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# Acetamidoquinone and Acetamidohydroxy Derivatives as Inhibitors for Both Dihydroxyacetamido Epoxidase and Dehydrogenase

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Abstract—A series of monohydroxy and dihydroxyacetanilides, acetamidoquinones and bromoacetamidoquinones have been synthesised and tested as substrates and/or inhibitors of highly purified dihydroxyacetamido epoxidase (DHAE) and dihydroxy acetamido dehydrogenase (DHADH) from Streptomyces LL-C10337. None was found to act as substrates but many selectively inhibit the enzymes. Kinetic analysis has shown that all the compounds act as reversible competitive inhibitors with respect to the substrates 2,5-dihydroxyacetanilide and 2,3-epoxy-1,4-benzoquinone-5-acetanilide. Monohydroxy acetanilides showed weak inhibition to these enzymes compared to the dihydroxy derivatives while the more powerful inhibitors were the benzoquinoneacetanilide and its 5-bromo equivalent. © 2002 Elsevier Science Ltd. All rights reserved.

#### Introduction

A large number of epoxyquinone antibiotics exist in nature as agents against murine leukaemia and associated diseases. Antitumour antibiotic (1), isolated and purified from cultures of *Streptomyces LL-C10037*<sup>1</sup> was found to have activity against murine leukaemia P388 with a 29% increase in the life span of treated mice. The biosynthetic origin of these molecules, originate from anthranilic acid with dihydroxyacetamide (2) and the corresponding epoxyquinone (3) the major precursors (Scheme 1).<sup>2–4</sup>

Two enzymes [dihydroxyacetamido epoxidase (DHAE) and dihydroxyacetamido dehydrogenase (DHADH)] were shown to play a major significant role during this biosynthetic pathway.<sup>5</sup>

Despite the strategic location of these enzymes relative to the epoxyquinone antibiotics, it is surprising that very little is known about them as far as their kinetic characteristics and inhibition. A useful tool for enzymologists has been to find and use specific inhibitors so as to follow the effects of these inhibitors on the enzyme action and probe structure–function relationships. Inhibitors can be divided into two groups: reversible and irreversible. Reversible inhibition is characterised by an equilibrium formed between the enzyme and the inhibitor that depends on the concentration of the inhibitor, value of the inhibitor constant ( $K_i$ ), and is time independent. The reversible inhibitor binds the enzyme non-covalently to modulate the enzymatic activity and this enzymic activity can be restored when the inhibitor is removed by a suitable procedure such as gel filtration or dialysis. On the contrary, irreversible inhibition is characterised by a progressive decrease of enzymatic activity with time and becomes complete when all the enzyme is combined with the inhibitor. After irreversible inhibition or dialysis.

The rational design of inhibitors based on the inhibition of the dihydroxyacetanilide epoxidase and dehydrogenase enzymes has attracted the attention of



Scheme 1. Biosynthesis of the semiquinone antitumour antibiotic (1) and epoxyquinone (3) from dihydroxyacetanilide (2) with dihydroxyacetanilide epoxidase (DHAE) and dihydroxyacetanilide dehydrogenase (DHADH).

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medicinal chemists in the search for new antitumour and antiviral agents. Furthermore these studies can also lead to views on their enzymatic mechanisms. As a continuation of research in these laboratories on enzymes associated with cell proliferation,<sup>6–8</sup> we decided to investigate the isolation, purification, kinetic properties and inhibition of both the dihydroxyacetanilide epoxidase and the dehydrogenase enzymes from *Streptomyces LL-C10037* species.

