V_{\max}$ and K_M values for GlcSBiz were obtained under initial velocity conditions using a non-linear regression fit to the Henri-Michaelis-Menten equation. K_M and K_i values were obtained by measuring the inhibition² of *p*-nitrophenolate production from the hydrolysis of pNPG under first-order conditions ($[p\text{NPG}] \approx 0.1 \text{ mM}$).

pL Dependence

The k_{cat}/K_M values $[(V_{\max}/K_M)/(\text{enz. conc.})]$ were determined at various pH/pD (i.e., pL) values. The buffer solutions consisted of 0.01 M buffer (citrate, pL 3.2–4.5; formate, pL 3.6–4.0, acetate, pL 3.5–5.8; MES¹, pL 5.5–7.0; HEPES¹, pL 6.8–8.4), prepared in deionized water (or D₂O) containing 0.01 M NaCl. pH measurements were made using a glass combination electrode (Accumet pH meter). pD values were estimated by adding 0.41 to the pH meter reading [12]. The data were fit to the following equation:

$$\log k_{\text{obs}} = \log [k^{\text{lim}} / (1 + 10^{(\text{p}K_1 - \text{pH})} + 10^{(\text{pH} + \text{p}K_2)})] \quad (1)$$

Results

GlcSBiz, its N-methyl derivative [GlcS(N-Me)Biz] and GlcSBox are very good substrates for sweet almond β -glucosidase, reacting two to three orders of magnitude more rapidly than *p*-nitrophenyl thioglucoside [6]. In fact, GlcSBiz is nearly as reactive as one of best substrates for the enzyme, the *O*-glucoside, pNPG (Table 1). As in the reaction with *O*-glycosides, the enzyme-catalyzed hydrolysis of GlcSBiz occurs with retention of configuration. Incubation of GlcSBiz ($\approx 30 \text{ mM}$) in D₂O (pD = 6.3, 0.01 M phosphate) with the almond enzyme ($\approx 13 \text{ units/mL}$), followed for about 20 minutes by ¹H-NMR (300 MHz), results in a product with the anomeric hydrogen signal occurring at 4.64 ppm (d, $J^{1,2} = 8.0$

¹Abbreviations: GlcSBiz, β -thioglucoside of 2-mercaptolbenzimidazole (2'-benzimidazolyl 1-thio- β -D-glucopyranoside); GlcSBox, β -thioglucoside of 2-mercaptolbenzoxazole; GlcS(N-Me)Biz, β -thioglucoside of 2-mercapto-N-methylbenzimidazole; GlcSTaz, β -thioglucoside of 2-mercaptothiazole; HEPES, N-(2-hydroxyethyl)-piperazine-N'-(2-ethansulfonate); MES, 2-(N-morpholino)ethanesulfonate; pNPG, *p*-nitrophenyl β -D-glucopyranoside; pNPGS, *p*-nitrophenyl 1-thio- β -D-glucopyranoside; SKIE, solvent kinetic isotope effect.

²When an alternate substrate is used to inhibit the reaction (in this case, pNPG hydrolysis), the apparent K_i is equal to the K_M of the alternate substrate [11].

Hz), corresponding to that of β -D-glucose. The rate of appearance of this peak equaled the rate of disappearance of the substrate anomeric hydrogen signal ($\delta = 5.03$ ppm, $J^{1,2} = 9.8$ Hz).

The N-methyl derivative, GlcS(N-Me)Biz, has a $k_{\text{cat}}/K_{\text{M}}$ value which is about 12% that of GlcSBiz, due to a larger K_{M} (Table 1). The benzoxazole derivative, GlcSBox, is about 16-times less reactive than GlcSBiz. The glucoside of the non-aromatic heterocycle, thiazolyl β -thioglucoside, which is known to be less reactive non-enzymatically [13],

GlcSTaz

was found *not* to be a substrate for the enzyme. Incubation of 20 mM GlcSTaz with the almond enzyme (0.2 mg/mL $\approx$ 5 units/mL, pH = 6.3) for 99 hr showed no detectable amount of glucose formation, indicating a value of $k_{\text{cat}}/K_{\text{M}} < 5.9 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$. This compound, however, does bind to the enzyme with a $K_{\text{i}} = 8.7 (\pm 0.8) \text{ mM}$.

