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Differential effects of bromination on substrates and inhibitors of kynureninase from *Pseudomonas fluorescens*

Christian Heiss,[a] Jay Anderson[a] and Robert S. Phillips*[a,b,c]

[a] *Department of Chemistry, University of Georgia, Athens, Georgia 30602-2556, USA*
[b] *Department of Biochemistry and Molecular Biology, Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602-2556, USA*
[c] *Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602-2556, USA*

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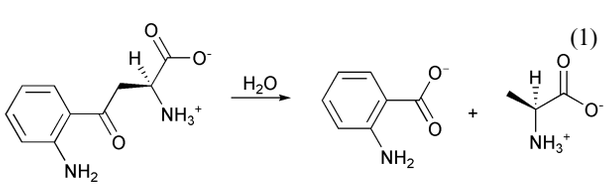
A series of brominated compounds has been synthesized and evaluated as substrates and inhibitors of kynureninase from *Pseudomonas fluorescens*. Both 3-bromo-L-kynurenine and 5-bromo-L-kynurenine were found to be substrates with similar k_{cat} values to L-kynurenine, but the K_m value for 3-bromo-L-kynurenine is very high (*ca.* 2 mM) compared to that for 5-bromo-L-kynurenine (11 μM) and L-kynurenine (25 μM). Both isomers of bromokynurenine react with kynureninase within the dead time of the stopped-flow instrument (*ca.* 1 ms) to form quinonoid intermediates with a λ_{max} of 494 nm that decay with rate constants of 300–600 s^{-1}, similar to L-kynurenine. The two diastereomers of 5-bromodihydro-L-kynurenine were also prepared, and are more potent inhibitors than dihydro-L-kynurenines. (4*R*)-5-Bromodihydro-L-kynurenine is one of the most potent inhibitors of *P. fluorescens* kynureninase found to date (K_i = 55 nM) and also acts as a slow substrate; the (4*S*)-epimer, on the other hand, shows no measurable substrate activity, but it is a potent competitive inhibitor with a K_i value of 170 nM. In contrast, brominated analogs of (*S*)-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide, (*S*)-(2-amino-4-bromophenyl)-L-cysteine *S*,*S*-dioxide and (*S*)-(2-amino-5-bromophenyl)-L-cysteine *S*,*S*-dioxide are competitive inhibitors of kynureninase, with K_i values of about 300 and 400 nm, respectively, about ten-fold higher than the value of 27 nM obtained for the parent compound. These results suggest that the binding modes of substrates and the various classes of inhibitors in the active site of kynureninase are different.

Introduction

Kynureninase [EC 3.7.1.1] is a pyridoxal 5′-phosphate (PLP) dependent enzyme that catalyzes a key step in the catabolism of tryptophan in *Pseudomonas fluorescens*, and some other bacteria,[1] the hydrolytic cleavage of L-kynurenine to give anthranilic acid and L-alanine [eqn. (1)]. As part of the biosynthetic pathway from L-tryptophan to NAD^+ in eucaryotes, kynureninase catalyzes the cleavage of 3-hydroxykynurenine to produce 3-hydroxyanthranilate. 3-Hydroxyanthranilate dioxygenase converts 3-hydroxyanthranilate to quinolinic acid, which is an *N*-methyl-D-aspartate (NMDA) receptor agonist and, hence, a postulated neurotoxin. Elevated levels of quinolinic acid have been implicated in the etiology of a number of neurological disorders such as Huntington's disease, AIDS-related dementia, stroke and other inflammatory brain diseases.[2–5] Effective and selective inhibition of human kynureninase thus represents a possible treatment of these diseases.



(1)

In a previous study, we prepared the two diastereomers of dihydro-L-kynurenine, and we found that the (4*S*)-diastereomer is a potent competitive inhibitor of bacterial kynureninase with a K_i value of 300 nM.[6] While the (4*R*)-diastereomer also displayed inhibitory activity (K_i = 1.5 μM), it is a slow substrate for kynureninase-catalyzed *retro*-aldol reaction to give *o*-aminobenzaldehyde and L-alanine. These results suggested that the kynureninase reaction proceeds through a *gem*-diolate intermediate, as had originally been proposed by Braunstein and Shemyakin.[7] In agreement with this hypothesis, we then found that (*S*)-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide (SAPCO), the structure of which resembles that of the postulated *gem*-diolate intermediate, is a very potent inhibitor with a K_i value of 70 nM.[8] In the present study, we have prepared brominated analogs of L-kynurenine, dihydro-L-kynurenine, and (*S*)-(2-aminophenyl)-L-cysteine-*S*,*S*-dioxide, and we compared the effects of bromination on substrate and inhibitor activity. Unexpectedly, the results of this study suggest that the substrates and different classes of inhibitors bind to the active site of kynureninase in different modes. These results are important for the design of potent and selective inhibitors of bacterial and human kynureninase.

Experimental

General

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AC 250 or AC 300, respectively. Optical rotations were measured with an Autopol IV polarimeter from Rudolph Research; $[a]_D^{20}$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Enzyme assays and kinetic experiments were performed with a Varian Cary 1E UV/visible spectrophotometer. HPLC measurements were carried out on an instrument with two Rainin Rabbit HP pumps and a LDC Milton Roy Spectromonitor 3000 variable wavelength detector using Gilson Unipoint software.

Enzyme

Kynureninase was purified from *E. coli* cells containing plasmid pTZ18U with the kynureninase gene of *P. fluorescens* as previously described.[9] The protein concentration of crude extracts was measured by the method of Bradford,[10] and the concentration of purified kynureninase was measured by absorbance at 280 nm ($A_{280}^{1\%}$ = 14).[11]

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Kinetic measurements

Kynureninase activity was measured by following the decrease in absorbance at 360 nm due to conversion of L-kynurenine to anthranilate ($\Delta\varepsilon = 4500\ M^{-1}\ cm^{-1}$). The competitive inhibition was measured by variation of [L-kynurenine] at several fixed concentrations of inhibitor. K_m, V_{max}, and K_i values were determined by fitting of initial rate data to eqns. (2) and (3)

$$v = V_{max}[S]/(K_m + [S]) \quad (2)$$

$$v = V_{max}[S]/\{K_m(1 + [I]/K_i) + [S]\} \quad (3)$$

using the compiled FORTRAN programs HYPER and COMP of Cleland.[12] Due to the very low K_m values for (4*R*)-5-bromo-dihydro-L-kynurenine, the K_m and V_{max} values could not be determined in the conventional manner, and were determined by running the reaction at a fixed concentration (35 μM) of substrate and fitting the time course of the increase in absorbance at 360 nm due to the formation of 2-amino-5-bromobenzaldehyde ($\Delta\varepsilon = 3900\ M^{-1}\ cm^{-1}$) to the integrated Michaelis–Menten relationship in eqn. (4).[13]

$$K_m \ln[S]/[S]_o + [S] - [S]_o = -V_{max}t \quad (4)$$

Stopped-flow kinetics

Rapid-scanning stopped flow measurements were performed on an OLIS RSM-1000 spectrophotometer, as described previously.[14] The reactions contained 1 mg mL^{-1} (22 μM) kynureninase in 0.05 M potassium phosphate, at pH 8.0 and 25 °C. Spectra were collected at a rate of 1000 s^{-1} over the wavelength range 320–570 nm. The spectra were fitted using the global analysis program, GlobalWorks, provided by OLIS[15] to obtain the singular value decomposition (SVD) spectra of intermediates and the rate constants for the reaction.

