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Aryl sulfamates are broad spectrum inactivators of sulfatases: Effects on sulfatases from various sources

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ABSTRACT

Aryl sulfamates were originally developed as inhibitors of steroid sulfatase, and have recently been shown to be powerful inactivators of a bacterial sulfatase, *Pa*AtsA from *Pseudomonas aeruginosa*. We demonstrate that a simple aryl sulfamate, 3-nitrophenyl sulfamate, can inactivate sulfatases from various sources including snail, limpet and abalone. In each case inactivation was time-dependent and active-site directed, as demonstrated by protection against inactivation by substrate. These results suggest that such easily acquired aryl sulfamates can be used as reliable biochemical reagents for the study of sulfatases from a diverse array of sources.

Sulfatases (EC 3.1.6) are hydrolytic enzymes found in a wide variety of lower and higher organisms.[1,2] One group of sulfatases cleave sulfate esters in a mechanism involving formylglycine (FGly), an active-site residue unique to these enzymes. FGly originates post-translationally from cysteine or serine found within a conserved pentapeptide motif (C/S)X(P/A)XR.[3–5] This sequence is required for posttranslational modification of the first cysteine or serine residue to FGly, and is essential for catalytic activity.

Estrone sulfatase (EC 3.1.6.1) is implicated in the progression of breast cancer in post-menopausal women,[6,7] and other sulfatases participate in bacterial pathogenesis,[8,9] and the evasion of plant defenses by grazing insects.[10] The design of sulfatase inhibitors is therefore highly attractive for applications in medicine and biotechnology. Many aryl sulfamates, phenolic esters of sulfamic acid (H_2NSO_2OH; Fig. 1), are potent inhibitors of human estrone sulfatase[11–13] and have recently been reported to be nanomolar inhibitors of a bacterial sulfatase, *Pseudomonas aeruginosa* arylsulfatase A (*Pa*AtsA).[1,14] For both enzymes, inactivation by aryl sulfamates was time-dependent, active-site directed and irreversible. These data are consistent with an inactivation mechanism involving covalent modification of the active site.

Several mechanistic alternatives for the inactivation of sulfatases by aryl sulfamates have been proposed.[1,11–14] In recent work we demonstrated that inactivation of *Pa*AtsA by aryl sulfamates occurs through cleavage of the ArO–S bond and that it exhibits >1 stoichiometry.[14] Although the exact identity of the inactivated species remains elusive, it seems likely that the reaction of an aryl sulfamate and the FGly residue is responsible for inactivation. Even though sulfamates are potent inactivators of estrone sulfatase and *Pa*AtsA, it is not known whether these easily acquired compounds are inactivators of other sulfatases, which could have widespread implications in a range of biotechnological applications.[1,15,16]

In this work, we have shown that a representative aryl sulfamate, 3-nitrophenyl sulfamate (which can be prepared in one step from commercially available 3-nitrophenol and chlorosulfonyl isocyanate[17]), efficiently inactivates three other sulfatases from various sources: snail (*Helix pomatia*), abalone entrails and keyhole limpet (*Patella vulgata*). The kinetics of inactivation of these sulfat-

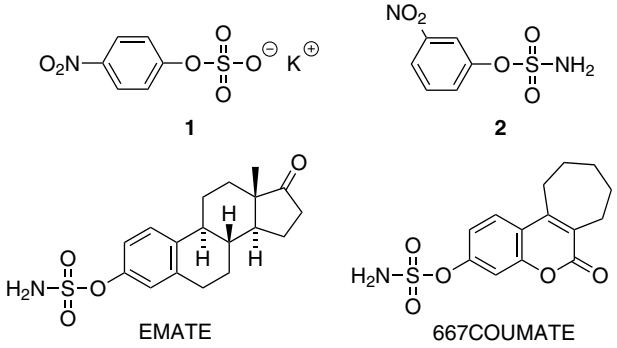

Figure 1. Structures of potassium 4-nitrophenyl sulfate **1** and inactivators 3-nitrophenyl sulfamate **2**, EMATE and 667COUMATE.

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ases were compared with those for PaAtsA,[14] demonstrating that aryl sulfamates are broad spectrum inactivators of sulfatases and providing new insight into the mechanism of inactivation by aryl sulfamates.

