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Substituents effects on activity of kynureninase from *Homo sapiens* and *Pseudomonas fluorescens*

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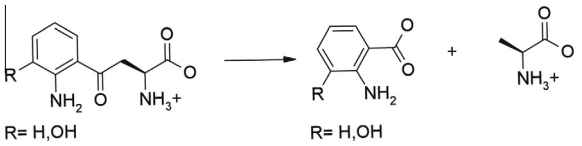
ABSTRACT

A series of substituted kynurenines (3-bromo-DL, 3-chloro-DL, 3-fluoro-DL, 3-methyl-DL, 5-bromo-L, 5-chloro-L, 3,5-dibromo-L and 5-bromo-3-chloro-DL) have been synthesized and tested for their substrate activity with human and *Pseudomonas fluorescens* kynureninase. All of the substituted kynurenines examined have substrate activity with both human as well as *P. fluorescens* kynureninase. For the human enzyme, 3- and 5-substituted kynurenines have k_{cat} and k_{cat}/K_m values higher than L-kynurenine, but less than that of the physiological substrate, 3-hydroxykynurenine. However, 3,5-dibromo- and 5-bromo-3-chlorokynurenine have k_{cat} and k_{cat}/K_m values close to that of 3-hydroxykynurenine with human kynureninase. The effects of the 3-halo substituents on the reactivity with human kynureninase may be due to electronic effects and/or halogen bonding. In contrast, for the bacterial enzyme, 3-methyl, 3-halo and 3,5-dihalokynurenines are much poorer substrates, while 3-fluoro, 5-bromo, and 5-chlorokynurenine have k_{cat} and k_{cat}/K_m values comparable to that of its physiological substrate, L-kynurenine. Thus, 5-bromo and 5-chloro-L-kynurenine are good substrates for both human as well as bacterial enzyme, indicating that both enzymes have space for substituents in the active site near C-5. The increased activity of the 5-halokynurenines may be due to van der Waals contacts or hydrophobic effects. These results may be useful in the design of potent and/or selective inhibitors of human and bacterial kynureninase.

1. Introduction

Kynureninase′ [EC 3.7.1.3] is a pyridoxal-5′-phosphate (PLP) dependent enzyme that catalyzes the hydrolytic cleavage of L-kynurenine to yield anthranilic acid and L-alanine (Eq. 1). This is a key enzyme in the kynurenine pathway of tryptophan catabolism in both bacteria and animals, and catalyzes the unique β,γ-cleavage of aryl substituted γ-keto-α-amino acids.[1] It has been found that the mammalian kynureninase cleaves 3-hydroxy-L-kynurenine more rapidly than L-kynurenine while the bacterial kynureninase from *Pseudomonas fluorescens* (PfKynase) cleaves L-kynurenine more rapidly than 3-hydroxy-L-kynurenine.[1] We showed that this difference in substrate specificity is due to differences in hydrogen bonding and steric interactions in the active sites of human and *P. fluorescens* enzymes.[2,3] Mutation of three active site residues (H104W, S332G, and N333T) of human kynureninase (HsKynase) to the corresponding residues in PfKynase gave a mutant enzyme that reacts exclusively with L-kynurenine, and exhibits undetectable activity for 3-hydroxy-L-kynurenine.[3]



(1)

These two distinct types of kynureninase have also been shown to differ in their regulation and their response to PLP. The Pseudomonas enzyme is induced by growing cells in the presence of L-tryptophan as carbon and nitrogen source,[4] and for this reason has been called 'inducible kynureninase' in the literature. Furthermore, it has been found that the inducible enzyme is reversibly inactivated by L-alanine, resulting in an abortive transamination reaction to give pyridoxamine-5′-phosphate and pyruvate from L-alanine[5]. However, the enzyme activity is restored either by addition of PLP or pyruvic acid; in the latter case there is a reverse transamination between pyridoxamine-5′-phosphate and the added pyruvate to give back PLP and alanine. On the other hand, the eukaryotic form of kynureninase is not induced by L-tryptophan, so it has been referred to as 'constitutive kynureninase′.[1] This

Abbreviations: HsKynase, *Homo sapiens* kynureninase, EC 3.7.1.3; PfKynase, *Pseudomonas fluorescens* kynureninase, EC 3.7.1.3.

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enzyme does not undergo abortive transamination in the presence of L-alanine or other amino acids. However, the rate of the hydrolytic cleavage reduces with time, indicating that the product, 3-hydroxyanthranilate, is a product inhibitor.[6]

The kynurenine pathway has been shown to be elevated in a number of neurodegenerative human diseases, including Huntington's Disease,[7,8] HIV-related dementia,[9] Parkinson's Disease[10] and Alzheimer's Disease,[11] resulting in accumulation of neurotoxic metabolites such as quinolinic acid. Furthermore, the R188Q allele of kynureninase has been linked to spontaneous hypertension in the Han Chinese population.[12] In addition, the kynurenine pathway is elevated in response to bacterial and viral infections, presumably to starve the pathogen of tryptophan, as a result of induction by γ-interferon.[13] However, *Chlamydia psittaci* has been shown to circumvent this response by expression of kynureninase in place of anthranilate synthase in the Trp operon.[14] Finally, kynureninase plays a role in the biosynthesis of PQS in *Pseudomonas aeruginosa*, and this signaling molecule is essential for pathogenicity.[15] Thus, both human and bacterial kynureninases emerge as potential drug targets. In the present work, we have compared the reaction of recombinant PfKynase and HsKynase with a range of ring-substituted kynurenines. The results show that both the enzymes respond similarly to substitutions at C-5, but differently to substitutions at C-3. These results may be useful for the design of potent and selective inhibitors of human and/or bacterial kynureninase.

2. Experimental methods

2.1. Materials

L-Kynurenine was obtained from Sigma–Aldrich, and 3-hydroxy-DL-kynurenine was a product of USBiochemicals. Common buffers and solvents were obtained from Fisher Scientific.

2.2. Instrumentation

^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra were recorded on a Varian 400 MHz instrument operating at 400, 100, and 384 MHz, respectively. HPLC measurements were carried out on a Thermo Spectrasystem P 2000 pump with a C_{18} reverse phase column connected to a Thermo UV 6000 diode array detector, at a flow rate of 1 ml/min and controlled by a Dell PC using Chromquest software. The solvent program was 0.1 M acetic acid/20% MeOH for the first 10 min, followed by a gradient to 70% MeOH over the next 10 min. GC–MS of the intermediate compounds was done on a Shimadzu GC-2010 instrument with a 2010QS detector. The steady state kinetic measurements and scanning kinetics measurements were performed on a Varian Cary 1E UV/Visible spectrophotometer equipped with a Peltier 6 × 6 thermoelectric cell block for temperature control. The UV instrument was controlled by a PC using software provided by Varian Instruments.