Synthesis of brominated substrates and inhibitors

(2*S*)-Methyl 2-acetamido-4-(2′-acetamidophenyl)-4-oxobutanoate (N^{α},*N*-diacetyl-L-kynurenine methyl ester) (1a)

A stream of ozone was passed through a solution of N^{α}-acetyl-L-tryptophan methyl ester (2.28 g, 8.76 mmol) in MeOH (100 mL) at −78 °C. After the reaction was completed (10% KI-trap), excess oxidizing species were destroyed by treatment with sat. $NaHSO_3$ solution (100 mL). The mixture was extracted with CH_2Cl_2, dried ($MgSO_4$), and evaporated. The residue was taken up in MeOH (80 ml) and treated with trifluoroacetic acid (5 mL). After stirring at room temperature overnight, the mixture was evaporated and the resulting oil taken up in $CHCl_3$ (100 mL). Acetic anhydride (10 mL) was added, the solution was stirred for 1 h, washed with saturated $NaHCO_3$ (10 × 30 mL), dried ($MgSO_4$), and evaporated. Recrystallization from ethyl acetate–hexanes gave N^{a},*N*-diacetyl-L-kynurenine methyl ester (**1a**) (1.36 g, 53%) as white needles, mp 163–164 °C (lit. 134–136 °C);[16] $[a]_D^{20}$ +141 (*c* = 0.5, $CHCl_3$) (Anal. Calcd for $C_{15}H_{18}N_2O_5$: C, 58.82; H, 5.92; N, 9.15. Found: C, 58.86; H, 5.94; N, 8.97%); ^{1}H-NMR ($CDCl_3$) δ 11.45 (s, 1H), 8.75 (d, *J* = 8.7 Hz, 1H), 7.88 (d, *J* = 7.8 Hz, 1H), 7.57 (t, *J* = 7.8 Hz, 1H), 7.12 (t, *J* = 7.3 Hz, 1H), 6.54 (d, *J* = 7.7 Hz, 1H), 4.97 (m, 1H), 3.76 (s, 3H), 3.75 (m, 2H), 2.23 (s, 3H), 2.04 (s, 3H).

(2*S*)-Methyl 2-acetamido-4-(2′-acetamido-5′-bromophenyl)-4-oxobutanoate (N^{α},*N*-diacetyl-5-bromo-L-kynurenine methyl ester) (2a)

N^{α},*N*-Diacetyl-L-kynurenine methyl ester (1.02 g, 3.32 mmol) was dissolved in acetic acid (20 mL) containing fused sodium acetate (1.5 g) and treated with bromine (4 mL). After 1 h at room temperature, the mixture was poured into ice–water, treated with 10% $NaHSO_3$ (20 mL), extracted with $CHCl_3$, dried ($MgSO_4$), and evaporated. The remaining acetic acid was removed by repeated evaporation with toluene. Recrystallization from MeOH–H_2O gave N^{α},*N*-diacetyl-5-bromo-L-kynurenine methyl ester (**2a**) (1.03 g, 80%) as pale yellow needles, mp 176–178 °C (lit.[17] mp 178 °C); $[a]_D^{20}$ +85.6 (*c* = 0.81, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 11.35 (s, 1H), 8.70 (d, *J* = 9.0 Hz, 1H), 7.98 (d, *J* = 2.3 Hz, 1H), 7.66 (dd, *J* = 9.2, 1.9 Hz, 1H), 6.49 (d, *J* = 7.6 Hz, 1H), 4.96 (m, 1H), 3.77 (s, 3H), 3.75 (m, 2H), 2.24 (s, 3H), 2.04 (s, 3H).

(2*S*)-2-Amino-4-(2′-amino-5′-bromophenyl)-4-oxobutanoic acid (5-bromo-L-kynurenine) (3)

N^{α},*N*-Diacetyl-5-bromo-L-kynurenine methyl ester (0.96 g, 2.5 mmol) was refluxed for 18 h with 6 M HCl (9 mL). The solution was evaporated and the yellow oily residue was purified by low pressure reverse phase column chromatography on C_{18} silica gel (10% MeOH–H_2O) to give 5-bromo-L-kynurenine (**3**) (0.57 g, 79%) as a waxy yellow solid, mp 213–217 °C (lit.[18] mp 233 °C for racemate); $[a]_D^{20}$ +62.5 (*c* = 0.28, dioxane–H_2O 1 : 1); ^{1}H-NMR (1% DCl–D_2O) δ 8.21 (d, *J* = 2.1 Hz, 1H), 7.77 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.22 (d, *J* = 8.6 Hz, 1H), 4.48 (t, *J* = 5.0 Hz, 1H), 3.84 (d, *J* = 5.1 Hz, 2H).

(2*S*,4*S*)- and (2*S*,4*R*)-2-Amino-4-(2′-amino-5′-bromophenyl)-4-hydroxybutanoic acid (5-bromodihydro-L-kynurenine) (4a + 4b)