Kinetic parameters for the hydrolysis of potassium 4-nitrophenyl sulfate **1**[17] by the four sulfatases are shown in Table 1. 4-Nitrophenyl sulfate was chosen as upon hydrolysis it releases 4-nitrophenol, which has a high extinction coefficient providing better sensitivity in enzymatic assays than 3-nitrophenyl sulfate. Nonetheless, the assays for limpet and abalone sulfatases suffered from poor sensitivity owing to the limited amounts of these enzymes available and thus α-cyclodextrin was added to enhance the sensitivity. α-Cyclodextrin lowers the pK_a value of 4-nitrophenol released in the hydrolytic reaction and thus increases the amount of the ionized form present at pH values close to or below the original pK_a value (7.14). Cyclodextrins have been successfully applied to enhance the sensitivity of cleavage of 4-nitrophenyl glycosides by glycosidases at low pH[18,19]; however, to the best of our knowledge this is the first time they have been used for the analysis of sulfatases. Addition of α-cyclodextrin (10 mM) resulted in a 2.2-fold increase in the extinction coefficient ($\Delta\varepsilon_{410}$) from 6530 to 14400 M^{-1} cm^{-1} at pH 7 (*cf.* $\Delta\varepsilon_{410}$ = 17 000 M^{-1} cm^{-1} in 0.4 M glycine–NaOH buffer, pH 10). The effect of α-cyclodextrin on the extinction coefficient is saturable (see Supporting Information) and does not affect the kinetic parameters of the abalone and limpet sulfatases.

To demonstrate the generality of aryl sulfamates as sulfatase inactivators, we selected 3-nitrophenyl sulfamate (**2**; Fig. 1), which was among the most potent inactivators identified against *Pa*AtsA[14] and, unlike 4-nitrophenyl sulfamate, is relatively stable at pH 7 (spontaneous decay rate constant at 37 °C is 0.0028 min^{-1}, corresponding to decomposition of 4.2% inactivator in 15 min).

Inactivation kinetics were assessed according to Eq. 1:

$$E + I \overset{K_i}{\rightleftharpoons} E.I \overset{k_{inact}}{\rightarrow} E - P \quad (1)$$

where E, I and E–P represent enzyme, aryl sulfamate and covalent adduct, respectively; K_i is the dissociation constant for the enzyme-sulfamate complex; and k_{inact} is the first-order rate constant for conversion of the non-covalent E.I complex to the E-P complex.

Inactivation of snail sulfatase obeyed pseudo-first-order kinetics (Fig. 2A), and a full kinetic analysis allowed determination of k_{inact} and K_i values (Table 2). The active-site directed nature of inactivation was unambiguously demonstrated by protection of snail sulfatase from inactivation using various concentrations of substrate **1** (Fig. 2B).

Substrate **1** competes with inactivator **2** according to Eq. (2):

$$\begin{array}{ccccc} E + I & \overset{K_i}{\rightleftharpoons} & E.I & \overset{k_{inact}}{\longrightarrow} & E\text{-}P \\ + & & & & \\ S & & & & \\ \| K_m & & & & \\ E.S & & & & \end{array} \quad (2)$$

A plot of the inverse pseudo-first-order rate constant, k^{-1}, against substrate concentration (Fig. 2B, inset) enabled determination of the inhibition constant for inactivator **2** (K_i = 40.3 μM), which compares favorably with that measured by full kinetic analysis (Table 2).

Inactivation of both abalone and limpet sulfatases was biphasic. In the case of limpet sulfatase (Fig. 3A and B), the first-order rate constants (k) were extracted from the initial rates of inactivation. With abalone sulfatase (Fig. 4A–C), the biphasic character was more pronounced and the first-order rate constants could not be extracted reliably from the initial phase of inactivation. Therefore, the rate constants for both the fast and slow phase of inactivation (in a ratio of 2.2:1) were determined by fitting the data to a double exponential decay Eq. (3):

$$v_{res}/v_0 = f_{fast} \times e^{(-k_{fast} \times t)} + (1 - f_{fast}) \times e^{(-k_{slow} \times t)} \quad (3)$$

Table 1
Kinetic parameters for hydrolysis of potassium 4-nitrophenyl sulfate **1** by sulfatases

Sulfatase	V_{max} (mmol min^{-1} mg^{-1} protein)	K_m (mM)	V_{max}/K_m (l min^{-1} mg^{-1})
*Pa*AtsA	6.1 ± 0.2	(0.77 ± 0.12) × 10^{-3}	7900 ± 1300
Snail	0.319 ± 0.003	2.03 ± 0.07	0.157 ± 0.006
Limpet[a]	0.72 ± 0.02	10 ± 1	0.070 ± 0.009
Abalone[a]	0.68 ± 0.01	10.2 ± 0.6	0.066 ± 0.004

[a] Limpet and abalone sulfatases are inhibited by phosphate buffer: 4% and 15% activity, respectively, in 5 mM K_2HPO_4/KH_2PO_4, pH 7.