# Results

# Enzyme isolation and purification

Dihydroxyacetanilide epoxidase. The steps for the purification of this epoxidase are summarised in Table 1. The specific activity of the crude homogenate was 391.4 nM  $g^{-1}$  gradually increasing by ammonium sulphate precipitation and Sephadex ion exchange chromatography to give a specific activity of 895.7 nM  $g^{-1}$ , a 22% yield and a 2.3-fold purification. The results of the chromatographic separations on DEAE-Sephadex are shown in Figure 1. Elution of the active enzyme in a sharp peak is readily accomplished by a gradient application in ionic strength to 1 M NaC1. Removal of extraneous proteins is achieved at low ionic strength. Other ionexchange resins were found to be totally inadequate in purifying the enzyme giving a broad elution peak over a wide range of ionic strength indicating a poor resolution. The pooled purified samples from the DEAE-Sephadex column were subjected to chromatography and molecular weight determination on Sephacryl S-400 equilibrated in phosphate buffer (50 mM, pH 7) containing glycerol (20%) and EDTA (0.2 mM) (Fig. 1, inset). Two peaks (one major) eluted. The first at Ve of 70 mls representing a  $M_r = 12,600$  and a second smaller peak at 85 mls corresponding to a  $M_r = 43,000$ . The column was standardised with proteins of known molecular weights–Blue Dextran ( $M_r = 2 \times 10^6$ , lactate dehydrogenase  $(M_r = 140,000)$ , Dnase  $(M_r = 63,000)$  and carboxypeptidase A ( $M_r = 34,300$ ). The final specific activity of the sample eluted from the Sephacryl S-400 was 3460.3 nM  $g^{-1}$  with an overall yield of 12% and a 8.8-fold purification (Table 1).

**Dihydroxyacetanilide dehydrogenase.** The steps for the purification of this dehydrogenase are summarised in Table 2. The specific activity of the crude homogenate was 438.3 nM  $g^{-1}$  gradually increasing by ammonium sulphate precipitation and DEAE–Sephadex ion exchange chromatography to give a specific activity of



**Figure 1.** Ion exchange chromatography of purified dihydroxyacetanilide epoxidase on DEAE–Sephadex. The absorbance is monitored at 280 nm and the NaCl gradient is indicated (-----). The inset represents chromatography of the epoxidase on Sephacryl S-400. The absorbance is monitored at 280 nm. Standard proteins eluted through the same column under the same conditions are superimposed: (1) Blue dextran ( $M_r = 2 \times 10^6$ ). (2) lactate dehydrogenase ( $M_r = 140,000$ ); (3) deoxyribonuclease ( $M_r = 63,000$ ); (4) carboxypeptidase A ( $M_r = 34,300$ ).

Table 1. Purification of dihydroxyacetanilide epoxidase by DEAE-Sephadex and Sephacryl S-400

Purification step	Protein			Enzyme			
	Vol. (mL)	Concn (mg mL <sup>-1</sup> )	Total (mg)	Activity (nM min <sup>-1</sup> )	Sp. Act (nM $g^{-1}$ )	Fold purific	Yield (%)
Crude	500	50	25,000	9804	391.4	1	100
$(NH_4)SO_4$	200	39.4	7880	6118.7	776.5	2	62.4
DEAE-Sephadex	160	15.2	2432	2176.5	895.7	2.3	22.2
Sephacryl-S400	38	8.9	338.2	1176.5	3460.3	8.8	12

Table 2. Purification of dihydroxyacetanilide dehydrogenase by DEAE-Sephadex and Sephacryl S-400

Purification step	Protein			Enzyme			
	Vol. (mL)	Concn (mg mL-1)	Total (mg)	Activity (nM min <sup>-1</sup> )	Sp. Act (nM g <sup>-1</sup> )	Fold purific	Yield %
Crude	325	30	9750	4273	438.3	1	100
(NH <sub>4</sub> )SO <sub>4</sub>	90	28	2520	1329	527.4	1.2	31.1
DEAE-Sephadex	60	23.5	1410	1176	834	1.9	27.5
Sephacryl-S400	16	8.3	130	515.4	4026.6	9.2	11.6

834 nM g<sup>-1</sup>, 27.5% overall yield and a 1.9-fold purification. The results of the chromatographic separations on DEAE–Sephadex are shown in Figure 2. Again the removal of extraneous proteins was affected by low ionic strength and pure active dehydrogenase enzyme eluted in a sharp peak by means of a gradient application of 1 M NaC1. Molecular weight determination was determined on Sephacryl S-400 equilibrated in phosphate buffer (50 mM, pH 7) (Fig. 2, inset). A single peak eluted at Ve = 61 mls accounting for a  $M_r$  = 134,000. The final specific activity of the dehydrogenase enzyme eluted from the S-400 column was 4026.6 nM g<sup>-1</sup> with an overall yield of 11.6% and a fold purification of 9.2 (Table 2).