2.3. Enzyme assay

PfKynase was purified from *Escherichia coli* DH5α cells containing plasmid pTZKyn, as described previously.[16] Recombinant HsKynase was purified from *E. coli* BL21(DE3) cells containing the human gene in plasmid pET100.[2] PfKynase activity was measured from the decrease in absorbance at 360 nm ($\Delta\varepsilon$ = 4500 M^{-1} cm^{-1}) upon conversion of kynurenine to anthranilic acid.[17] Similarly, the HsKynase activity was measured from the decrease in absorbance at 370 nm ($\Delta\varepsilon$ = 4500 M^{-1} cm^{-1}) upon conversion of 3-hydroxy-DL-kynurenine to 3-hydroxyanthranilic acid.[2] The reaction mixtures for these measurements contained 100 μM of the substrate, L-kynurenine, or 3-hydroxy-DL-kynurenine, in 30 mM potassium phosphate buffer pH 8, containing 40 μM of pyridoxal-5′-phosphate at 37 °C, and the absorbance decrease was followed for 10 min.

2.4. Kinetics measurements

The scanning kinetics measurements to determine the absorbance change for the 3-substituted substrate analogs was done using the human enzyme, since 3-hydroxykynurenine is the natural substrate for the human enzyme.[1] The scanning kinetics measurements to determine the absorbance change for the 5-substituted substrate analogs were done using the *Pseudomonas fluorescens* enzyme since 5-substituted kynurenines have been shown previously to be good substrates for the *P. fluorescens* enzyme.[18] Depending on the reactivity of the substrate, repetitive scans were performed at intervals ranging from 5 to 30 min until no further changes were observed. The K_m, V_{max}, and V_{max}/K_m values were determined by fitting of the initial rate data to Equation 2 using the compiled FORTRAN program of Cleland, HYPERO.[19] The fitted values of V_{max}, and V_{max}/K_m were divided by the respective extinction coefficients in Table 1 and by the enzyme concentration to give the k_{cat} and k_{cat}/K_m values. The concentration of the enzymes was determined using the A_{280} = 1.43 for a 1 mg/mL solution.[20] The molecular weights used to calculate the molar enzyme concentrations were 46.5 kDa for PfKynase[16] and 57 kDa for HsKynase.[2]

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

2.5. Synthesis of kynurenine analogues

2.5.1. Ethyl 2-acetamido-2-carboethoxy-5-oxopentanoate 2-chlorophenylhydrazone (3a)

To a suspension of 9.71 g of diethyl acetamidomalonate in 20 ml benzene was added 97 mg MeONa, with stirring. After 5 min, the suspension was cooled in an ice-water bath, and 3.6 ml of acrolein was added dropwise in about 20–25 min while maintaining the temperature below 5 °C. After completion of addition, the reaction was warmed to ambient temperature and stirred for about 2 h, when a clear pale yellow solution resulted. At the end of 2 h of stirring, 2.7 ml of AcOH was added, followed by a solution of 7 g of 2-chlorophenylhydrazine in 14 ml benzene, resulting in a clear orange solution, which was warmed to 55–60 °C, for about 30 min and the heat was removed. The reaction was left stirring for 2.5 days at ambient temperature, then it was concentrated in vacuo, to give a reddish brown oil. Yield, 14 g (72%). ^{1}H NMR: MeOH-d_4, δ 7.40 (1H, d, J = 8.9 Hz), 7.29 (1H, t, J = 5.4 Hz), 7.19 (1H, d, J = 8.0 Hz), 7.13 (1H, t, J = 7.8 Hz), 6.69 (1H, t, J = 8.0 Hz), 4.20 (4H, q, J = 7.2 Hz), 2.56 (1H, t, J = 7.3 Hz), 2.22 (1H, m), 2.02

Table 1
λ_{max} and $\Delta\varepsilon$ used for substituted kynurenines

Compound	λ_{max} (nm)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	Anthranilate product λ_{max} (nm)
L-Kynurenine (**1**)	360	4500	310
3-Hydroxy (**2**)	370	4500	320
3-Chloro (**3**)	365	4650	323
3-Fluoro (**4**)	360	3050	308
3-Methyl (**5**)	362	3440	316
3-Bromo (**6**)	360	4500	323
5-Bromo (**7**)	370	4000	323
5-Chloro (**8**)	370	4330	323
3,5-Dibromo (**9**)	378	4480	330
5-Bromo-3-chloro (**10**)	379	3850	336

(3H, s), 1.20 (6H, t, J = 7.0 Hz); ^{13}C NMR: MeOH-d_4, δ 13.4, 21.6, 26.9, 62.5, 66.5, 114, 116.5, 119.3, 127.7, 129.1, 129.2, 141.9, 142.4, 168, 171.2. MS (EI): 142 (100), 144 (32).

2.5.2. Ethyl 2-acetamido-2-carboethoxy-5-oxopentanoate 2-methylphenylhydrazone (5a)

Using the procedure above, 16 g (69%) of a reddish brown oil was obtained from 7.5 g of 2-methylphenylhydrazine. ^{1}H NMR: MeOH-d_4, δ 7.31 (1H, d, J = 8.5 Hz), 7.23 (1H, t, J = 4.9 Hz), 7.05 (1H, t, J = 7.7 Hz), 6.98 (1H, d, J = 8.0 Hz), 6.67 (1H, t, J = 7.7 Hz), 4.21 (4H, q, J = 7.0 Hz), 2.54 (2H, t, J = 7.7 Hz), 2.23 (2H, m), 2.02 (3H, s), 1.98(3H, s), 1.2 (6H, t, J = 7.0 Hz); ^{13}C NMR: MeOH-d_4, δ 13.4, 16.6, 21.5, 26.8, 30.1, 62.5, 66.6, 112.5, 118.9, 120.8, 126.7, 130.2, 140.7, 143.9, 168, 171.3.

2.5.3. Ethyl 7-chloroindole-3-(3-acetamido-3-carboethoxy)butanoate (3b)

Compound **3a** (14 g) was heated in 85 ml 10% aqueous H_2SO_4 for about 2 h with vigorous stirring, when a dark brown mixture resulted. The reaction was cooled to 55–60 °C, 100 ml EtOAc was added, and the mixture was stirred for about 10 min to dissolve the semisolid product completely. A solution of 21 g NaCl in 50 ml H_2O was added, and stirring was continued at ambient temperature for about 10 min. The organic layer was separated, and the aqueous layer was extracted with 75 ml EtOAc. The combined organic layers were washed once with 75 ml of brine, then dried over anhydrous Na_2SO_4. The solvent was removed in vacuo to give a brown semisolid. Yield = 11.0 g (82%) ^{1}H NMR: $CDCl_3$, δ 8.38 (1H, br s), 7.39 (1H, d, J = 7.6 Hz)), 7.18 (1H, d, J = 7.6 Hz), 7.02 (1H, t, J = 8 Hz), 6.95 (1H, d, J = 2.4 Hz), 6.62 (1H, br s), 4.24 (4H, m), 3.84 (2H, s), 1.99 (3H, s), 1.28 (6H, t, J = 7.2 Hz); ^{13}C NMR: MeOH-d_4 δ 13.3, 19.9, 28.2, 62.5, 68.1, 109.4, 116.7, 117.1, 119.7, 120.8, 125.1, 130.2, 133.4, 167.8, 171.5. MS (EI): 164 (100), 166 (25), 321 (52), 323 (19), 380 (10, M^+), 382 (3).