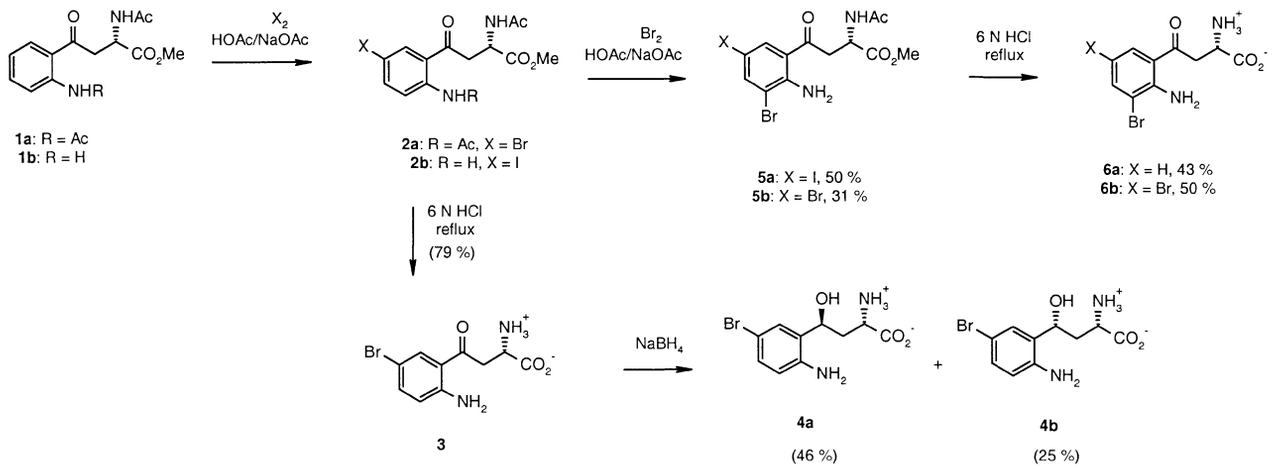
5-Bromo-L-kynurenine (143 mg, 0.5 mmol) was suspended in H_2O (50 mL) and treated with $NaBH_4$ (50 mg, 1.3 mmol). The mixture was stirred overnight, brought to pH 3 by addition of 1 M HCl, and loaded onto a Dowex-50 ion exchange column. Elution with 1 M NH_3 yielded a 2 : 1 mixture of (2*S*,4*S*) and (2*S*,4*R*)-diastereomers (100 mg, 69%) (Anal. Calcd. for $C_{10}H_{13}BrN_2O_3 \cdot 0.5H_2O$: C, 40.29; H, 4.73; N, 9.40. Found: C, 40.04; H, 4.50; N, 9.17%). The mixture was separated by HPLC (C_{18}, 2.5 × 25 cm, isocratic at 5% MeOH–0.1%TFA) in 4 mg portions. (2*S*,4*S*)-Isomer (**4b**): mp > 360 °C; $[a]_D^{20}$ −53.5 (*c* = 0.40, 1% NaOH); ^{1}H-NMR (1.0% NaOD–D_2O) δ 7.36 (d, *J* = 2.1 Hz, 1H), 7.21 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.69 (d, *J* = 8.6 Hz, 1H), 3.31 (dd, *J* = 8.0, 5.0 Hz, 1H), 2.07 (m, 1H), 1.75 (m, 1H); ^{13}C-NMR (1.0% D_2O) δ 185.3, 162.6, 145.0, 133.3, 131.6, 121.7, 113.2, 69.9, 55.9, 43.4. (2*S*,4*R*)-Isomer (**4a**): mp > 360 °C; $[a]_D^{20}$ +8.2 (*c* = 0.44, 1% NaOH); ^{1}H-NMR (1.0% NaOD–D_2O) δ 7.39 (d, *J* = 2.1 Hz, 1H), 7.25 (dd, *J* = 8.6, 2.3 Hz, 1H), 6.74 (d, *J* = 8.6 Hz, 1H), 3.27 (t, *J* = 6.9 Hz, 1H), 2.00 (m, 1H), 1.89 (m, 1H); ^{13}C-NMR (1.0% D_2O) δ 185.2, 162.5, 145.1, 133.4, 131.6, 121.6, 113.1, 71.0, 56.4, 43.4.

(2*S*)-Methyl 2-acetamido-4-(2′-amino-5′-iodophenyl)-4-oxobutanoate (N^{α}-acetyl-5-iodo-L-kynurenine methyl ester) (2b)

N^{α}-Acetyl-L-kynurenine methyl ester (**1b**) (1.5 g, 5.7 mmol)[19] was dissolved in acetic acid (50 mL) containing fused sodium acetate (3.75 g) and treated with iodine (3.6 g, 14.2 mmol). After 1 h at room temperature, the mixture was poured into ice–water, treated with 10% $NaHSO_3$ (20 mL), extracted with $CHCl_3$, dried ($MgSO_4$), and evaporated. Residual acetic acid was removed by repeated evaporation with toluene. Passage of the crude product through a short column of silica gel and elution with ethyl acetate gave N^{α}-acetyl-5-iodo-L-kynurenine methyl ester (**2b**) (1.15 g, 52%) as pale yellow needles, mp 156–157 °C; $[a]_D^{20}$ +131 (*c* = 0.56, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.93 (d, *J* = 1.9 Hz, 1H), 7.49 (dd, *J* = 8.8, 1.9 Hz, 1H), 6.56 (d, *J* = 7.9 Hz, 1H), 6.46 (d, *J* = 8.8 Hz, 1H), 4.95 (m, 1H), 3.74 (s, 3H), 3.71 (dd, *J* = 3.9, 18.2 Hz, 1H), 3.52 (dd, *J* = 18.2, 3.9 Hz, 1H), 2.02 (s, 3H).

(2*S*)-2-Amino-4-(2′-amino-3′-bromophenyl)-4-oxobutanoic acid (3-bromo-L-kynurenine) (6a)

N^{α}-Acetyl-5-iodo-L-kynurenine methyl ester (400 mg, 1.03 mmol) was dissolved in acetic acid (8 mL) containing fused



Scheme 1

sodium acetate (600 mg) and treated with bromine (0.051 mL, 160 mg, 1.0 mmol). After 30 min at room temperature, the mixture was poured into ice–water and the precipitate (380 mg) was filtered and dried. The product was a mixture of N^{α}-acetyl-3-bromo-5-iodo-L-kynurenine methyl ester (**5a**) (242 mg, 50%) and N^{α}-acetyl-3,5-dibromo-L-kynurenine methyl ester (**5b**) (135 mg, 31%). The mixture (309 mg) was refluxed in 6 M HCl overnight. Low pressure reverse phase chromatography on C_{18}-silica gel (10–20% MeOH–H_2O) gave (2*S*)-2-amino-4-(2′-amino-3′-bromophenyl)-4-oxobutanoic acid (3-bromo-L-kynurenine) (**6a**) (52 mg, 43%), mp 210–212 °C (lit.[20] >175 °C); $[\alpha]_D^{20}$ +63.2 (*c* = 0.5, dioxane–H_2O, 1 : 1); ^{1}H-NMR (1% DCl–D_2O) δ 7.85 (d, *J* = 8.1 Hz, 1H), 7.75 (d, *J* = 7.7 Hz, 1H), 6.71 (t, *J* = 7.9 Hz, 1H), 4.34 (t, *J* = 4.9 Hz, 1H), 3.81 (d, *J* = 5.0 Hz, 1H).

(*S*)-(4-Bromo-2-nitrophenyl)-L-cysteine (7)

Triethylamine (1.4 mL) was added dropwise to a solution of 1.0 g L-cysteine hydrochloride hydrate, 0.894 g 4-bromo-2-nitrofluorobenzene, and 4 mL of DMF. After 40 minutes, the resultant yellow solid was broken up in 20 mL of water and filtered. The crude product was recrystallized from 300 mL of hot water per g of product to yield 1.18 g (58%) bright yellow crystals of (*S*)-(4-bromo-2-nitrophenyl)-L-cysteine (**7**). Anal. Calcd. for $C_9H_9BrN_2O_4S$: C, 33.66; H, 2.82; N, 8.72. Found: C, 33.83; H, 2.85; N, 8.68%. ^{1}H-NMR (1% DCl–D_2O) δ 8.26 (d, *J* = 2.2 Hz, 1H), 7.72 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.45 (d, *J* = 8.7 Hz, 1H), 4.24 (t, *J* = 4.7 Hz, 1H), 3.66 (dd, *J* = 4.0, 15 Hz, 1H), 3.47 (dd, *J* = 7.7, 15 Hz, 1H).

