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Research article

Comparative inhibition by substrate analogues 3-methoxy- and 3-hydroxydesaminokynurenine and an improved 3 step purification of recombinant human kynureninase.

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Abstract

Background: Kynureninase is a key enzyme on the kynurenine pathway of tryptophan metabolism. One of the end products of the pathway is the neurotoxin quinolinic acid which appears to be responsible for neuronal cell death in a number of important neurological diseases. This makes kynureninase a possible therapeutic target for diseases such as Huntington's, Alzheimer's and AIDS related dementia, and the development of potent inhibitors an important research aim.

Results: Two new kynurenine analogues, 3-hydroxydesaminokynurenine and 3-methoxydesaminokynurenine, were synthesised as inhibitors of kynureninase and tested on the tryptophan-induced bacterial enzyme from *Pseudomonas fluorescens*, the recombinant human enzyme and the rat hepatic enzyme. They were found to be mixed inhibitors of all three enzymes displaying both competitive and non competitive inhibition. The 3-hydroxy derivative gave low K_i values of 5, 40 and 100 nM respectively. An improved 3-step purification scheme for recombinant human kynureninase was also developed.

Conclusion: For kynureninase from all three species the 2-amino group was found to be crucial for activity whilst the 3-hydroxyl group played a fundamental role in binding at the active site presumably via hydrogen bonding. The potency of the various inhibitors was found to be species specific. The 3-hydroxylated inhibitor had a greater affinity for the human enzyme, consistent with its specificity for 3-hydroxykynurenine as substrate, whilst the methoxylated version yielded no significant difference between bacterial and human kynureninase. The modified purification described is relatively quick, simple and cost effective.

Background

Kynureninase (EC 3.7.1.1) is a pyridoxal-5'-phosphate (PLP) dependent enzyme which catalyses the β, γ-hydrolytic cleavage of the amino acids kynurenine (**1**, R = H) and 3-hydroxykynurenine (**1**, R = OH) to give either anthranilic acid (**2**, R = H) or 3-hydroxyanthranilic acid (**2**, R=OH) and alanine (**3**) (Figure 1) [1].

It is one of the enzymes in the kynurenine pathway of tryptophan metabolism [2], a pathway which is currently eliciting considerable interest due to the neurological activities of some of its endproducts particularly the excitotoxin quinolinic acid [3]. Quinolinic acid is an agonist of NMDA (N-methyl-D-aspartate) receptors and increased levels of quinolinic acid cause overstimulation resulting

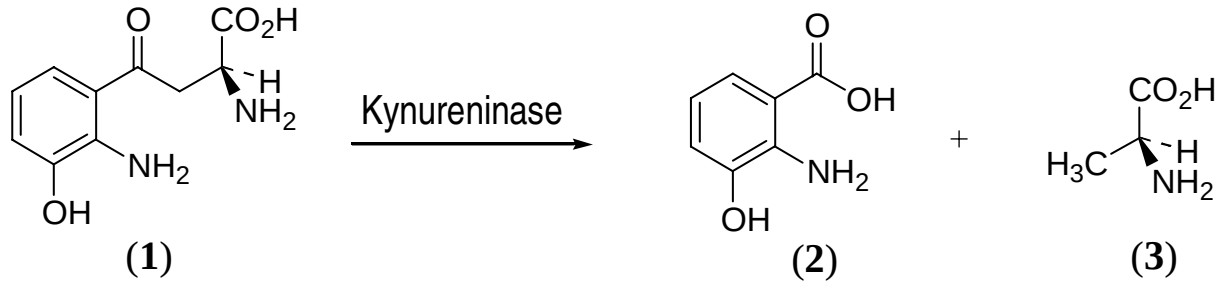

Figure 1
Reaction catalysed by kynureninase

in eventual neuronal cell death [1,3]. This phenomenon has been demonstrated in patients that are neurophysiologically impaired and manifest supraphysiological levels of this potent neurotoxin in their brain and spinal column fluids, although it is still unclear whether the levels can become high enough to be excitotoxic [4]. The design and synthesis of potent and selective inhibitors of kynureninase could prove to be useful in the development of the successful treatment regimen for neurological disorders such as septicemia, AIDS (Acquired Immune Deficiency Syndrome) related dementia, Lyme disease, Huntington's and Alzheimer's disease[1,4].

In our laboratory, we have synthesised a series of substrate analogues of kynurenine as inhibitors of the enzyme. We report here, the results of our studies using two of these compounds, methoxylated and hydroxylated desaminokynurenine derivatives, as inhibitors of the bacterial enzyme (*Pseudomonas fluorescens*), rat hepatic kynureninase and recombinant human enzyme, expressed in a baculovirus/insect cell system. In an earlier report [5] from our laboratory, the findings indicated that the dihydroxydesamino derivative markedly inhibited both mammalian enzymes with a $K_i \approx 250$ nM. This finding thus prompted additional research into the effects of the monohydroxy substituted desaminokynurenine. It is known that the bacterial and mammalian enzyme differ in their substrate-selectivity [6] and therefore it is possible that the potency of inhibitory compounds is species dependent. In addition an improved 3 step purification of the recombinant human enzyme is also outlined.

Results

Purification

Recombinant human kynureninase was successfully purified to homogeneity in a modified three step procedure (Figure 2).