2.5.4. Ethyl 7-methylindole-3-(3-acetamido-3-carboethoxy)butanoate (5b)

Compound **5a** (16 g) was reacted in 96 ml of 10% aqueous H_2SO_4 and worked up as above for **3a**. Yield = 11 g (72%) of a brown semisolid. ^{1}H NMR: MeOH-d_4, δ 7.22 (1H, d, J = 7.8 Hz), 6.97 (1H, s), 6.91 (1H, d, J = 7.7 Hz), 6.87 (1H, t, J = 6.4 Hz), 4.18 (4H, m), 3.76 (2H, s), 2.45 (3H, s), 1.95 (3H, s), 1.22 (6H, t, J = 7.1 Hz); ^{13}C NMR: MeOH-d_4, δ 13.3, 15.9, 21.6, 28.3, 62.4, 68.2, 108.4, 115.6, 119, 120.8, 121.8, 123.7, 128.1, 135.9, 167.9, 171.3. MS (EI): 144 (100), 301 (40), 360 (10, M^+).

2.5.5. Ethyl 2-acetamido-2-carboethoxy-5-oxo-5-(3-chloro-2-aminophenyl)pentanoate (3c)

A solution of **3b** (11 g) in 100 ml MeOH was cooled to below −70 °C using a dry ice-acetone bath. Ozone gas was then passed at 0.5 psig through the reaction mixture for about 90 min. The reaction was then quenched with an aqueous solution of 44 g sodium bisulfite in 200 ml H_2O, when a yellow suspension resulted. The mixture was then stirred for about 10–15 min to allow it to reach ambient temperature. The MeOH was removed in vacuo, 100 ml H_2O was added, and it was extracted with two 75 ml portions of EtOAc. The combined organic layers were washed with 75 ml saturated brine solution, treated with charcoal, filtered over Celite, and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave 5.5 g (50%) of a semisolid, which after recrystallization from 2-propanol gave 4.0 g of the product as a pale yellow solid, mp 177–178 °C. ^{1}H NMR: $CDCl_3$, δ 8.34 (2H, br d), 7.62 (2H, d, J = 7.6 Hz), 7.27 (1H, t, J = 7.6 Hz), 7.1 (1H, s), 4.27 (4H, q, J = 7.0 Hz), 4.23 (2H, s), 2.01 (3H, s), 1.26 (6H, t, J = 7.0 Hz); ^{13}C NMR: MeOH-d_4, δ 13.5, 20.1, 36.5, 62.2, 70.1, 116.2, 118.6, 122.1, 124.3, 130.6, 142.1, 170.4, 173.1, 205.3. MS (EI): 154 (100), 156 (32), 252 (90), 254 (30), 384 (21, M^+), 386 (7).

2.5.6. Ethyl 2-acetamido-2-carboethoxy-5-oxo-5-(3-fluoro-2-formamidophenyl)pentanoate (4c)

Ozonolysis of **4b** (7.8 g), prepared as described previously,[21] in 64 ml MeOH with workup as described for **3b** gave 3.5 g (45%) of the product as a brown oil. ^{1}H NMR: MeOH-d_4, δ 7.62 (1H, d, J = 7.7 Hz), 7.12 (1H, dd, J = 7.9, 4.0 Hz), 7.05 (1H, m), 6.35 (1H, br s), 4.27 (4H, q, J = 7 Hz), 4.24 (2H, s), 2.01 (3H, s), 1.25 (6H, t, J = 7 Hz); ^{13}C NMR: MeOH-d_4, δ 13.8, 24.2, 36.5, 66.5, 72.5, 119.3, 121.4, 123.2, 127.6, 138.4, 162.5, 169.5, 172.2, 204.1 ^{19}F NMR: MeOH-d_4, δ −137.6 (dd, J = 11.7, 4 Hz). MS (EI): 138 (100), 236 (60), 368 (10, M^+).

2.5.7. Ethyl 2-acetamido-2-carboethoxy-5-oxo-5-(2-formamido-3-methylphenyl)pentanoate (5c)

Ozonolysis of **5b** (11 g) in 110 ml MeOH as described for **3a** gave 6.1 g (55%) of the product as a semisolid. Recrystallization from 2-propanol gave 4.5 g of the product as a pale yellow solid, mp 183–185 °C. ^{1}H NMR: $CDCl_3$, δ 9.23 (1H, s), 8.30 (1H, s), 7.71 (1H, d, J = 7 Hz), 7.46 (1H, d, J = 8 Hz), 7.28 (1H, t, J = 7.6 Hz), 4.28 (2H, s), 4.26 (4H, q, J = 7 Hz), 2.28 (3H, s), 1.96 (3H, s), 1.25 (6H, t, J = 7 Hz); ^{13}C NMR: $CDCl_3$, δ 13.9, 19.5, 22.9, 43.9, 62.9, 63.9, 125.5, 126.2, 128.0, 128.5, 130.3, 136.2, 158.9, 167.2, 169.7, 200.1. MS (EI): 162 (100), 232 (20), 259 (18), 392 (8, M^+).

2.5.8. 3-Chloro-DL-kynurenine (3)

Compound **3c** (4.0 g) in 40 ml of 6 N HCl was refluxed for 4 h, then cooled to ambient temperature, and concentrated in vacuo. The resulting semisolid was dissolved in 20 ml H_2O, treated with charcoal at ambient temperature for 15 min, and filtered through Celite. The filtrate was brought to pH 6.5 using 2 N NaOH, when a solid precipitated. The solid 3-chloro-DL-kynurenine was collected by filtration, washed with about 5 ml H_2O, and allowed to air dry overnight. Yield, 1.2 g (48%), mp 216–218 °C. ^{1}H NMR: (1% DCl/D_2O) δ 7.7 (1H, d, J = 8 Hz), 7.6 (1H, d, J = 8 Hz), 6.7 (1H, t, J = 8 Hz), 4.2 (1H, t, J = 5 Hz), 3.7 (2H, m); ^{13}C NMR: (1% DCl/D_2O) δ 43.2, 55.4, 119.1, 121.8, 123.3, 125.2, 131.4, 143.6, 172.1, 204.2.