With the family 3 β -glucosidase from *Aspergillus niger*, GlcSBiz and its N-methyl derivative are also good substrates. At pH = 6.3 (25°C) the values of the second-order rate constants ($k_{\text{cat}}/K_{\text{M}}$) are $1.1 (\pm 0.3) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for GlcSBiz and $1.3 (\pm 0.5) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for GlcS(N-Me)Biz. These values can be compared with the value of $k_{\text{cat}}/K_{\text{M}} = 9.6 (\pm 0.8) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for the hydrolysis of pNPG with this enzyme.

The pH dependence of the hydrolysis of GlcSBiz, catalyzed by the family 1 β -glucosidase from sweet almond (Fig. 1), is bell-shaped, similar to that for the hydrolysis of the thiophenyl glucoside, *p*-nitrophenyl β -D-thioglucoside (pNPSG) [6]. The dependence of the second-order rate constant on pL shows an excellent fit to eq. 1 ($r^2 = 0.98$ in H_2O and 0.99 in D_2O) to eq. (1), with kinetic pK_{a} s of 4.2 and 7.9. Surprisingly, the limiting second-order rate constant, $k_{\text{cat}}/K_{\text{M}}$, is nearly identical in H_2O [$= 23.1 (\pm 0.4) \text{ mM}^{-1}\text{s}^{-1}$] and in D_2O [$= 24.0 (\pm 0.5) \text{ mM}^{-1}\text{s}^{-1}$] yielding a SKIE of D_2O ($k_{\text{cat}}/K_{\text{M}}$) = $0.96 (\pm 0.03)$. In order to see if this might reflect a cancellation of isotope effects on k_{cat} and on K_{M} , we examined the effect of D_2O on the steady state kinetics for the hydrolysis of GlcSBiz around the pL optima. The kinetic parameters are summarized in Table 2. In H_2O , over the range of 5.5 pH 6.3, $k_{\text{cat}} = 60 (\pm 1) \text{ s}^{-1}$ and in D_2O , over the range of 5.9 pD 6.6, $k_{\text{cat}} = 47 (\pm 3) \text{ s}^{-1}$. This yields a value for the SKIE of $\text{D}_2\text{O} k_{\text{cat}} = 1.28 (\pm 0.06)$, and a SKIE on K_{M} [$= 1.28 (\pm 0.09)$] identical to that on k_{cat} .] An isotope effect was also found on the K_{M} (K_{i}) of the N-methyl-2-mercaptobenzimidazole thioglucoside by looking at the inhibition of pNPG hydrolysis. At pH = 6.0, the K_{i} is $46 (\pm 7) \text{ mM}$ and at pD = 6.3 it is $20.3 (\pm 0.4) \text{ mM}$, yielding a value for the solvent isotope effect on the K_{M} of GlcS(N-Me)Biz of $\text{D}_2\text{O} K_{\text{M}} = 2.3 (\pm 0.4)$.

Discussion

The thioglucosides with the heteroaromatic leaving groups, 2-mercapto-benzimidazole, N-methyl-2-mercaptobenzimidazole and 2-mercaptobenzoxazole, are unusually good substrates for almond β -glucosidase. Because of this high reactivity, it was easy to confirm that the hydrolysis of GlcSBiz proceeds with retention of configuration. This is the first thioglucoside substrate for which such a confirmation has been made with a β -glucosidase.

The thioglucosides examined here are the most reactive thioglucosides for β -glucosidase, hydrolyzing a couple of orders of magnitude more rapidly than *p*-nitrophenyl thiophenylglucoside [6]. *p*-Nitrophenyl β -thioglucoside (thiol leaving group $pK_a = 4.5$) is about 100-times more reactive than phenyl β -thioglucoside (thiol leaving group $pK_a = 6.5$) [14]. Since the reactivity of thiol glucosides with the enzyme depends on the basicity of the leaving group, it could be argued that the high reactivity of GlcSBiz is due to a strong thiol acidity of the leaving group. This would require a pK_a of less than 2 in order to account for the observed second-order rate constant for GlcSBiz (or <3 for GlcSBox) compared to that of the thiophenyl glucosides. However, a reasonable estimate³ of the SH acidity of the minor thiol tautomer of (neutral) 2-mercaptobenzimidazole is closer to $pK_a \approx 8$.