# Irreversible inhibition studies

Dihydroxyacetanilide epoxidase. Incubation of the epoxidase with inhibitor resulted in progressive loss of

0.15

**Figure 2.** Ion exchange chromatography of purified dihydroxyacetanilide dehydrogenase on DEAE–Sephadex. The absorbance is monitored at 280 nm and the arrow indicates elution with 1 M NaCl. The inset represents chromatography of the dehydrogenase on Sephacryl S-400. The absorbance is monitored at 280 nm. Standard proteins eluted through the same column under the same conditions are superimposed: (1) Blue dextran ( $M_r = 2 \times 10^6$ ); (2) lactate dehydrogenase ( $M_r = 140,000$ ). enzyme activity. As shown (Fig. 3) the inactivation of the enzyme by 4-acetamidophenol showed a 31% inhibition after 30 min while 2-acetamido-phenol had little or no effect. The 2,3-dihydroxyacetanilide and 3,4dihydroxyacetanilide produced inhibition at 37 and 41%, respectively, while more powerful inhibitors were 1,4-benzoquinoneacetanilide (57%) and its bromoanalogue (72%).

**Dihydroxyacetanilide dehydrogenase.** Incubation of the dehydrogenase with inhibitor also resulted in progressive loss of enzyme activity (results not shown). The inactivation of the enzyme by 4-acetamidophenol showed a 25% inhibition after 30 min, 2-acetamidophenol (10%), 2,3-dihydroxyacetanilide (31%), and 3,4-dihydroxyacetanilide (41%) while the quinone inhibitors were 1,4-benzoquinone acetanilide (50%) and its bromoanalogue (65%).

# **Reversible inhibition studies**

**Dihydroxyacetanilide epoxidase.** The evidence of a reversible inhibitory mechanism was realised after characteristic Lineweaver–Burk plots (Fig. 4) were produced, indicating that all of the inhibitors studied were competitive with respect to the natural substrate 2,5-dihydroxyacetanilide. A replot of the slopes of this plot versus inhibitor concentration is linear. (Fig. 4, inset) and  $K_i$ , the inhibitor constant, can be calculated by extrapolation of the graph slope versus inhibitor concentration (Fig. 4, inset). All the relevant kinetic



**Figure 3.** Inactivation of dihydroxyacetanilide epoxidase by 2-acetamidophenol ( $\bigcirc$ ); 4-acetamidophenol ( $\bigcirc$ ); 2,3-dihydroxacetanilide ( $\blacktriangle$ ); 3,4-dihydroxyacetanilide ( $\bigtriangleup$ ); 1,4-benzoquinoneacetanilide ( $\blacksquare$ ); 5-bromo-1,4-benzoquinone ( $\Box$ ).

Table 3. Inhibitor constants  $(K_i)$  of various inhibitors with respect to dihydroxyacetanilide epoxidase and dihydroxyacetanilide dehydrogenas

Dihydroxyacetanilide epor	xidase	Dihydroxyacetanilide dehydrogenase		
Inhibitor	$K_i$ (nM)	Inhibitor	K <sub>i</sub> (nM)	
2-Acetamidophenol	nd	2-Acetamidophenol	nd	
4-Acetamidophenol	20	4-Acetamidophenol	24	
2,3-Dihydroxyacetanilide	27	2.3-Dihydroxyacetanilide	32	
3,4-Dihydroxyacetanilide	18	3.4-Dihydroxyacetanilide	26	
1,4-Benzoquinoneacetanilide	12	1.4-Benzoguinoneacetanilide	19	
5-Bromo-1,4-benzoquinone 4		5-Bromo-1,4-benzoquinone	9	





**Figure 4.** Competitive inhibition of dihydroxyacetanilide epoxidase by 5-bromo-1,4-benzoquinone: 0 nM ( $\odot$ ), 3.3 nM ( $\blacksquare$ ), 6.7 nM ( $\blacktriangle$ ), and 10 nM ( $\bigcirc$ ). The secondary plot represents the slopes of the primary plot versus concentration of 5-bromo-1,4-benzoquinone.

parameters for the inhibitors are represented (Table 3). With variable substrate concentrations values for  $K_{\rm m}$  and  $V_{\rm max}$  for the epoxidase were determined at 110 nM and 0.8 nM min<sup>-1</sup>, respectively. The single substituted hydroxy-acetanilide showed relatively weak inhibition when compared to the dihydroxy-acetanilides. The bromo-1,4-quinone was the most potent inhibitor ( $K_{\rm i}$ =4 nM).