2.5.9. 3-Fluoro-DL-kynurenine (4)

Compound **4c** (3.5 g) was hydrolyzed in 32 ml of 6 N HCl and worked up as described for **3c**. Yield, 0.65 g (31%), mp 205–210 °C. ^{1}H NMR: (1% DCl/D_2O) δ 7.48 (1H, d, J = 7.2 Hz), 7.10 (2H, m), 4.10 (1H, t, J = 5.0 Hz), 3.49 (2H, d, J = 4.7 Hz); ^{13}C NMR: (1% DCl/D_2O) δ 46.5, 53.2, 119.2, 121.6, 123.1, 128.2, 140.6, 159.2, 176.5, 204.2; ^{19}F NMR: MeOH-d_4, δ −126 (dd).

2.5.10. 3-Methyl-DL-kynurenine (5)

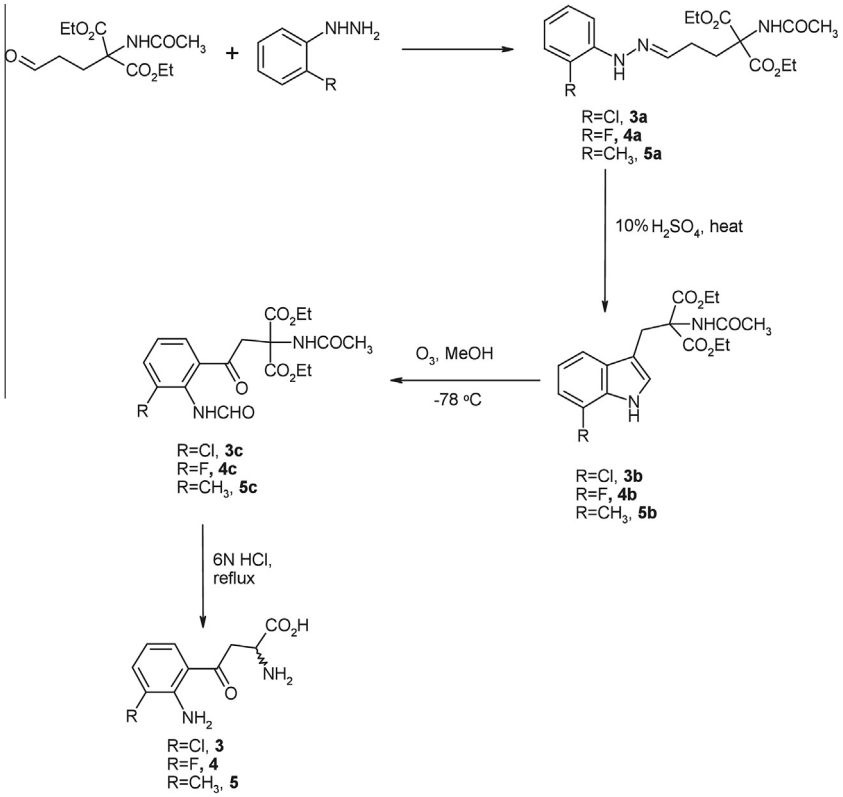
Compound **5c** (6.1 g) was hydrolyzed in 54 ml of 6 N HCl and worked up as described for **3c**. Yield, 2.1 g (75%) of a yellow solid, mp 215–217 °C. ^{1}H NMR: (1% DCl/D_2O) δ 7.46 (1H, d, J = 7.6 Hz), 7.10 (1H, d, J = 7.2 Hz), 6.97 (1H, t, J = 7.6 Hz), 4.00 (1H, t, J = 5.2 Hz), 3.43 (2H, d, J = 5.2 Hz), 1.81 (3H, s); ^{13}C NMR: (1% DCl/D_2O) δ 16.2, 39.1, 47.2, 126.6, 129.3, 130, 134.3, 138, 142.4, 170.6, 201.

2.5.11. 3-Bromo-L-kynurenine (6)

3-Bromo-L-kynurenine was prepared as described previously.[18]

2.5.12. 5-Bromo-L-kynurenine (7)

5-Bromo-L-kynurenine was prepared using our previously published procedure.[18]



Scheme 1. Synthesis of 3-substituted kynurenines.

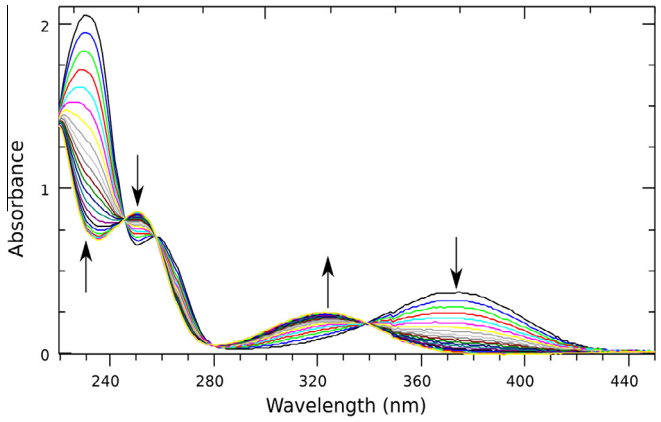


Figure 1. Scanning kinetics for 0.1 mM 5-bromo-L-kynurenine reaction with HsKynase. The arrows indicate the direction of absorbance change in the reaction.

2.5.13. 5-Chloro-*N*,*N*$^{\alpha}$-diacetyl-L-kynurenine methyl ester (8a)

N,*N*$^{\alpha}$-Diacetyl-L-kynurenine methyl ester (2 g), prepared by ozonolysis of *N*-acetyl-L-tryptophan methyl ester and acetylation,[18] was dissolved in 40 ml acetic acid and stirred at room temperature. A solution of 2 g N-chlorosuccinimide dissolved in 12 ml HCl–saturated AcOH was added, and the reaction was stirred at room temperature for 1 h. The reaction was quenched with a solution of 6 g sodium bisulfite dissolved in 24 ml water. The reaction was then extracted with two 40 ml portions of chloroform, and the combined organic layers were washed with 40 ml water and then with 40 ml brine. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed in vacuo to give a semisolid, which was recrystallized from 2-propanol to give the product as pale yellow needles. Yield, 1.6 g (72%), mp, 185–187 °C. ^{1}H NMR: $CDCl_3$ δ 11.35 (1H, br s), 8.76 (1H, d, J = 9 Hz), 7.84 (1H, s, J = 2.4 Hz), 7.53 (1H, dd, J = 9, 2.4 Hz), 6.50 (1H, d, J = 7.6), 4.96 (1H, dd, J = 11.6, 4 Hz), 3.80 (1H, m), 3.77 (3H, s), 3.67 (1H, m), 2.24 (s, 3H), 2.05 (s, 3H) ^{13}C NMR: $CDCl_3$ δ 22.8, 24.8, 40.5, 51.3, 54.6, 121.2, 122.3, 128.5, 129.8, 134.3, 135.6, 168.5, 169.3, 170.1, 200.3.