(*S*)-(2-Amino-4-bromophenyl)-L-cysteine *S*,*S*-dioxide (9)

(*S*)-(4-Bromo-2-nitrophenyl)-L-cysteine (0.300 g) was added to a mixture of 10 mL 88% formic acid and 2 mL 30% H_2O_2. The solution was stirred for one week, then the solvent was removed *in vacuo* at 30 °C.[21] The syrupy product was triturated with water to give 0.331 g (82%) pale yellow-to-white crystals of (*S*)-(4-bromo-2-nitrophenyl)-L-cysteine *S*, *S*-dioxide (**8**). (*S*)-(4-Bromo-2-nitrophenyl)-L-cysteine *S*,*S*-dioxide (0.301 g) was dissolved in 25 mL of glacial acetic acid, 1.00 g Zn powder was added, and the mixture was stirred for 24 hours. The reaction mixture was filtered through Celite to remove remaining Zn and precipitated $Zn(OAc)_2$, and the filtrate was evaporated *in vacuo*. Trituration with water and methanol gave 0.16 g (67%) of a cream-colored powder of (*S*)-(2-amino-4-bromophenyl)-L-cysteine *S*,*S*-dioxide (**9**). MS (ESI): *m*/*z* 323, 325 (M + H)$^+$. ^{1}H-NMR (1% DCl–D_2O) δ 7.47 (d, *J* = 8.7 Hz, 1H), 7.13 (d, *J* = 1.7 Hz, 1H), 6.98 (dd, *J* = 8.7, 1.7 Hz, 1H), 4.53 (t, *J* = 4.7 Hz, 1H), 3.96 (m, 2H).

(*S*)-(2-Amino-5-bromophenyl)-L-cysteine *S*,*S*-dioxide (12)

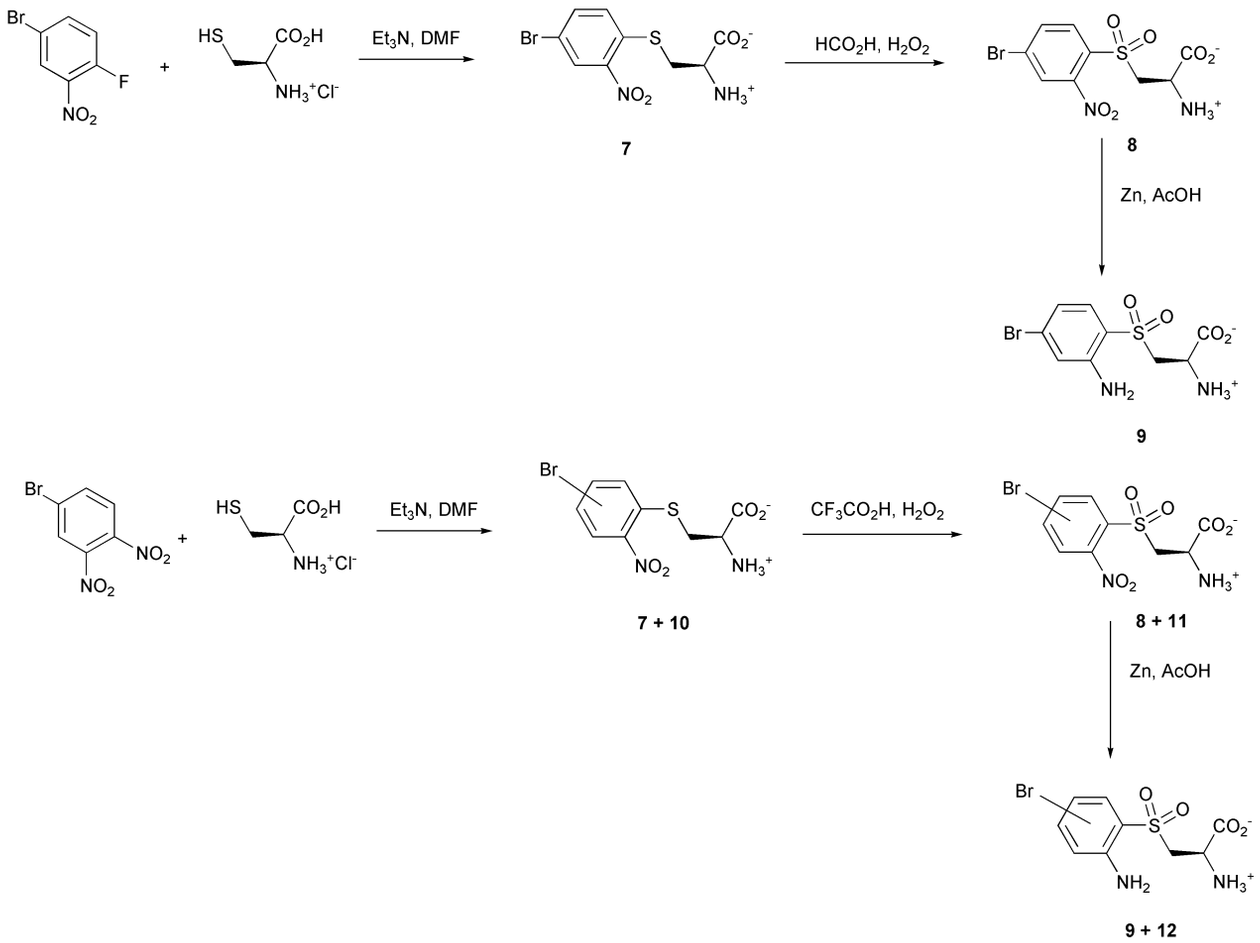
Triethylamine (3.5 mL) was added dropwise to a solution of 2.24 g L-cysteine hydrochloride hydrate, 2.00 g 1-bromo-3,4-dinitrobenzene,[22] and 9 mL DMF. After 40 minutes, a red–orange solid formed, which was broken up in 10 mL of water and filtered to give the crude product. Recrystallization of the crude product from 400 mL hot water per g gave 1.3 g (28.5%) bright yellow crystals. MS (ESI): *m*/*z* 321, 323 (M + H)$^+$. This product was found to be a mixture of (*S*)-(5-bromo-2-nitrophenyl)-L-cysteine (**10**) and (*S*)-(4-bromo-2-nitrophenyl)-L-cysteine (**7**) by NMR. ^{1}H-NMR of **10**: (1% DCl–D_2O) δ 8.04 (d, *J* = 8.4 Hz, 1H), 7.81 (d, *J* = 2.0 Hz, 1H), 7.56 (dd, *J* = 8.8, 2.0 Hz, 1H), 4.37 (dd, *J* = 3.6, 6.8 Hz, 1H), 3.75 (dd, *J* = 4.8, 16 Hz, 1H), 3.60 (dd, *J* = 8.0, 16 Hz, 1H).