**Dihydroxyacetanilide dehydrogenase.** A reversible competitive inhibition was also seen with the dehydrogenase enzyme (results not shown) and the relevant kinetic parameters for these inhibitors are represented (Table 3).

#### Discussion

Current techniques for the preparative isolation of proteins use the chromatrographic recognition of a variety of physical parameters. The ideal separation method employs an easily identifiable physical parameter unique to the protein of interest.<sup>10</sup> Gel filtration, salt fractionation, affinity chromatography with biospecific or group specific adsorbent, ion exchange chromatography and preparative electrophoresis have proven to be versatile separation techniques. Seldom does any of these procedures provide sufficient resolution or characterisation to obtain a homogeneous protein from a complex biological material. For this reason purification procedures usually combine some or all of these techniques to increase purity in a series of chromatographic or electrophoretic steps. Each of these steps, however, suffer from several disadvantages. The resolving power of gel filtration is limited by chromatographic factors and cannot yield fractions of discrete molecular size. Ionexchange chromatography, a technique based on the nature and degree of available ionisable groups on the protein molecule<sup>11</sup> is not sufficiently characteristic and many proteins elute under similar conditions. The interaction between proteins and biospecific affinity adsorbents may be too strong, causing problems in desorption or too weak making an inefficient adsorbent. Group specific affinity adsorbents have eliminated this problem<sup>12</sup> but in doing so they have decreased the highly specific recognition and purification parameters of biological specificity and thus the degree of purification. In some protocols that follow ion-exchange chromatography and/or sucrose gradient centrifugation there have been complaints of rather low yields and decreased resolution with modification of the properties of the purified protein. Purification and fractionation of proteins is facilitated by their different isoelectric points and the possibility of producing a pH gradient using an ion-exhange column has provided techniques for separation with resolution and recovery comparable to those of electrophoretic systems. With ampholyte displacement column chromatography a pH gradient is produced either by mixing buffers of different pH or by employing the buffering action of an ion-exchanger and a running buffer initially adjusted to one pH through a column adjusted to another pH. The advantages of this system are that ordinary chromatographic equipment may be used; there is no restriction on the size of the column, mixtures of ordinary buffers may be used and proteins are not subjected to more extreme pH values than their pI.<sup>13</sup> As part of a study in these laboratories we required large amounts of purified protein and in view of the foregoing discussion an improved method for purification was sought. Earlier attempts to purify involved 'classical' biochemical methods such as salt formation, gel filtration and polymer partitioning. None of these techniques nor any combination of them would achieve substantial purification of the enzymes with a reasonable yield. Our attempts to purify the epoxidase enzyme by DEAE-Cellulose afforded the enzyme in a yield of 6.2%, a fold purification of 6.8 and a specific activity of 2383.3 nM  $g^{-1}$ . Furthermore, attempted purification on hydroxyapatite affinity chromatographic resin lead to poor yields (4.1%), a fold purification of 3 and a specific activity of 1152.7 nM  $g^{-1}$ .

By using a combination of DEAE–Sephadex followed by Sephacryl S-400 we managed to purify the epoxidase enzyme with a specific activity of 3460 nM g<sup>-1</sup>, 8.8-fold purification and a 12% overall yield. In comparison the dihydroxyacetanilide-dehydrogenase was purified to specific activity of 4026.6 nM g<sup>-1</sup>, 9.2-fold purification and 11.6% yield.