2.5.14. 5-Chloro-L-kynurenine (8)

5-Chloro-*N*$^{\alpha}$,*N*-diacetyl-L-kynurenine methyl ester (1.6 g) was refluxed in 15 ml of 6 N HCl for 4 h. After concentration, the resulting semisolid was taken up in 12 ml water, and treated with charcoal at room temperature for 10 min. The solution was filtered through Celite, and the filtrate was brought to pH 6.5 with 6 N NaOH, when the product precipitated as a pale yellow solid. Yield, 0.70 g (61%), mp, 216–218 °C. ^{1}H NMR: (1% DCl–D_2O) δ 8.05 (1H, d, J = 2.4 Hz), 7.61 (1H, dd, J = 9, 2.6 Hz), 7.27 (1H, d, J = 8.2 Hz), 4.44 (1H, t, J = 8.7 Hz), 3.80 (2H, m) ^{13}C NMR: (1% DCl–D_2O) δ 41.8, 53.6, 118.2, 120.1, 125.3, 130.5, 137.8, 150.1, 170.5, 201.8.

2.5.15. 3,5-Dibromo-L-kynurenine (9)

L-Kynurenine (30.0 mg, 0.144 mmol) and NaOAc (26 mg) were dissolved in 1 ml AcOH, and 16.4 μL of Br_2 was added with stirring. Within an hour, the orange color disappeared and a yellow precipitate formed. The reaction was diluted with water to about 10 ml after 4 h, then filtered to give a light yellow solid, which was washed with water and dried in vacuo over P_2O_5 to give 43.3 mg (82%). ^{1}H NMR: D_2O/DCl, δ 7.97 (1H, d, J = 2 Hz), 7.82 (1H, J = 2 Hz), 4.40 (1H, t, J = 5 Hz), 3.75 (2H, m). MS (ESI): 364.8 (50), 366.8 (100), 368.8 (50), $(M+1)^{+}$.

2.5.16. 5-Bromo-3-chloro-DL-kynurenine (10)

3-Chloro-DL-kynurenine (**3**) (50.0 mg, 0.21 mmol) and NaOAc (19 mg) were suspended in 1.5 ml AcOH, and 11.9 μL of Br_2 was added with stirring. Within an hour, the orange color disappeared and a thick yellow precipitate formed. After 4 h, the reaction

mixture was diluted with water to about 10 ml, then filtered to give a light yellow solid, which was washed with water and dried in vacuo over P_2O_5 to give 54.9 mg (82%). ^{1}H NMR: D_2O/DCl, δ 7.74 (1H, d, J = 2.6 Hz), 7.52 (1H, d, J = 2.6 Hz), 4.32 (1H, t, J = 5 Hz), 3.64 (2H, m). MS (ESI): 320.8 (80), 322.8 (100), 324.8 (25), $(M+1)^+$.

2.6. Modeling of 3,5-dibromo-L-kynurenine in HsKynase

The structure of HsKynase with bound 3-hydroxyhippuric acid (PDB accession number 3E9K) was used to generate the model of 3,5-dibromo-L-kynurenine bound to HsKynase (Figs. 2 and 3). The stucture of the ligand was modified using PyMOL[22] to that of 3,5-dibromo-L-kynurenine, by substitution of bromine at the 5-position, adding the α-amino group, changing the 3-OH to a bromine, and changing the amide nitrogen to a methylene carbon. In a docking experiment, the structure of HsKynase (PDB accession number, 2HZP) was used to dock 3,5-dibromo-L-kynurenine using Autodock-Vina[23] using PyMOL with the PyMOL Autodock-Vina plugin.

3. Results and discussion

3.1. Synthesis of kynurenine analogues

Electrophilic substitution of kynurenine selectively at the 3-position, *ortho* to the amino group, is problematic. Previously, we tried to block the 5-position by iodination,[18] but still obtained a mixture of 3-bromo-5-iodo and 3,5-dibromo products after bromination. Acid-catalyzed hydrolysis and protiodeiodination of the former product gave a mixture of 3-bromokynurenine and 3,5-dibromokynurenine, which were separated by reversed-phase chromatography.[18] In the present work we have developed a novel route to 3-substituted kynurenines which starts from the readily available *ortho*-substituted anilines, and uses a Fischer cyclization of a phenylhydrazone to introduce the side chain, similar to older methods for synthesis of substituted tryptophans.[24] We found that increasing the aqueous sulfuric acid to 10% in the Fischer cyclization, rather than the 5% in the published procedure,[24] gave higher yields of indole product. Ozonolysis of the indole product, followed by hydrolysis and decarboxylation, provides the racemic 3-substituted kynurenines in good yield (Scheme 1). In contrast to the 3-position, direct halogenation of kynurenine at the 5-position, *para* to the amino group, is facile, so we brominated and chlorinated suitably protected L-kynurenine, prepared by ozonolysis of the corresponding protected L-tryptophan, to obtain 5-bromo and 5-chlorokynurenines, similar to our previous procedure.[18] Our route is more efficient than the previously published route to 5-chloro-L-kynurenine, which involved synthesis of the racemate and resolution.[25] 3,5-Dibromo-L-kynurenine and 5-bromo-3-chloro-DL-kynurenine were prepared by direct bromination of L-kynurenine and 3-chloro-DL-kynurenine with 2 or 1 equiv, respectively, of Br_2 in glacial acetic acid. All of the kynurenines used in the kinetic studies were found to be at least 95% pure by HPLC analysis (see Supplementary data).

3.2. Kinetic analysis of kynurenine analogues

All of the analogues were examined as substrates for HsKynase and PfKynase by repetitive UV/visible scanning of solutions. A representative scanning kinetic graph for the reaction of 100 μM 5-bromo-L-kynurenine with HsKynase is shown in Figure 1. The reaction shows a time-dependent decrease in the substrate absorbance at λ_{max} of 370 nm, an increase in product absorbance at 320 nm, and clear isosbestic points at 245, 258, 280, and 338 nm, indicating that there are only two absorbing species in the reaction, the reactant, 5-bromo-L-kynurenine, and the product, 5-bromoanthranilate. The absorbance difference at 370 nm between the