The most likely explanation for the high reactivity of the heteroaromatic thioglucosides with β -glucosidase is “remote activation”, i.e., protonation on the ring nitrogen either before or during cleavage of the anomeric C-S bond leading to formation of the glucosyl-enzyme intermediate:

One indirect piece of evidence that the acid on the enzyme which protonates the leaving group is not the same residue which must be protonated when the enzyme catalyzes the hydrolysis of other substrates, can be seen in the pH profile (Fig. 1). This bell-shaped dependence of k_{cat}/K_M on pH has an acidic limb (corresponding to the ionization of the glutamic acid nucleophile) characterized by a pK_a of 4.2. This is nearly identical to that seen with the *O*-glucoside, pNPG ($pK_{a1} = 4.4$ [17]) and the analogous *S*-glycoside ($pK_{a1} = 4.5$ [6]). However, the basic limb in the pH-profile for hydrolysis of GlcSBiz is characterized by a pK_a (≈ 7.9)⁴ which is about 1 unit *higher* than that seen with other substrates ($pK_{a2} = 6.7$ with pNPG [16] and with pNPSG [6]).

It was surprising that we did not detect a significant solvent kinetic isotope effect in the hydrolysis of GlcSBiz catalyzed by almond β -glucosidase [D_2O (k_{cat}/K_M) = $0.96 (\pm 0.03)$]. This, of course, could be due to a fortuitous cancellation of isotope effects on k_{cat} and K_M as have been seen with other glycohydrolases (e.g., [18]). Indeed, at the pL optima, the hydrolysis of GlcSBiz to the enzyme does show a very small, but significant, solvent isotope effect on k_{cat} [$=1.28 (\pm 0.06)$]. For this substrate, the rate-limiting step is most likely glucosylation of the enzyme⁵ and the SKIE indicates proton transfer in, or before, this step. This is not the case with other substrates such as pNPG [17], phenyl β -glucoside [18],

³ This is based on the linear free energy relationship for thiol (RSH) acidity: $pK_a = 10.22 - 3.50\sigma^*$ [15] and a value of $\sigma^* = 0.66$ for the 2-benzimidazolyl group [16].

⁴ While the accuracy of this value is limited by the inability to look at the kinetics at higher pL values (enzyme instability), it is clear that pK_{a2} is greater than 6.7.

methyl β -glucoside [19] or the thioglucoside, pNPSG [6], all of which show no solvent kinetic isotope effect ($D_2O k_{cat} \approx 1$).

The magnitude of the SKIE for the β -glucosidase-catalyzed hydrolysis of GlcSBiz is unusually small compared to most enzymatic reactions for which a solvent kinetic isotope effect is observed [20]. One possible explanation for this is that there may only be a small degree of proton transfer from the acid group in the enzyme to leaving (mercaptobenzimidazole) group in the transition state. If this is the case, however, it's hard to see how there can be much rate enhancement provided by the enzyme. Another explanation is that proton transfer is concerted with cleavage of the anomeric C-S bond. However, this is a typical situation that usually results in solvent kinetic isotope effects of ~ 2 –4. Furthermore, according to the tenets of Jenck's "libido rule" [21], ..proton transfer is not required to be concerted with respect to C-S bond cleavage in this case. The mercaptobenzimidazole moiety does not undergo a large change in pK_a as the C-S bond is cleaved (the pK_a of benzyimidazolium group on the substrate is about 3.4⁶ and the pK_a of 2-mercaptobenzimidazole is ca. 6.2 [22]), and the pK_a acid catalyst (≈ 7.9) is outside this range. Thus, a concerted proton transfer is not likely. Therefore, a "pre-equilibrium" protonation of bound substrate is expected. The isotope effect for this proton transfer is small. We can look at this equilibrium proton transfer as:

where AH represents the protonated acid on the enzyme ($pK_a \approx 7.9$), S represents the bound substrate and SH^+ represents the bound substrate containing a protonated benzimidazole moiety ($pK_a \approx 3.4$). The solvent isotope effect for the equilibrium proton transfer between two groups with $\Delta pK_a \approx 4.5$ is expected⁸ to be on the order of $10^{(0.033)(4.5)} = 1.4$. As the C-S bond is cleaved, and the protonated benzimidazole moiety becomes less acidic, an inverse isotope effect will occur. (For a group which undergoes a change in pK_a from 3.4 to 6.2, or $\Delta pK_a = -2.8$, the estimated equilibrium isotope effect is $DK = 10^{(0.033)(-2.8)} = 0.8$). While these estimates are too imprecise to describe the transition state structure for the thioglycosidic bond cleavage with any precision, the observed SKIE ($D_2O k_{cat} = 1.3$) is consistent with a stepwise proton transfer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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⁵ The k_{cat} value of pNPG (which is slightly greater than that of GlcSBiz) is *lower* than that of some more reactive phenyl β -glucosides [17]. For example, 3,4-dinitrophenyl β -glucoside has a value of k_{cat} about 5-times larger than that of pNPG. Also, although there is a bit more uncertainty in this ratio, k_{cat} of GlcS(N-Me)Biz is about twice as large as the value for GlcSBiz.

⁶ The estimate of this $pK_a (= 3.4)$ is based on a series of linear free energy relationships for the ionization of protonated 2-substituted benzimidazoles ($\rho^* = -1$) and the estimation of $\sigma^* (= 2.28)$ for the β -1-thioglycosyl group. (See sup. material 2)

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β -Thioglucosides of 2-mercaptobenzimidazole and analogs are good substrates for β -glucosidase.

The reaction proceeds with retention of configuration.

A solvent kinetic isotope effect is seen in k_{cat} but not in $k_{\text{cat}}/K_{\text{m}}$.

The enzyme activates these substrates *via* remote site protonation.

Figure 1.

pH/pD Dependence of the sweet almond β -glucosidase-catalyzed hydrolysis of GlcSBiz (0.01 M buffer, 0.01 M NaCl, H₂O or D₂O, 4.6% v/v DMSO, 25°C). The curves are the theoretical fit based on the parameters evaluated by a fit to the logarithmic form of the equation, eq. (1). The parameters are: $(k_{cat}/K_M)^{lim} = 23.1 (\pm 0.4) \text{ mM}^{-1}\text{s}^{-1}$, $pK_{a1} = 4.19 (\pm 0.03)$, $pK_{a2} = 7.86 (\pm 0.04)$ in H₂O and $(k_{cat}/K_M)^{lim} = 24.0 (\pm 0.5) \text{ M}^{-1}\text{s}^{-1}$, $pK_{a1} = 4.51 (\pm 0.03)$, $pK_{a2} = 8.12 (\pm 0.05)$ in D₂O.

Table 1Reactivity of Heteroaryl β -Thioglucosides with Sweet Almond β -Glucosidase^a

substrate	k_{cat} , s ⁻¹	K_{M} , mM	$k_{\text{cat}}/K_{\text{M}}$, M ⁻¹ s ⁻¹
[pNPG	71 ($\pm$ 4)	2.5 ($\pm$ 0.1)	2.84 ($\pm$ 0.03) $\times 10^4$
GlcSBiz	59 ($\pm$ 4)	2.5 ($\pm$ 0.3)	2.39 ($\pm$ 0.02) $\times 10^4$
GlcS(N-Me)Biz	136 ^b	47 ($\pm$ 8)	2.9 ($\pm$ 0.1) $\times 10^3$
GlcSBox	3.8 ^b	2.7 ($\pm$ 0.4)	1.4 ($\pm$ 0.1) $\times 10^3$

^a pH = 6.3 (0.01 M MES, 0.01 M NaCl, 4.6% v/v DMSO), 25°C^b calculated from $k_{\text{cat}}/K_{\text{M}}$ value (measured under pseudo first-order conditions) and K_{M}

Table 2

Solvent Isotope Effects on GlcSBiz Hydrolysis^a

pH	H ₂ O			D ₂ O		
	K _M , mM	k _{cat} s ⁻¹	pD	K _M , mM	k _{cat} s ⁻¹	D ₂ O k _{cat}
5.5	2.67 (±0.13)	61 (±3)	5.9	2.15 (±0.16)	49.3 (±0.9)	1.24 (±0.07)
6.0	2.6 (±0.2)	59 (±4)	6.3	2.1 (±0.2)	47 (±2)	1.26 (±0.10)
6.3	2.5 (±0.2)	59 (±2)	6.6	1.90 (±0.15)	44 (±3)	1.34 (±0.10)

^a 0.01 M MES, 0.01 M NaCl, 4.6% v/v DMSO, 25°C; mean value of SKIE over this pL range is $D_2O k_{cat} = 1.28 (\pm 0.06)$