This article has shown that all the inhibitors studied inhibit the epoxidase and the dehydrogenase reversibly and competitively with respect to the respective substrates. A kinetic analysis of the inhibition of the enzyme by the bromobenzoquinone acetanilide is presented (Fig. 4) using standard graphical techniques of Lineweaver–Burk.<sup>14</sup> The lines are drawn by computer calculation for linear competitive inhibition [eq (2)] and least square analysis:<sup>15</sup>

$$v^{-1} = V^{-1} \max \left( K_{\rm m} \left( 1 + K_{\rm i}^{-1} . {\rm I} \right) \right) {\rm S}^{-1} + V_{\rm max}^{-1}$$
 (2)

The data demonstrates reversible competitive inhibition for the system, implying that the inhibitors bind at the substrate binding site of the enzymes. The potency of a particular inhibitor in the interaction with the enzymes is determined by the dissociation constant for the enzyme–inhibitor complex. The fit into the active site of the enzyme, reflected by the values of  $K_i$  is largely determined by the size, structure and configuration of the inhibiting molecule. The capability of the inhibitor to bind non-covalently at, or close to, the active site could also influence these values.

Detection of spectroscopic changes in a protein upon binding with ligands or inhibitors is one of the simplest methods to study induced conformational changes. In the present investigation the changes in optical properties of the enzyme upon binding with the inhibitors may be due to the presence of non polar regions around the substrate binding site of the enzyme. The inhibitors themselves cannot, however, increase the basicity of the micro-environment of the binding locus. It is assumed that the binding of inhibitors in the active site leads to exposure of new groups and a transmission of conformational changes from one subunit to another. The dihydroxyacetanilide, quinone and bromoquinone bind strongly to a hydrophobic domain on the enzyme surface increasing the rigidity of the molecule and, at the same time, influencing the solvating properties of the catalytic site and hence accelerating the binding process.

The strong inhibitory power of the benzoquinones especially the bromobenzoquinone are reflected in the electrophilic centres and Michael acceptors, creating hydrophobic binding between the inhibitor and the lipophilic region of the enzymes. The possibility that the epoxidase and dehydrogenase enzymes were metalloenzymes was ruled out from data obtained with the potential chelating 2,3- and 3,4-dihydroxyacetanilide inhibitors giving only limited inhibitions.

#### Conclusion

In summary, the inhibition of both the dihydroxyacetamido epoxidase and the dihydroxyacetamido dehydrogenase, which have been isolated and purified from *Streptomyces* species, has been investigated and evaluated with respect to a series of monohydroxy and dihydroxyacetanilides, and acetamidoquinones and its bromo analogue. All inhibitors were reversibly competitive and the most powerful was the halogenated acetamidoquinone reflected in a  $K_i$  value of 4 nM.

#### Experimental

# Dihydroxyacetamido epoxidase

**Isolation and purification.** Cells from S. LL-C10037<sup>9</sup> were harvested at 120 h by centrifugation (13,800g, 30 min, 4°C). They were washed with KC1 (1 M) followed by NaC1 (0.8 M) to remove surface proteases, suspended in potassium phosphate buffer (pH 7, 0.01 M) and sonicated (2 min) with cooling. Centrifugation (26,700g, 20 min) yielded a cell-free extract (CFE) (10% v/v from the original broth).

The CFE was brought to 0.01% with protamine sulphate (to remove nucleic acids) by a dropwise addition of a 2% aqueous solution. The resulting solution was stirred (30 min) and centrifuged (38,400g, 4°C, 20 min). The supernatant was brought to 52% saturation by the addition of solid ammonium sulphate, stirred (60 min), the precipitate brought to 72% saturation with a further addition of ammonium sulphate and after stirring for a further 60 min the active pellet was collected by centrifugation (38,400g, 4°C, 20 min). These active fractions were applied to a DEAE-Sephadex column equilibrated with the same phosphate-glycerol buffer. After the  $A_{280}$  had returned to baseline the column was eluted (1 mL/min) with a gradient containing the buffer and 0-1 M NaC1 in this buffer. Active fractions were collected, dialysed, freeze dried and the residue dissolved in phosphate buffer (50 mM, pH 7, 11.5 mL) containing 20% glycerol and 0.2 mM EDTA to remove metal ions and reduce disulphide formation. This was then applied to a Sephacryl S-400 column that had been equilibrated in the same buffer and fractions collected (7.0 mL) at a flow rate of 13 mL  $h^{-1}$ . Active fractions were pooled dialysed against distilled water, freeze dried and the residue redissolved in buffer.