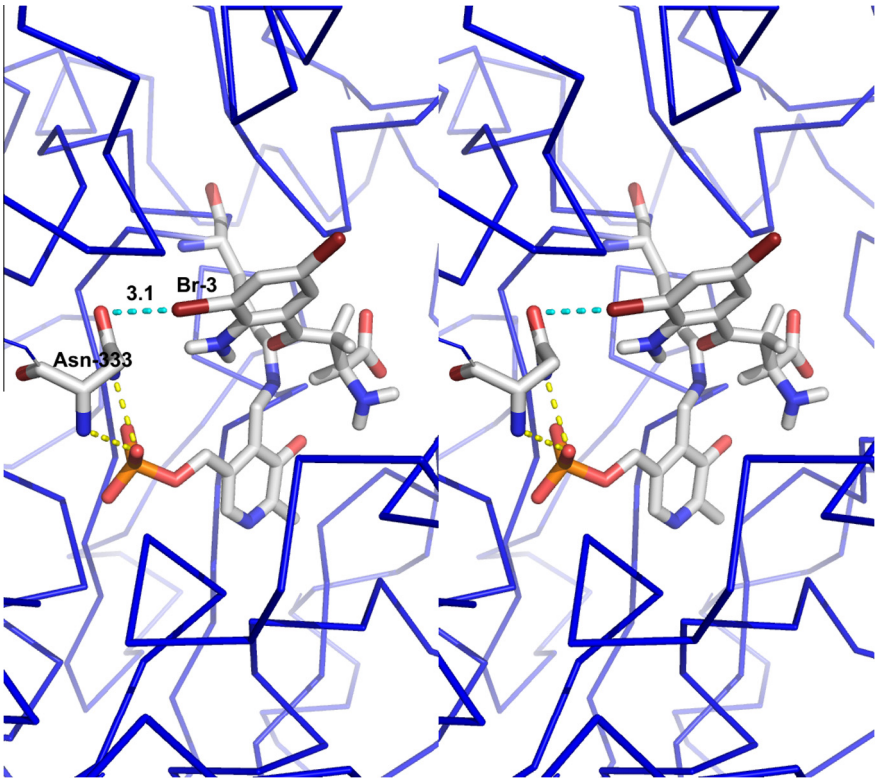


Figure 2. Model of 3,5-dibromo-L-kynurenine in the active site of HsKynase. Crossed-eye stereo view showing the PLP, Asn-333, and the bound substrate. The distance between the Asn-333 γ-carbonyl-O and the 3-bromo substituent is 3.1 Å. Hydrogen bonds are shown in yellow, and the proposed halogen bond in cyan. The figure was created with PyMOL.[22]

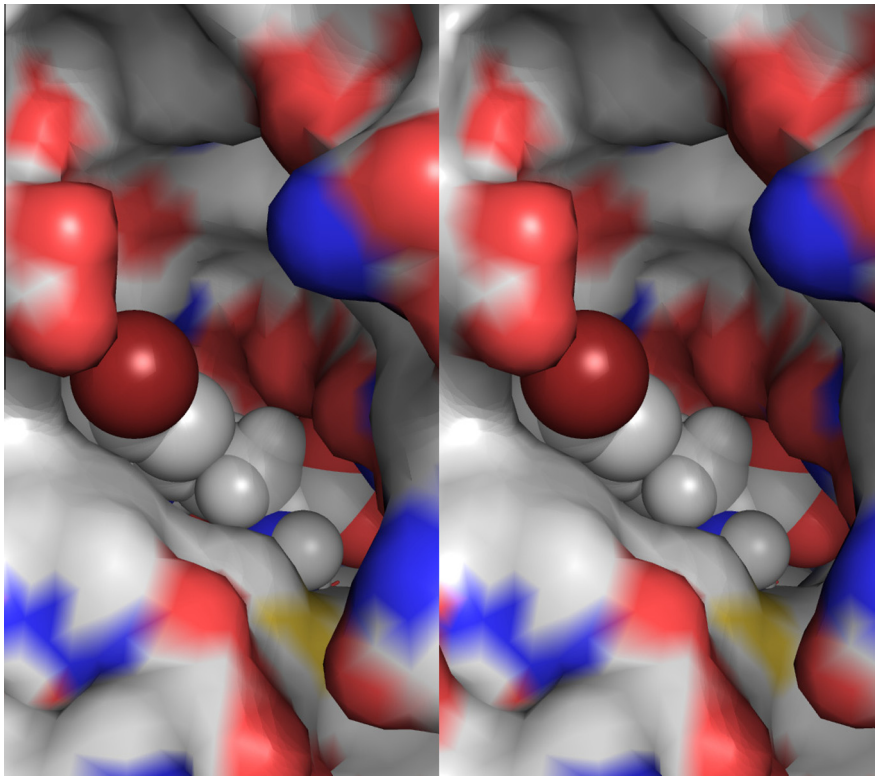


Figure 3. Model of 3,5-dibromo-L-kynurenine in the active site of HsKynase. Crossed-eye stereo view of the active site surface with 3,5-dibromo-L-kynurenine shown in spheres. The 5-bromo substituent can be seen as the red sphere projecting up out of the active site into the channel. The figure was created with PyMOL.[22]

initial and final spectra were used to determine the value of $\Delta\varepsilon$ (Table 1). All of the compounds examined showed a decrease in the kynurenine substrate λ_{max} at 360–379 nm and an increase in the anthranilate product λ_{max} at 308–336 nm (Table 1). The other substrates were analyzed in the same way to obtain $\Delta\varepsilon$ for each compound, the values of which are listed in Table 1.

In previous work, we compared the reactivity of wild-type HsKynase and several active site mutants with L-kynurenine and 3-hydroxy-DL-kynurenine.[3] We found that wild-type HsKynase has about a 265-fold preference in k_{cat}/K_m for 3-hydroxy-DL-kynurenine (Table 2). In contrast, PfKynase shows a 24-fold preference for L-kynurenine over 3-hydroxy-DL-kynurenine (Table 3). All of the substituted kynurenines that we studied in the present work are substrates for both HsKynase and PfKynase, with varying degrees of efficiency. However, there are some interesting similarities and differences in the effects of substituents and positions for the two enzymes. Although some compounds studied were racemates, we found previously that the K_m value for racemic β-benzoylalanine is twice that of the L-enantiomer, indicating that the D-enantiomer does not significantly inhibit.[26] That the D-isomers are also not substrates is supported by the results of the scanning kinetics experiments described above, where for the DL compounds the absorbance decreases are half of the initial substrate absorbance. For those substrates, the $\Delta\varepsilon$ was calculated based on the L-amino acid concentration.