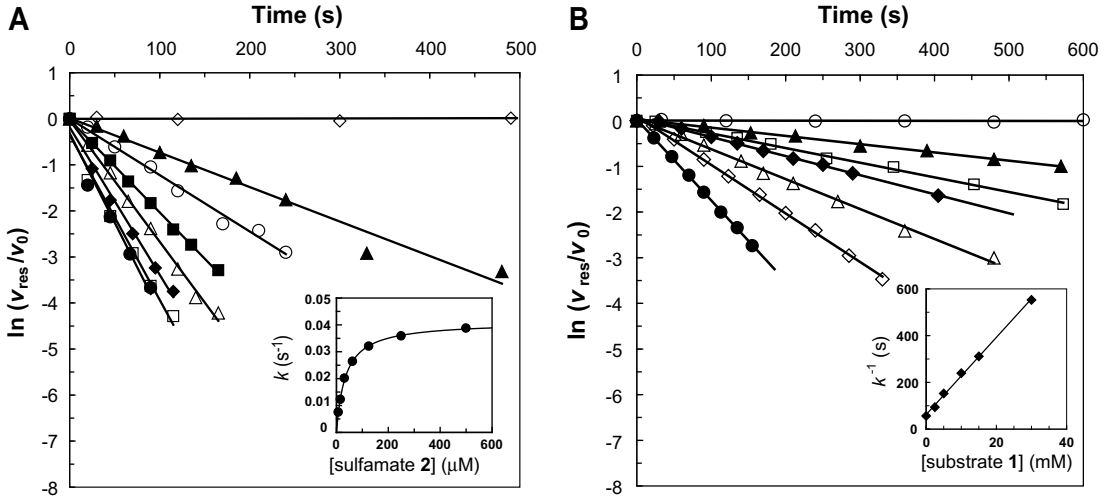

Figure 2. Inactivation of snail sulfatase by 3-nitrophenyl sulfamate **2** at pH 7. (A) Semi-logarithmic plot of inactivation by 500 μM (●), 250 μM (□), 125 μM (♦), 62.5 μM (△), 31.3 μM (■), 15.6 μM (○), 7.8 μM (▲), and 0 M (◇) sulfamate **2**. Inset: Replot of inactivation rates. (B) Semi-logarithmic plot of protection against inactivation by **2** (35 μM) using 0 M (●), 2.5 mM (◇), 5 mM (△), 10 mM (♦), 15 mM (□), and 30 mM (▲) potassium 4-nitrophenyl sulfate **1** as substrate; (○) denotes control. Inset: Plot of inverse pseudo-first-order rate constant of inactivation (k^{-1}) against concentration of **1**.

Table 2
Inactivation parameters for inhibition of sulfatases by 3-nitrophenyl sulfamate **2**

Sulfatase	k_{inact} (s^{-1})	K_i (mM)	$t_{1/2}$ (s)	k_{inact}/K_i (s^{-1} mM^{-1})
*Pa*AtsA[a]	0.063 ± 0.007	(0.13 ± 0.03) × 10^{-3}	11	480 ± 110
Snail	0.0411 ± 0.0004	0.035 ± 0.001	17	1.19 ± 0.06
Limpet	0.038 ± 0.002	0.98 ± 0.16	18	0.038 ± 0.006
Abalone, fast	0.070 ± 0.005	1.6 ± 0.3	10	0.043 ± 0.009
Abalone, slow	0.0027 ± 0.0002	5.0 ± 1.0	257	0.0005 ± 0.0001

[a] See Ref. 14.