# Assay

This enzyme was assayed by following the consumption of the dihydroxyacetamide (2) and the production of the epoxyquinone (3) simultaneously by HPLC on a Beckman Gold and a C<sub>18</sub> reverse-phase column with H<sub>2</sub>O/ MeCN (85/15) as the mobile phase. The assay (400  $\mu$ L) contained aliquots of substrate, phosphate buffer and enzyme and was incubated at 30 °C for 5 min. The enzymatic reaction was terminated by the addition of trifluoroacetic acid/acetonitrile/water/(1:1:8, 100  $\mu$ L) (TFA/MeCN/H<sub>2</sub>O).

# Kinetic study

The assay mixture consisted of phosphate buffer (1 M, pH 6.5, 50 µL), MeOH (40 µL), 2,5-dihydroxyacetanilide (DHA) (60  $\mu$ M, 25  $\mu$ L), enzyme (40  $\mu$ L) and distilled water (245 µL). The enzyme reaction was quenched after 5 min incubation with trifluoroacetic acid/MeCN/H<sub>2</sub>O (1:1:8) (100  $\mu$ L) and the activity of the enzyme assayed, by the production of the epoxyquinone (EQ) (3) at 225 nm using HPLC with a  $C_{18}$  reversephase column and H<sub>2</sub>O/MeCN (85:15) mobile phase. Enzyme activity was determined from a standard curve prepared from 2.0, 5.0, 10.0 and 20.0  $\mu L$  of a 1 mM solution of epoxyquinone (3) on an HPLC C<sub>18</sub> reversephase column with  $H_2O/MeCN$  (85:15) mobile phase. The slope of the curve represented an integration area of 1 nmol  $\mu L^{-1}$ . The rate of the enzymatic reaction (v)  $(nM min^{-1})$  was determined from equation 1.

$$v = (\mathrm{E}\mathrm{Q}_{\mathrm{t}}^* V_{\mathrm{t}}) / (\mathrm{E}\mathrm{Q}_{\mathrm{s}}^* V_{\mathrm{i}}^* t) \tag{1}$$

where  $EQ_t$  is the integration area of the epoxyquinone (3) produced,  $EQ_s$  the integration area of the epoxy-



Scheme 2. Synthesis of 2,3-dihydroxyacetanilide (4).

quinone standard (nmol  $\mu L^{-1}$ ),  $V_t$  is the total volume of the assay (500  $\mu L$ ),  $V_i$  is the injection volume (10  $\mu L$ ) and *t* is the assay time (5 min).

In order to obtain values for  $K_{\rm m}$  and  $V_{\rm max}$ , a series of kinetic experiments were performed with the variation in the concentration of the substrate (DHA).

# **Inhibition studies**

Assay mixtures were set up containing phosphate buffer (pH 6.5, 100 mM, 50  $\mu$ L), MeOH (40  $\mu$ L), enzyme (50  $\mu$ L), 2,5-dihydroxyacetanilide (2,4 mM L<sup>-1</sup> in phosphate buffer, 0–50  $\mu$ M), inhibitor (10 mM L<sup>-1</sup> in phosphate buffer, 0–10 nM) and water to a total volume of 400  $\mu$ L. After 5 min incubation terminating solution (TFA/MeCN/H<sub>2</sub>O, 1:1:8, 100  $\mu$ L) was added. The rate of the enzymatic reaction  $\nu$  (nmol min<sup>-1</sup>) was determined by HPLC using a C<sub>18</sub> reverse-phase column with a mobile phase (MeCN/H<sub>2</sub>O, 85/15) and using eq (1) above.

#### Dihydroxyacetamido dehydrogenase

**Isolation and purification.** The protamine sulphate of cell free extract (CFE) (see Dihydroxyacetanilide epoxidase above) was brought to 40% saturation by the addition of solid ammonium sulphate, the suspension stirred (60 min) and the precipitate removed by centrifugation (13,800g, 10 min). The resultant supernatant was brought to 60% saturation ammonium sulphate, stirred (60 min) and the active pellet collected by centrifugation (38,400g, 10 min). All subsequent purification steps involving column chromatography on DEAE– Sephadex and Sephacryl S-400 were identical as for the epoxidase enzyme.