Bulky substitutents at the 3-position of kynurenine result in lower k_{cat} and k_{cat}/K_m values for PfKynase (Table 3), but higher values for HsKynase (Table 2). This is consistent with the known preference for kynurenine over 3-hydroxykynurenine for PfKynase,[1,3] and vice-versa for HsKynase. However, 3-F-kynurenine exhibits kinetic parameters similar to those of kynurenine for both PfKynase and HsKynase. Thus, the human enzyme seems to prefer a large, polar substituent at the 3-position, and in this case fluorine does

Table 2
Substrates for human kynureninase

Kynurenine substituent	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Relative k_{cat}/K_m
None (**1**)[a]	0.23	465	1
3-Hydroxy-DL (**2**)[a]	3.5	1.23×10^5	265
3-Chloro-DL (**3**)	0.67 ± 0.063	$(8.2 \pm 1.3) \times 10^3$	18
3-Fluoro-DL (**4**)	0.23 ± 0.022	$(2.7 \pm 0.5) \times 10^3$	6
3-Methyl-DL (**5**)	0.33 ± 0.025	$(1.8 \pm 0.2) \times 10^3$	4
3-Bromo-L (**6**)	1.9 ± 0.3	$(4.4 \pm 0.3) \times 10^3$	10
5-Bromo-L (**7**)	0.63 ± 0.022	$(1.5 \pm 0.1) \times 10^4$	33
5-Chloro-L (**8**)	0.47 ± 0.022	$(1.1 \pm 0.1) \times 10^4$	24
3,5-Dibromo-L (**9**)	1.2 ± 0.1	$(7.9 \pm 1.3) \times 10^4$	170
5-Bromo-3-chloro-DL (**10**)	1.3 ± 0.1	$(6.5 \pm 2.0) \times 10^4$	140

[a] From Ref. 3.

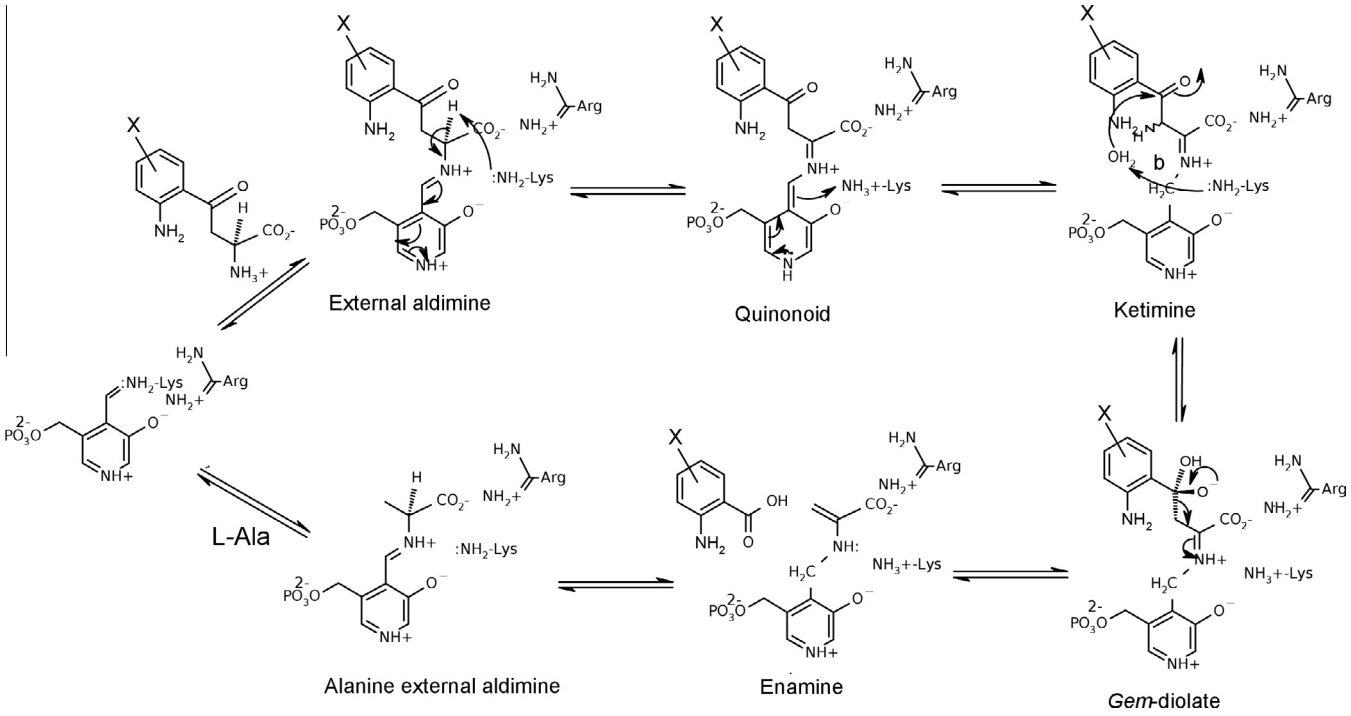
Table 3
Substrates for *P. fluorescens* kynureninase

Kynurenine substituent	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Relative k_{cat}/K_m
None (**1**)[a]	16	6×10^5	1
3-Hydroxy-DL (**2**)[a]	1	2.5×10^3	0.042
3-Chloro-DL (**3**)	0.71 ± 0.042	$(1.1 \pm 0.1) \times 10^4$	0.018
3-Fluoro-DL (**4**)	6.9 ± 0.5	$(9.0 \pm 0.8) \times 10^4$	0.15
3-Methyl-DL (**5**)	1.5 ± 0.1	$(2.2 \pm 0.3) \times 10^4$	0.037
3-Bromo-L (**6**)[b]	11	5.5×10^3	0.0092
5-Bromo-L (**7**)	11.9 ± 0.7	$(3.3 \pm 0.9) \times 10^6$	5.5
5-Chloro-L (**8**)	8.7 ± 0.4	$(1.8 \pm 0.3) \times 10^6$	3.0
3,5-Dibromo-L (**9**)	0.23 ± 0.01	$(2.1 \pm 0.5) \times 10^4$	0.035
5-Bromo-3-chloro-DL (**10**)	0.30 ± 0.02	$(2.2 \pm 0.5) \times 10^4$	0.037

[a] From Ref. 30.
[b] From Ref. 18, at 25 °C.