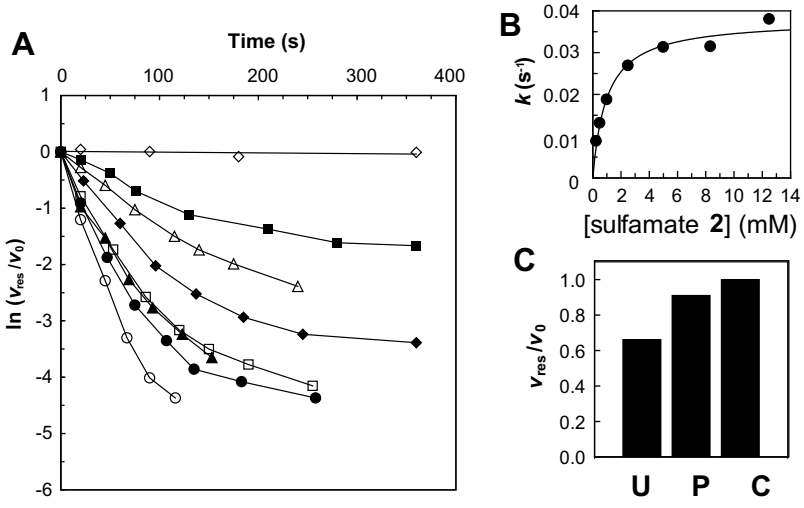

Figure 3. Inactivation of limpet sulfatase by 3-nitrophenyl sulfamate **2** at pH 7. (A) Semi-logarithmic plot of inactivation by 12.5 mM (○), 8.3 mM (▲), 5 mM (●), 2.5 mM (□), 1 mM (♦), 0.5 mM (△), 0.25 mM (■), and 0 M (◇) sulfamate **2**. (B) Replot of inactivation rates. (C) Protection against inactivation by **2** (1 mM) using substrate **1** (79 mM). Residual enzyme activity (v_{res}) was assayed after 20 s from addition of **2**. C denotes control, P protected and U unprotected reaction with v_{res}/v_0 ratio 1, 0.91 and 0.66, respectively.

and are shown in Table 2. The first-order rates for protection of limpet and abalone sulfatases by substrate **1** against 3-nitrophenyl sulfamate-induced inactivation could not be accurately determined as substrate was rapidly depleted in the inactivation reaction mixture. Evidence for substrate protection is therefore reported as a ratio of v_{res}/v_0 at a single time point (Fig. 3C and 4D, respectively).

The mode of inactivation seen for the four sulfatases deserves comment. While inactivation of *Pa*AtsA[14] and snail sulfatases by **2** obeyed pseudo-first-order kinetics (Fig. 2A), limpet and abalone sulfatases were inactivated in a biphasic manner (Fig. 3A, 4A). This latter behavior is reminiscent of the inactivation of estrone sulfatase by EMATE, COUMATE and 667COUMATE (Fig. 1).[11–13] Two possible explanations for the biphasic kinetics of inactivation can