The above mixture (0.200 g) was added to a solution of 5 mL of trifluoroacetic acid and 1 mL of 30% H_2O_2. After 48 hours, the pale yellow solution was evaporated *in vacuo* at 30 °C.[21] Trituration with water resulted in pale yellow-to-white crystals, MS (ESI): *m*/*z* 353, 355 (M + H)$^+$. The product was dissolved in 25 mL of glacial acetic acid, 1.00 g Zn powder was added, and the mixture was stirred for 24 hours. The reaction mixture was then filtered through Celite to remove remaining Zn and precipitated $Zn(OAc)_2$, and the filtrate was evaporated *in vacuo*. Trituration of the residue with water and methanol gave 0.080 g (43.7%) of a tan powder. HPLC analysis (C_{18}, 10% MeOH) showed that this product was a 2 : 1 mixture of (*S*)-(2-amino-5-bromophenyl)-L-cysteine *S*,*S*-dioxide (**9**) and (*S*)-(2-amino-5-bromophenyl)-L-cysteine *S*,*S*-dioxide (**12**). MS (ESI): *m*/*z* 323, 325 (M + H)$^+$; ^{1}H-NMR of **12**: (1% DCl–D_2O) δ 7.77 (d, *J* = 2.2 Hz, 1H), 7.55 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 4.54 (dd, *J* = 6.4, 4.4 Hz, 1H), 3.99 (m, 2H).

Results

Synthesis of brominated substrates and inhibitors

5-Bromo-L-kynurenine (**3**) was prepared as summarized in Scheme 1, by acid hydrolysis of N^{α},*N*-diacetyl-5-bromo-L-kynurenine methyl ester (**2a**), obtained by a procedure similar to that described by Casnati *et al.*[17] 5-Bromo-L-kynurenine was then reduced to 5-bromodihydro-L-kynurenine with $NaBH_4$ following a method we previously described for preparation of dihydro-L-kynurenine.[5] A 2 : 1 mixture of (4*R*)-5-bromodihydro-L-kynurenine (**4a**) and (4*S*)-5-bromodihydro-L-kynurenine (**4b**) was obtained, which was separated using preparative HPLC. N^{α}-Acetyl-5-iodo-L-kynurenine methyl ester (**2b**) was prepared from N^{α}-acetyl-L-kynurenine methyl ester (**1b**) according to Scheme 1. Attempts to obtain 5-iodo-L-kynurenine by hydrolysis of N^{α}-acetyl-5-iodo-L-kynurenine methyl ester were unsuccessful, because the iodine was substituted by a



Scheme 2

proton under the strongly acidic conditions needed for hydrolysis. This observation, however, indicated to us that iodine could be used as an easily removable protecting group in the convenient synthesis of 3-bromo-L-kynurenine (Scheme 1). Thus, N^{α}-acetyl-5-iodo-L-kynurenine methyl ester (**2b**) was subjected to bromination conditions, and the product was found to be a 2 : 1.1 mixture of N^{α}-acetyl-3-bromo-5-iodo-L-kynurenine methyl ester (**5a**) and N^{α}-acetyl-3,5-dibromo-L-kynurenine methyl ester (**5b**). The mixture was subjected to hydrolysis, and the resulting mixture of 3-bromo-L-kynurenine and 3,5-dibromo-L-kynurenine was readily separated by atmospheric pressure reverse phase liquid chromatography on C_{18} silica gel. This synthesis of 3-bromo-L-kynurenine is shorter and more efficient than the only other one reported in the literature.[20]

A mixture of (*S*)-(2-amino-4-bromophenyl)-L-cysteine *S*,*S*-dioxide (4-Br-SAPCO) (**9**) and (*S*)-(2-amino-5-bromophenyl)-L-cysteine *S*,*S*-dioxide (5-Br-SAPCO) (**12**) was prepared by the reaction of L-cysteine with 1-bromo-3,4-dinitrobenzene (obtained by nitration of 3-nitrobromobenzene by the method of Mangini[22]), followed by oxidation with H_2O_2 in trifluoroacetic acid (Scheme 2). The final step, reduction of the nitro group, was performed with Zn in glacial acetic acid, instead of Pd and H_2 in formic acid, as we had used previously,[8] to avoid hydrogenolysis of the aryl bromide. This reduction method was also found to produce a cleaner product than catalytic hydrogenation in the preparation of SAPCO. We have found that the catalytic reduction performed in formic acid, as described in our original procedure,[8] gives variable amounts of N^2-formyl SAPCO. We had hoped that the nucleophilic substitution reaction of cysteine with 1-bromo-3,4-dinitrobenzene would show regioselectivity in favor of the desired 5-isomer. However, NMR analysis of the brominated *o*-nitrophenyl-L-cysteine product clearly showed two isomeric products. HPLC analysis of the final product mixture demonstrated that it contained 67% of the (*S*)-(2-amino-4-bromophenyl)-L-cysteine *S*,*S*-dioxide (**9**) and only 33% of the desired (*S*)-(2-amino-5-bromophenyl)-L-cysteine *S*,*S*-dioxide (**12**), since we had the authentic 4-bromo isomer as a standard. (*S*)-(2-Amino-4-bromophenyl)-L-cysteine *S*,*S*-dioxide (**9**) was prepared as shown in Scheme 2 from commercially available 4-bromo-2-nitrofluorobenzene.

Steady-state kinetic studies

5-Bromo-L-kynurenine is an excellent substrate for kynureninase, with a k_{cat}/K_m value about half that of L-kynurenine, whereas 3-bromo-L-kynurenine also has a high turnover, but k_{cat}/K_m is reduced about 100-fold due to a very high K_m (Table 1). Thus, the bromo substitutent at the 5-position has much less effect on activity than the 3-bromo substitutent. Of the diastereomers of 5-bromodihydro-L-kynurenine, only (4*R*)-5-bromodihydro-L-kynurenine acts as a slow substrate, with a low k_{cat} value of 4.6×10^{-3} s^{-1}. Similarly, we found previously that (4*R*)-dihydro-L-kynurenine is a substrate, while the (4*S*)-diastereomer is an inhibitor.[5] However, both diastereoisomers of 5-bromodihydro-L-kynurenine are potent competitive inhibitors of kynureninase, with K_i values of 170 and 55 nM, respectively, for the (4*S*) and (4*R*)-epimers (Table 1). In contrast, (4*S*)-dihydro-L-kynurenine is a more potent inhibitor (K_i = 300 nM) than the (4*R*)-epimer (K_i = 1.5 mM).[5] The K_i value of 4-Br-SAPCO is 300 nM (Table 1), while that of the 2 : 1 mixture of 4- and 5-bromo isomers is 327 nM. Assuming that the observed K_i is a weighted average for the 2 : 1 mixure of the 4-bromo and 5-bromo isomers, the K_i for 5-Br-SAPCO was estimated to be 400 nM (Table 1). In this study, we also repeated the measurement of the K_i for the parent compound, SAPCO, and we found it to be 27 nM, somewhat lower than we reported previously (70 nM).[8] This may be due to the change in the reduction procedure