not substitute well for oxygen, despite their similar steric and electronic properties. In contrast, the catalytic efficiency of the bacterial enzyme is affected the least by introducing a 3-F substituent, and all larger 3-substituents show dramatic decreases in reactivity (Table 3). We demonstrated previously that there is a potential steric clash between the 3-substituent of the substrate and Trp-102 in PfKynase,[3] which could explain the effect of substituents. For PfKynase, the catalytic efficiency is better for the 3-methyl rather than a 3-Cl or 3-Br substituent. For HsKynase, the k_{cat}/K_m value is the least for 3-methyl than any other 3-substituent, but it is still fourfold better than unsubstituted kynurenine. The relative k_{cat}/K_m values for 3-chloro-DL-kynurenine and 3-bromo-L-kynurenine are 17.5 and 8.6 for HsKynase, respectively (Table 2). Thus, having a Cl or Br substituent at the 3-position of kynurenine increases the substrate activity for HsKynase much more than smaller or nonpolar substituents, such as methyl or F. In previous structural work, we found that the 3-OH of an inhibitor, 3-hydroxyhippurate, can form a hydrogen bond with the γ-carbonyl O of Asn-333 in the active site of HsKynase.3 Previously, we had found a similar result in docking 3-hydroxykynurenine into the active site of HsKynase.[2] The roughly 10-fold increase in k_{cat}/K_m for HsKynase obtained by substituting a chlorine or bromine at the 3-position of kynurenine suggests that halogen bonding[27,28] may be occurring. This is consistent with a model of 3,5-dibromokynurenine in the active site of Hskynase, generated from the 3-hydroxyhippurate structure, which shows there is 3.1 Å between the γ-carbonyl O of Asn-333 and the 3-Br substituent of 3,5-dibromokynurenine (Fig. 2), an optimal distance for halogen bonding.[27] Halogen bonding involves a donor electron-rich atom interacting with the positive end of the C–X bond, the so-called 'sigma hole'.[28] Halogen bonding is minimal with fluorine, and maximal with bromine and iodine.[27,28] The smaller positive effects of fluorine and methyl substituents at C-3 for k_{cat}/K_m with HsKynase may be due to van der Waals forces or hydrophobic interactions. We note that 3-chlorokynurenine is a relatively weak inhibitor of rat kynurenine aminotransferase.[25]

In contrast to the 3-position, chloro and bromo substituents at the 5-position have stimulating effects on k_{cat}/K_m for both PfKynase and HsKynase (Tables 2 and 3). 5-Halokynurenines are also good inhibitors of rat kynurenine aminotransferase.[25] We found that 5-bromokynurenine was an excellent substrate for PfKynase in our previous work.[18] The values of k_{cat} and k_{cat}/K_m found in the present work are significantly higher than those reported previously, at least in part because of the higher temperature (37 °C vs 25 °C) used in this work. However, there is a significantly larger increase in k_{cat}/K_m (20- to 30-fold) for HsKynase than for PfKynase (3- to 5-fold) with the 5-halo derivatives. The effects of halogenation at the 3- and 5-positions are at least partially additive, since 3,5-dibromo-L-kynurenine and 5-bromo-3-chloro-DL kynurenine are excellent substrates for HsKynase, with k_{cat}/K_m values higher than any other substrate except the physiological substrate, 3-OH-kynurenine (Table 2). The effect of the 5-halo substituent on activity with both kynureninases is likely due to van der Waals contacts and/or hydrophobic effects, since these substituents occupy the tunnel leading from the active site to the outside, as can be seen from the model of 3,5-dibromokynurenine in the active site of HsKynase (Fig. 3). In addition, electronic effects may contribute to the effects of substituents on the activity with HsKynase (Fig. 4), since there is a reasonable Hammett correlation of the log(relative k_{cat}/K_m) for all substituents at both C-3 and C-5, with $\rho = +1.73 \pm 0.74$, with the major outlier being the physiological 3-OH substituent, probably as a result of hydrogen bonding. Both C-3 and C-5 substituents are *meta* to the reactive substrate carbonyl, so σ_m values were used in creating the plot. However, there is no significant correlation for the substituent effects on PfKynase activity ($\rho = -0.55 \pm 1.42$), consistent with steric effects playing a predominant role at C-3, and van der Waals and/or hydrophobic effects at C-5. We found previously that 4-substituted (*para* to the reactive carbonyl) β-benzoylalanines, which exhibit rate-determining formation of benzoate products, show a nonlinear Hammett relationship for k_{cat} and k_{cat}/K_m with PfKynase, indicating a change in rate-determining step with substituents.[29] We interpreted this as resulting from electron-withdrawing substituents favoring formation of the gem-diol intermediate (Scheme 2), but reducing its breakdown to the carboxylic acid product. Conversely, for electron-donating substituents, the formation of the gem-diol is reduced, but its breakdown is increased. In contrast, for kynuren-



Scheme 2. Mechanism of kynureninase.

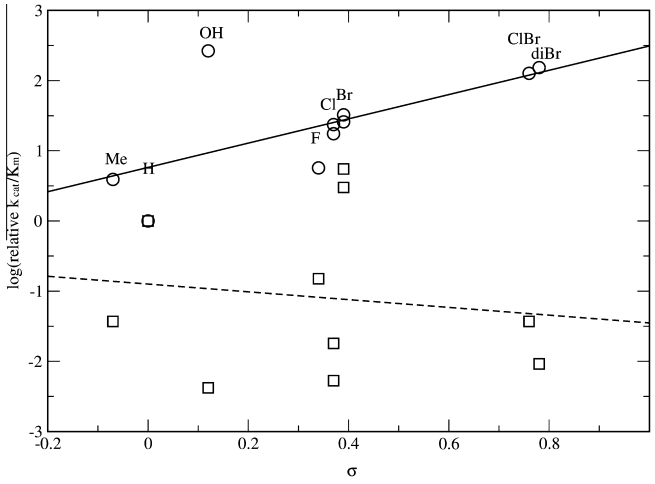


Figure 4. Hammett plot of log(relative k_{cat}/K_m) for PfKynase (squares) and HsKynase (circles). The lines are the regression fits, with $y = +1.73 \times x + 0.76$ (solid line) for HsKynase and $y = -0.55 \times x - 0.90$ (dashed line) for PfKynase.

ine with PfKynase, the rate-determining step is release of the second product, Ala, not the first product, anthranilate.[30] However, in the present work the effects of halogenation on the reactivity of HsKynase substrates (Fig. 4) suggests that the formation of the gem-diol may be rate-determining.

4. Conclusion

These results will be useful in the design of potent and specific inhibitors of HsKynase or PfKynase that may be useful as drugs. Since the known potent inhibitors of PfKynase and HsKynase are proposed to be transition state analogues,[18,31–33] the effects of substituents on k_{cat}/K_m are expected to correlate better with inhibitory potency than K_m.[34] Thus, halogen substituents in the 5-position are well tolerated by both enzymes, which is consistent with the X-ray structures. In our previous work, we found that both diastereomers of 5-bromodihydrokynurenine inhibit better than the corresponding dihydrokynurenines.[18] Furthermore, our result imply that in the design of inhibitors for HsKynase, a hydroxyl group at the 3-position is not absolutely necessary for good activity, since the 3,5-dihalo analogues have excellent substrate activity. Since kynurenine aminotransferase is inhibited well by 5-halokynurenines, but not 3-halokynurenines, it is likely that compounds can be made that are selective for HsKynase inhibition. The application of these results to selective inhibitor design is currently underway in our laboratory.

Supplementary data

Supplementary data (^{1}H NMR, MS and HPLC chromatograms of the final compounds used in the kinetics studies) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.039.

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