# Assay

The dehydrogenase was assayed by the disappearance of the epoxyquinone (3) using HPLC with a  $C_{18}$  reversephase column and H<sub>2</sub>O/MeCN (85:15) mobile phase, monitoring the effluent at 225 nm. The assay solution (400 µL) contained NADPH, substrate and enzyme, incubated at 30 °C, 5 min, then the enzymatic activity quenched with 100 µL terminating solution (MeCN/ H<sub>2</sub>O/TFA). Comparative assays were performed by monitoring the disappearance of the absorbance of NADPH at 340 nm.

#### Kinetic studies

The assay mixture contained NADPH (2.0 mM, 100  $\mu$ L), epoxyquinone (16.26 mM, 100  $\mu$ L), Tris buffer (100 mM, pH 8.5, 1.7 mL), enzyme (100  $\mu$ L, 132  $\mu$ g/mL). The enzyme activity was monitored by the decrease in A<sub>340</sub>.

# **Inhibition studies**

The assay mixture contained NADPH (2.0 mM, 100  $\mu$ L), epoxyquinone (0–20 mM, 100  $\mu$ L), Tris buffer (200 mM, pH 8.5, 1.7 mL), enzyme (100  $\mu$ L, 132  $\mu$ g mL<sup>-1</sup>), and inhibitor (0–10 nM, 10  $\mu$ L). The enzyme activity was monitored by the decrease in A<sub>340</sub>.

#### Synthesis of inhibitors

2,3-Dihydroxyacetanilide (4) (Scheme 2). 2,3-Dimethoxybenzoic acid (Aldrich) (1.82 g, 10 mM) was dissolved in dry benzene (10.0 mL). To this was added thionyl chloride (1.45 g, 1.0 mL 12 mM) and the whole was heated under reflux (2 h). The solvents and lower boiling liquids were removed under reduced pressure and the residue dissolved in dry acetone (10 mL). This solution was then added to a cold (5°C) stirred solution of sodium azide (780 mg, 12 mM) and water (10 mL) maintaining the temperature below 15 °C. Stirring was continued (60 min) and then the whole was extracted into benzene. The benzene solution was heated (60 °C, 2 h) (gas evolution), evaporated and then treated with concentrated hydrochloric acid (15 mL) and heated at 80°C, 18 h. The solution was cooled, neutralised (NaHCO<sub>3</sub>) and thoroughly extracted into ethylacetate, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated to afford crude 2,3-dimethoxy-aniline (1.303 g, 85%). All attempts to purify this by distillation under reduced pressure lead to considerable loss in product. The crude dimethoxyaniline was dissolved in dichloromethane (50.0 mL) containing triethylamine (1.4 mL), cooled to 5 °C then treated dropwise with a 33% (v/v) dichloromethane solution of acetylchloride (4 mL). After stirring (60 min) the reaction mixture was quenched with water and extracted into ethyl acetate. The solution was dried  $(Na_2SO_4)$  and the solvent removed to afford  $^{13}C$ 2,3-dimethoxy acetanilide. NMR  $(CDC1_3,$ 100 MH<sub>z</sub>) δ 112.81–156.98, aromatic C; 170.7 amide C; 56–57.2 (2×OCH<sub>3</sub>); 24.24 (C–CH<sub>3</sub>).

This dimethoxyanilide (380 mg, 2 mM) was dissolved in dry dichloromethane and cooled to -78 °C (dry ice/ acetone) under a stream of nitrogen. Boron tribromide (5.0 mL, 1 M solution in dichloromethane, 5 mM) was added slowly. After the addition the pale-yellow/green solution was allowed to reach room temperature. The reaction mixture was quenched with brine (10%), extracted into methylene chloride, dried (Na<sub>2</sub>SO<sub>4</sub>) and the solvents evaporated to produce a light brown oil (300 mg, 90%) identified as 2,3-dihydroxyacetanilide. <sup>13</sup>C NMR (CDC1<sub>3</sub>, 100 MH<sub>z</sub>)  $\delta$  113.26–146.84 (aromatic C); 170.7 (amide C) 24.24 (C–*CH*<sub>3</sub>).

**3,4-Dihydroxyacetanilide (5)** (Scheme 3). 3,4-Dimethoxyaniline (Aldrich) (1.47 g, 10 mM) was dissolved in dichloromethane (5.0 mL) containing triethylamine (1.4



Scheme 3. Synthesis of 3,4-dihydroxyacetanilide (5).