Table 1 Kinetic parameters for kynureninase inhibitors and substrates

Compound	K_i/nM	k_{cat}/s^{-1}	K_m/μM	(k_{cat}/K_m)/M^{-1} s^{-1}
L-Kynurenine	—	6.9	25	2.8×10^5
5-Br-L-Kyn	—	2.1	12	1.8×10^5
3-Br-L-Kyn	—	11	2.0×10^3	5.5×10^3
(4*S*)-H_2-L-Kyn[a]	300	—	—	—
(4*R*)-H_2-L-Kyn[a]	1500	0.6	1.5	4.0×10^5
(4*S*)-5-Br-H_2-L-Kyn	170	—	—	—
(4*R*)-5-Br-H_2-L-Kyn	55	4.6×10^{-3}	5.2×10^{-2}	8.8×10^4
SAPCO	27	—	—	—
4-Br-SAPCO	300	—	—	—
5-Br-SAPCO	400	—	—	—

[a] From ref. 6.

noted above, which avoids formation of the N^2-formyl derivative.

Stopped-flow kinetic studies

The reactions of 3-bromo- and 5-bromo-L-kynurenine with kynureninase were examined by rapid scanning stopped-flow spectrophotometry. In the reaction of 5-bromo-L-kynurenine, there is an absorption peak at 494 nm (Fig. 1A) due to a quinoid intermediate, formed within the dead time (*ca.* 1 ms) of the stopped-flow instrument, which undergoes rapid decay in two phases, at about 600 s^{-1}, and 22 s^{-1}, very similar to the behavior of L-kynurenine that we observed previously.[14] These rate constants do not change significantly over the concentration range of 5-bromo-L-kynurenine from 0.08 to 0.64 mM. Addition of 10 mM benzaldehyde and 5-bromo-L-kynurenine together in these reactions results in the rapid formation ($k_{obs} = 78 \pm 10$ s^{-1}) of a stable absorbance peak at 496 nm, similar to that we observed previously with L-kynurenine (k_{obs} = 65 s^{-1})[14] (Fig. 1B). This new intermediate is formed by rapid trapping of the enamine intermediate, after elimination of 5-bromoanthranilate, and subsequent deprotonation at C4′ to form a quasi-stable quinonoid complex. Rapid-scanning stopped-flow kinetic experiments with 1 mM 3-bromo-L-kynurenine also showed the rapid formation, within the dead time, and subsequent decay, of an intermediate at 494 nm, with two phases, k_{obs} = 300 s^{-1} and 21 s^{-1} (Fig. 2A). However, the amplitude of the absorbance changes with 3-bromo-L-kynurenine is smaller than with 5-bromo-L-kynurenine. Addition of 10 mM benzaldehyde to the reactions of 3-bromo-L-kynurenine also results in the formation of a new quinonoid intermediate with $k_{obs} = 56 \pm 5$ s^{-1} (Fig. 2B).

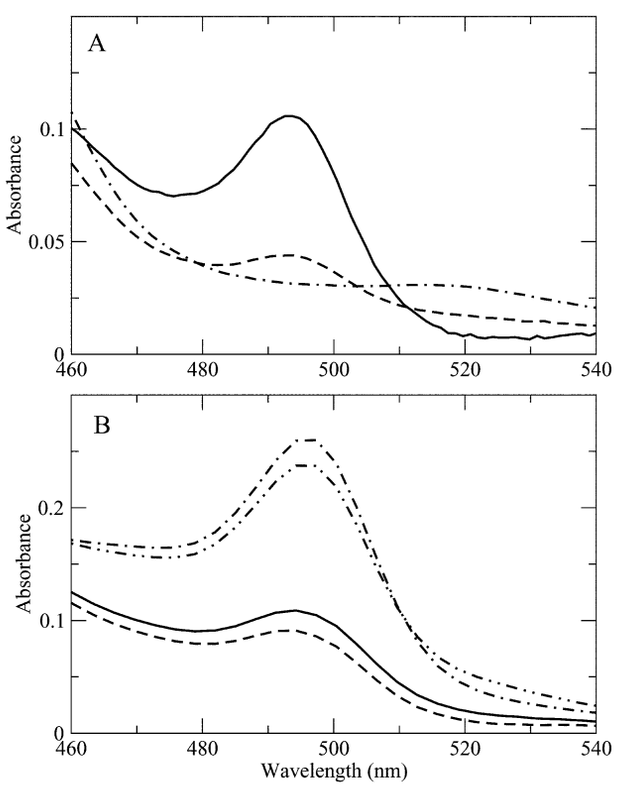


Fig. 1 A. Rapid-scanning stopped flow fitted SVD spectra of the reaction of kynureninase with 0.64 mM 5-bromo-L-kynurenine. Solid line, initial spectrum; dashed line, intermediate spectrum; dashed and dotted line, final spectrum. B. Rapid-scanning stopped flow fitted SVD spectra of the reaction of kynureninase with 0.64 mM 5-bromo-L-kynurenine in the presence of 10 mM benzaldehyde. Solid line, initial spectrum; Dashed line, first intermediate spectrum; dashed and dotted line, second intermediate spectrum; dashed and double dotted line, final spectrum.

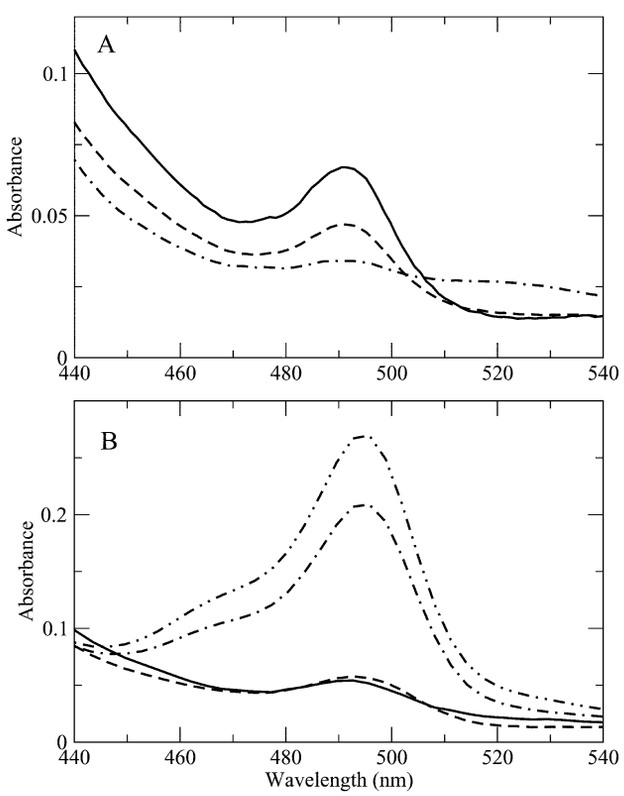


Fig. 2 A. Rapid-scanning stopped flow fitted SVD spectra of the reaction of kynureninase with 1 mM 3-bromo-L-kynurenine. Solid line, initial spectrum; dashed line, intermediate spectrum; dashed and dotted line, final spectrum. B. Rapid-scanning stopped flow SVD spectra of the reaction of kynureninase with 1 mM 3-bromo-L-kynurenine in the presence of 10 mM benzaldehyde. Solid line, initial spectrum; Dashed line, intermediate spectrum; dashed and dotted line, second intermediate spectrum; dashed and double dotted line, final spectrum.