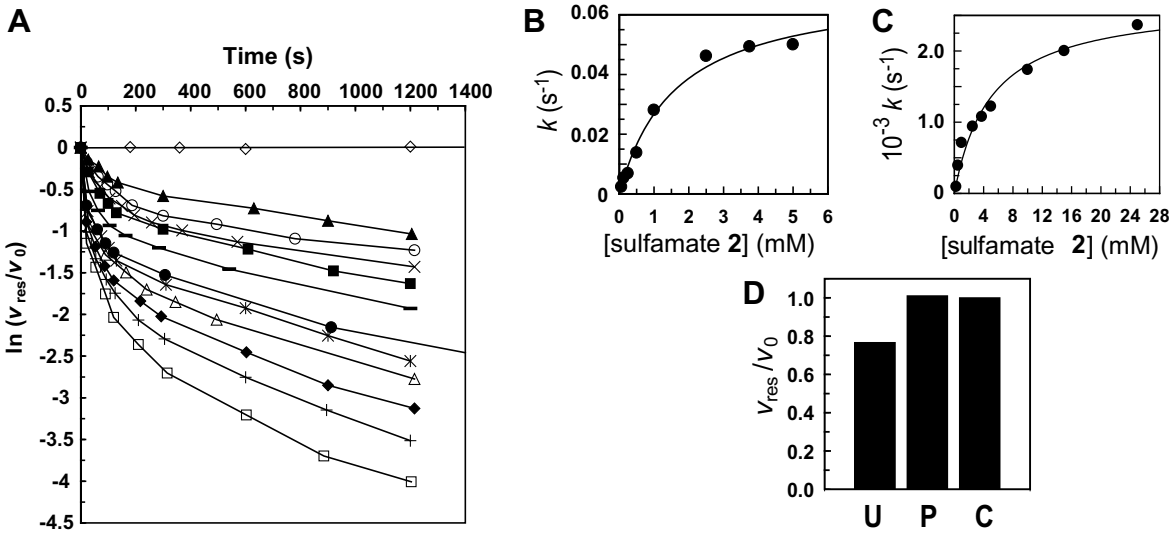

Figure 4. Inactivation of abalone sulfatase by 3-nitrophenyl sulfamate **2** at pH 7. (A) Semi-logarithmic plot of inactivation by 25 mM (□), 15 mM (+), 10 mM (♦), 5 mM (△), 3.75 mM (*), 2.5 mM (●), 1 mM (–), 0.5 mM (■), 0.25 mM (×), 0.125 mM (○), 0.0625 mM (▲) and 0 M (◇) sulfamate **2**. (B and C) Replot of rates of fast and slow fractions of inactivation, respectively. (D) Protection against inactivation by **2** (0.5 mM) using substrate **1** (30 mM). Residual enzyme activity (v_{res}) was assayed after 25 s from addition of **2**. C denotes control, P protected and U unprotected reaction with v_{res}/v_0 ratio 1, 1.01 and 0.77, respectively.

be advanced. The first explanation is that the crude enzyme preparations used in this work as well as that for estrone sulfatase contain at least two different sulfatase enzymes (or isozymes) that possess different parameters of inactivation. Thus, the first fast phase of inactivation reports on an enzyme possessing a high k_{inact}/K_i value, whereas the second slower phase reports on a different enzyme with a lower k_{inact}/K_i value. An alternative explanation for biphasic inactivation kinetics is that inactivation by 3-nitrophenyl sulfamate results in sulfamoylation of multiple enzyme residues through two independent processes.[12,14] Thus, one process leads to the loss of enzyme activity by specific sulfamoylation of the active-site FGly residue. The second process results in direct sulfamoylation of other enzyme residues, possibly conserved active-site nucleophiles such as Lys or His.[14] This second process leads to a non-specifically sulfamoylated enzyme with altered parameters of inactivation that is then inactivated by specific sulfamoylation of the active-site FGly.

It is also worthwhile commenting on the differences in the efficiency of inactivation (in terms of the k_{inact}/K_i ratio) seen for the four sulfatases. This value ranges from 480 to 0.038 s^{-1} mM^{-1} for sulfatases from different origins, declining in the order *Pa*AtsA > snail > abalone (fast fraction) ≈ limpet sulfatase. This correlates well with the relative affinity of these sulfatases for potassium 4-nitrophenyl sulfate as reflected by the increasing K_m value from nanomolar (*Pa*AtsA) to millimolar (abalone and limpet sulfatases), Table 1. Therefore, the varying k_{inact}/K_i ratio for these enzymes is likely related to differences in their affinity for the nitrophenyl group. Indeed, closer inspection of these data reveals a good correlation of inactivator K_i value with the K_m value for each enzyme. These data suggest that the ability of aryl sulfamates to inactivate sulfatases is a common phenomenon.

Sulfatases mediate a wide variety of biological processes including developmental cell signaling, hormone regulation, pathogenesis and cellular degradation. Studies of these enzymes are limited by a lack of specific reagents, especially inhibitors. This study demonstrates that aryl sulfamates are time-dependent, active-site-directed inactivators of a range of sulfatases from various sources. The efficiency of inactivation (k_{inact}/K_i) for individual sulfatases correlated with the K_m values for substrate hydrolysis. Thus, for sulfatases able to catalyze the hydrolysis of a specific aryl sulfate, we anticipate that the corresponding aryl sulfamate should act as an inhibitor. We have already demonstrated that aryl sulfamates can be used as active-site titrants for sulfatases,[14] and there is great potential for their broad-scale use as small molecule tools in dissecting sulfatase-specific processes in complex biological systems. The generality of aryl sulfamates as sulfatase inactivators suggests their potential application in the treatment of sulfatase-related dysfunctions aside from therapy of hormone-dependent breast cancer.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.059.

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