Scheme 4. Synthesis of 2-acetamido-1,4-benzoquinone (7) and 5-bromo-2-acetamido-1,4-benzoquinone (6).

mL). The reaction mixture was cooled to 5 °C, treated dropwise with a 33% (v/v) dichloromethane solution of acetyl chloride (4 mL). After stirring (60 min) the reaction mixture was quenched with water, extracted with ethyl acetate, dried (Na<sub>2</sub>SO<sub>4</sub>) and the solvents removed under reduced pressure to afford crude 3,4-dimethoxy-acetanilide (1.42 g, 82%). <sup>13</sup>C NMR (CDC1<sub>3</sub>, 100 MH<sub>z</sub>)  $\delta$  101.91–151.18 (aromatic C), 170.45 (amide C); 55.85, 55.95 (2×OCH<sub>3</sub>, 23.95 (C–*CH*<sub>3</sub>).

The crude anilide (380 mg, 2 mM) was dissolved in dry dichloromethane and cooled to  $-78 \,^{\circ}\text{C}$  (dry ice/acetone) under a stream of nitrogen. Boron tribromide (5.0 mL, 1 M solution in dichloromethane, 5 mM) was added slowly. After the addition the pale-yellow/green solution was allowed to reach room temperature. The reaction mixture was quenched with brine (10%) and extracted into dichloromethane. This was dried (Na<sub>2</sub>SO<sub>4</sub>) and the solvents evaporated to produce an oil (275 mg, 85%). <sup>13</sup>C NMR (CDC1<sub>3</sub>, 100 MH<sub>z</sub>)  $\delta$  111.46–146.74 (aromatic C); 170.45 (amide c); 23.95 (C–*CH*<sub>3</sub>).

**2-Acetamido-5-bromo-1,4-benzoquinone (6)** (Scheme 4). 2,5-Dimethoxy acetanilide (195 mg, 1 mM) was dissolved in dichloromethane (10.0 mL) and glacial acetic acid (1.0 mL) added. The whole was treated with slight excess bromine (176 mg), 55  $\mu$ L, 1.1 mM) and the paleorange solution stirred for a further 30 min. The organic layer was washed with sodium dithionite then sodium bicarbonate dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to yield the final product (120 mg, 66%). <sup>13</sup>C NMR (CDC1<sub>3</sub>,

 $100 \text{ MH}_{z}$ )  $\delta$  109.07–151.52 (aromatic C); 108.44 (C–Br) 56.4 (2×OCH<sub>3</sub>); 170.7 (amide c); 24.24 (C–CH<sub>3</sub>).

This bromoacetanilide (68.5 mg, 250  $\mu$ M) was dissolved in acetonitrile (10.0 mL) then treated with excess ceric ammonium nitrate (CAN) (1.1 g) in water (10.0 mL). After stirring (30 min) a yellow crystalline solid precipitated. This was extracted into ethyl acetate, washed with sodium chloride, dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to afford a yellow crystalline material (52 mg, 85%), mp 138 °C. <sup>13</sup>C NMR (CDC1<sub>3</sub>, 100 MH<sub>z</sub>)  $\delta$  181.43 (Br–C– *CO*); 177.55 (Br–C=C–*CO*); 171.76 (amide C); 136.32 (C=*C*–NH); 134.12 (Br–C=*C*–CO), 132.72 (Br–*C*=); 24.1 (C–*C*H<sub>3</sub>).

**2-Acetamido-1,4-benzoquinone** (7) (Scheme 4). 2,5 Dimethoxyacetanilide (250  $\mu$ M) was dissolved in acetonitrile (10.0 mL) then treated with excess ceric ammonium nitrate (CAN) (1.1 g) in water (10.0 mL). After stirring (30 min), the crystalline solid was collected, extracted into ethyl acetate, dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to afford a solid product (194 mg, 81%) mp 126 °C. <sup>13</sup>C NMR (CDC1<sub>3</sub>, 100 MH<sub>z</sub>)  $\delta$  136.32–132.72 (aromatic C); 181.43–177.55 (aromatic CO); 171.76 (amide C); 24.1 (C–CH<sub>3</sub>).

#### **References and Notes**

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