SAPCO also reacts with kynureninase to form a prominent absorption band at about 500 nm, but at a slower rate than L-kynurenine (Fig. 3A).[8] This intermediate is stable in the steady-state, but decays after consumption of SAPCO, which is a very slow substrate for β-elimination.[8] 4-Br-SAPCO forms a similar absorption band, with λ_{max} at 505 nm (Fig. 3B). The rate constants of formation of the quinonoid intermediates are essentially identical for both SAPCO and 4-Br-SAPCO, k_{obs} = 260 s^{-1} (compare curves in Fig. 3B).

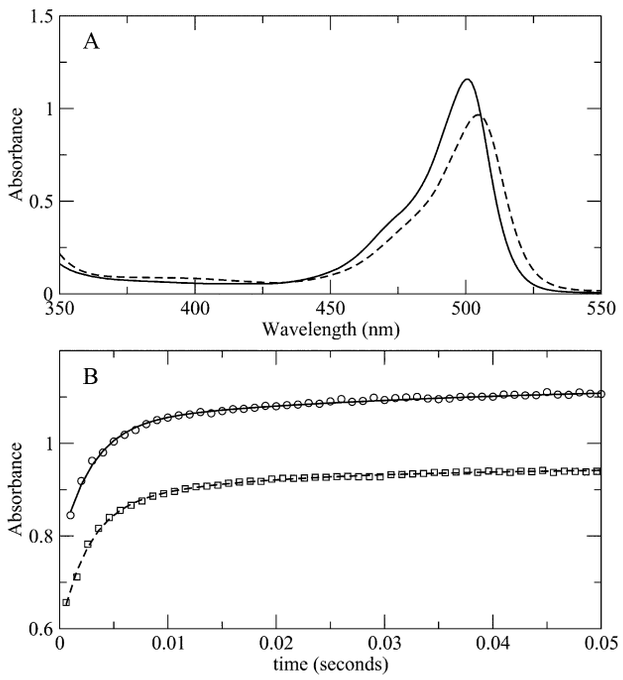


Fig. 3 A. Spectra of kynureninase in the presence of SAPCO and 4-Br-SAPCO. The spectra are the fitted SVD spectra from the rapid-scanning stopped-flow experiment. B. Time courses at 500 nm for the reaction of SAPCO and 4-Br-SAPCO with kynureninase. Circles: SAPCO; squares, 4-BrSAPCO. The lines are the fitted curves from the exponential fits with $k = 260\ s^{-1}$.

Discussion

We initially prepared 3-bromo- and 5-bromo-L-kynurenine as potential heavy atom derivatives to use in the determination of the three-dimensional structure of *P. fluorescens* kynureninase by X-ray crystallography.[23] Our method of preparation of 3-bromokynurenine is more direct and efficient than the previously reported method.[20] We determined substrate activity in solution to see if the binding of the brominated compounds to kynureninase crystals was possible. We found that 5-bromo-L-kynurenine has k_{cat} and k_{cat}/K_m values comparable to those of L-kynurenine (Table 1), while the k_{cat}/K_m value for 3-bromo-L-kynurenine is 127-fold lower, primarily due to the very high value of K_m for the latter compound (2 mM). This result is consistent with previous studies, where we demonstrated that 3-hydroxy-DL-kynurenine has a k_{cat}/K_m value about 80-fold less than that of L-kynurenine.[24] Thus, a bulky substitutent such as bromo or hydroxy at the C-3 position is not well accommodated by bacterial kynureninase, but a corresponding bromo substitutent at C-5 has very little effect on substrate activity (Table 1). This implies that there is adequate space in the active site of bacterial kynureninase near the 5-position of bound L-kynurenine to accommodate a bulky substituent, but not the 3-position. The mechanism of kynureninase, based on previous steady-state and stopped-flow experiments,[14,24] is shown in Scheme 3. The kynurenine quinonoid intermediate (EQ_1) is formed within the dead time of the mixer (*ca.* 1 ms), and decays rapidly to form a PLP ketimine (EK1). Upon hydration of the carbonyl, cleavage of the C_β–C_γ bond can occur to give anthranilate and a PLP enamine (EE). It is this enamine species which can react in an aldol-like manner with benzaldehyde.[6,25] All of the steps up to the formation of anthranilate, the first irreversible step, can contribute to k_{cat}/K_m. Thus, for the simplified kinetic mechanism shown in eqn. (5), k_{cat}/K_m is given by eqn. (6).

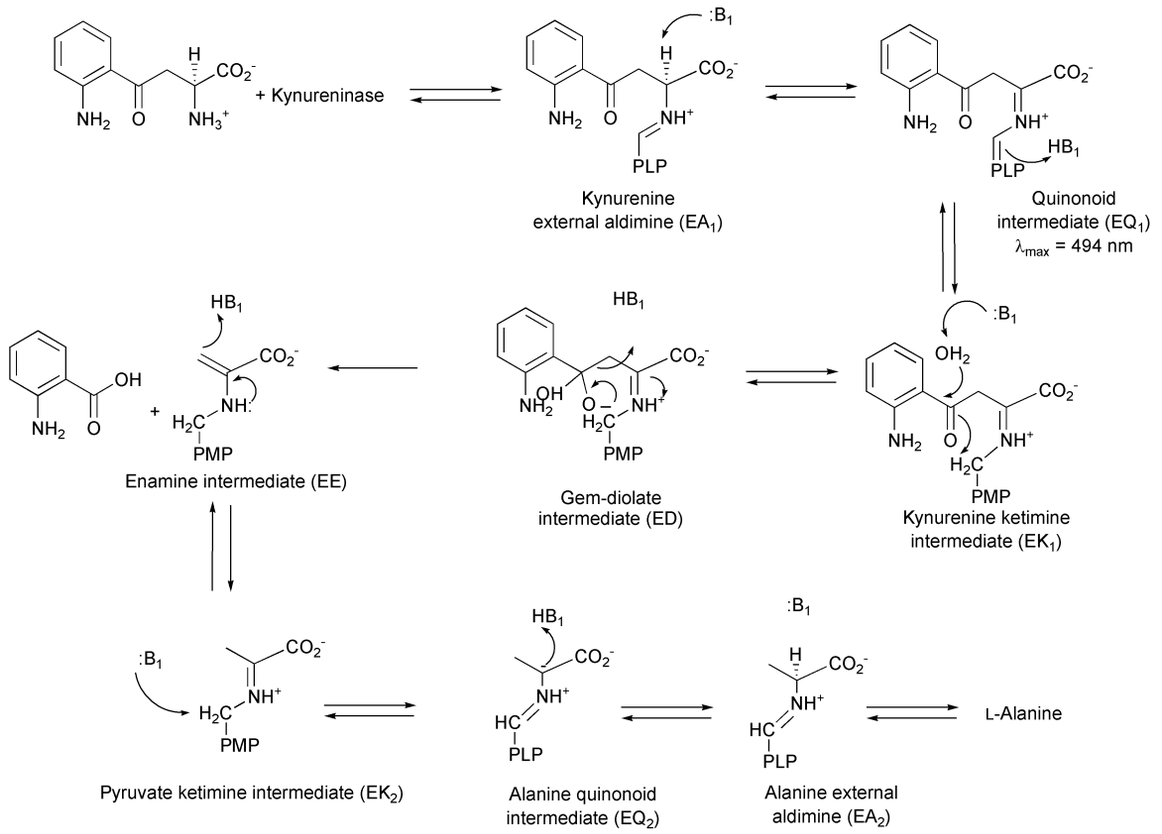
$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{-1}} EP+Q \rightleftharpoons E+P \qquad (5)$$

$$k_{cat}/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2}) \qquad (6)$$

In this mechanism the formation of the external aldimine and quinonoid intermediates are combined in k_{+1}, since both take place within the deadtime of the stopped-flow instrument. It is interesting that there is very little effect of bromine substitution at either the 3- or 5-position on the rate of formation and decay of the 494 nm quinonoid intermediate in stopped-flow kinetic experiments (Figs. 1 and 2). The initial formation of the external aldimine is very fast for the C-3 brominated compound, since the quinonoid intermediate is still completely formed in the dead time of the stopped-flow mixer, so it is unlikely that the k_{+1} for external aldimine formation has been decreased significantly. Furthermore, k_{+2}, the formation of anthranilate, is not significantly affected by the presence of bromine, as determined by the stopped-flow experiments in the presence of benzaldehyde, which directly measure the rate of carbon–carbon bond cleavage by trapping the enamine.[14] Thus, the off rate, k_{-1}, for release of substrate from the external aldimine must be increased at least 50-fold by the presence of the 3-bromo substitutent in order to cause the observed k_{cat}/K_m to decrease from $2.8 \times 10^5\ M^{-1}\ s^{-1}$ to $5.5 \times 10^3\ M^{-1}\ s^{-1}$. We demonstrated previously that steady-state turnover for L-kynurenine is limited by the slow release of L-alanine from the pyruvate ketimine (EK2 in Scheme 3);[24] hence, it is not surprising that all of the kynurenines examined exhibit similar values of k_{cat} (Table 1).

We next reduced 5-bromo-L-kynurenine in order to make a more active inhibitor for potential use in our X-ray structural analysis. In previous work, we showed that the diastereoisomers of dihydro-L-kynurenine differ in their properties, in that the (4*R*)-epimer acts as a substrate (K_m = 1.5 mM) and the (4*S*)-epimer is a potent competitive inhibitor (K_i = 300 nM).[6] The behavior of the two diastereomers of 5-bromodihydro-L-kynurenine parallels that of the unsubstituted dihydro-L-kynurenines in that the (4*R*)-epimer acts as a substrate and the (4*S*)-epimer does not. Surprisingly, in contrast to the minimal effect on k_{cat}/K_m observed for substrate bromination at C-5, both brominated dihydrokynurenines are significantly more potent inhibitors than the parent compounds. Apparently, the presence of bromine results in favorable dipolar or van der Waals forces that enhance binding affinity of the dihydro-L-kynurenines. Furthermore, the brominated (4*R*)-epimer is a more potent inhibitor (K_i = 55 nM) than the (4*S*)-epimer (K_i = 170 nM), as shown in Table 1. Thus, there is a 27-fold increase in the binding of the (4*R*)-epimer with 5-bromination, but only a 1.8-fold increase in binding for the (4*S*)-epimer. This suggests that the aromatic rings of the diastereomeric 5-bromodihydrokynurenines have different modes of binding in the active site.

(*S*)-(2-Aminophenyl)-L-cysteine *S*,*S*-dioxide (SAPCO) was previously reported to be a potent inhibitor of bacterial kynureninase.[8] We were interested to see if bromination would also enhance the activity of this compound in the same way as it does for dihydro-L-kynurenine. If so, we expected to observe a K_i value in the low nM range for 5-Br-SAPCO. We prepared pure 4-Br-SAPCO and a 2 : 1 mixture of the 4-Br and 5-Br



Scheme 3

isomers of SAPCO. From this, we were able to estimate that the K_i values for these compounds are 300 and 400 nM, respectively (Table 1), compared to 27 nM for the unsubstituted compound. Thus, bromination at either C-4 or C-5 results in a 10-fold reduction in binding affinity for SAPCO, rather than the increase in binding seen with dihydro-L-kynurenines. This suggests that the binding mode of the aromatic ring for these compounds is different again from those for both the dihydro-L-kynurenines and L-kynurenine. This is surprising, since we initially designed SAPCO as a transition state analog inhibitor based on the properties of the dihydrokynurenines.[6] However, SAPCO accumulates a quinonoid intermediate (Fig. 3), while the kynurenines only exhibit a transient quinonoid species (Figs. 1 and 2). In our previous work, we applied COMFA to develop a model of inhibitor and substrate binding to the active site of kynureninase.[8] This model required that the aromatic rings of the substrate and different classes of inhibitors are bound in the same way in the active site. However, the present data clearly cannot be accommodated by a single mode of binding of the aromatic rings of these compounds. Unfortunately, attempts to observe the bound brominated compounds in kynureninase crystals by soaking the crystals with the inhibitor have been unsuccessful to date. The three dimensional structure of bacterial kynureninase has been ultimately solved using a combination of heavy atom derivatives and molecular replacement.[23]

The results of this work show that bromine, and likely other halogen, substitution on dihydrokynurenines can enhance the kynureninase inhibitory activity significantly. The effects of the bromo substituent may be due to dipolar effects, van der Waals effects, or a combination of both, with active site residues. A series of substitutents will need to be examined in order to determine the nature of these effects. The inhibition of mammalian kynureninase by SAPCO and some substituted analogs was reported by Drysdale and Reinhard.[26] In that study, fluorine or methoxy substitution in the 3-position resulted in slightly improved inhibition, but the best activity (IC_{50} = 11 μM) was observed with the 5-methyl derivative. More recently, Walsh *et al.* reported that 2-amino-4-(3′-hydroxyphenyl)-4-hydroxybutanoic acid, which is similar in structure to the dihydrokynurenines examined in this work, is a potent inhibitor of human and rat kynureninase, with K_i values around 100 nM.[27] It will be of interest to see if halogenation also results in an increase in the inhibitory activity of dihydrokynurenines for human kynureninase.

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