Synthesis of novel inhibitors

The two new inhibitors, 3-hydroxydesaminokynurenine (4) and 3-methoxydesaminokynurenine (5) (Figure 3) were successfully synthesised using adaptations of previously reported methods [7]. The spectral data for the two final inhibitors confirmed their structures and analysis by reverse phase HPLC was used to confirm their purity.

Inhibition studies

From the results obtained in table 1 it is clear that there is significant difference in the degree of inhibition with the hydroxylated when compared to the methoxylated inhibitor. The type of inhibition is also mixed in all instances as indicated by the Lineweaver-Burk (Figure 4) and Dixon plots (Figure 5) [7]. Previously [8] it has been shown that the recombinant human enzyme is also inhibited similarly when treated with 3,5-dihydroxydesaminokynurenine, which was also the case with both inhibitors, used in this study.

Discussion

The improved purification of recombinant human kynureninase has allowed the rapid production of stocks of purified enzyme for inhibition studies. The 3 step purification is a significant improvement over the previously published procedure [9] which involved 6 steps. This pro-

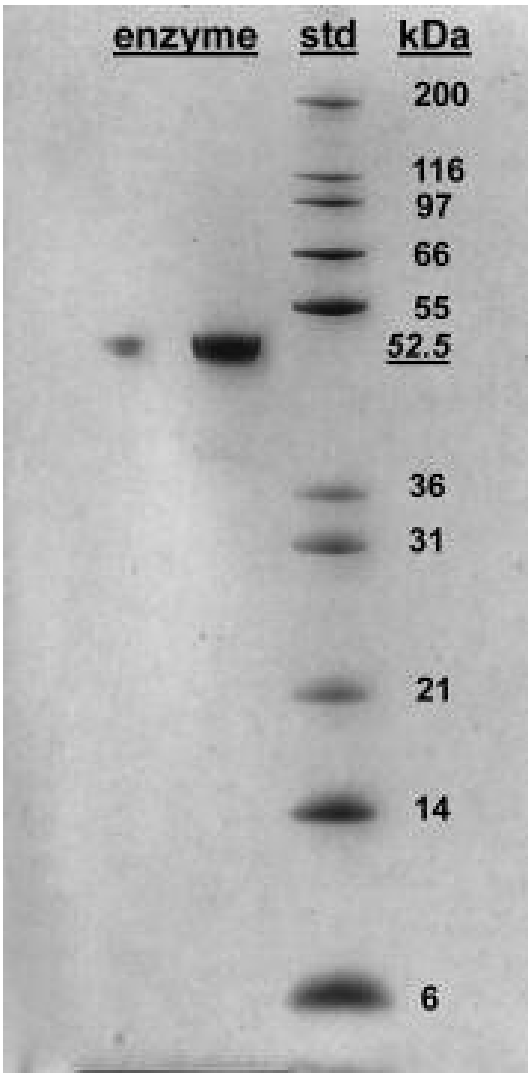

Figure 2
Discontinuous gel electrophoresis of human kynureninase. 10% SDS-Page gel image of purified recombinant kynureninase (20 μg) at 52.4 kDa in the presence of PLP. This NuPAGE Novex Bis-Tris Gel (prepacked) was produced using the Xcell SureLock Mini-Cell from Invitrogen. The pertained Mark 12 standards were also acquired from Invitrogen. Run conditions were 200 V (constant)/35 minute in MES buffer with expected current of 100–125 mA/gel at start going to 60–80 ma/gel at the end.

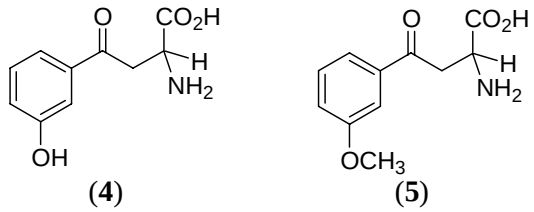

Figure 3
Structures of new inhibitors

Table 1: Comparative inhibition of kynureninase from different sources by synthetic substrate analogues.

Source	K_I (4) / (nM)	K_I (8) / (μM)
Bacterial	100	10
Rat	40	175
Human	5	15

The inhibition constants (K_I) were determined from the figures 4 and 5 for 3-hydroxydesaminokynurenine **(4)** and those for 3-methoxydesaminokynurenine **(5)** were determined in a similar fashion (graphs not shown).

tocol can be performed in less than 60 hrs thereby reducing the overall time involved in keeping the enzyme at 4°C. Other major advantages are the elimination of one of the harsh strong anion steps and the time consuming ammonium sulfate precipitation, that invariably result in significant losses of enzyme. The net result is a substantial increase in the overall yield of kynureninase (up to 3 fold) when compared to first published protocol [9].

Human kynureninase was previously shown to possess two substrate binding sites [9], a regulatory non-catalytic site plus catalytic site, and it can be assumed that it is this phenomenon that determines the mixed pattern of inhibition common to all three enzymes. The two inhibitors reflect interesting differences in their affinity for kynureninase from the three sources. It is clear that racemic 3-hydroxydesaminokynurenine (4) shows most potent inhibition with the recombinant human kynureninase giving a K_i of 5 nM (Table 1; fig. 5 inset). This is in good agreement with the observed specificity of the human enzyme, which was found to be completely specific for 3-hydroxykynurenine (1, R = OH) (K_m = 3.0 ± 0.10 μM) with no appreciable substrate activity for kynurenine. The hydroxyl group at the 3-position thus appears to be an important recognition element. When this hydroxyl group is then methylated to give (5) there is a very signif-

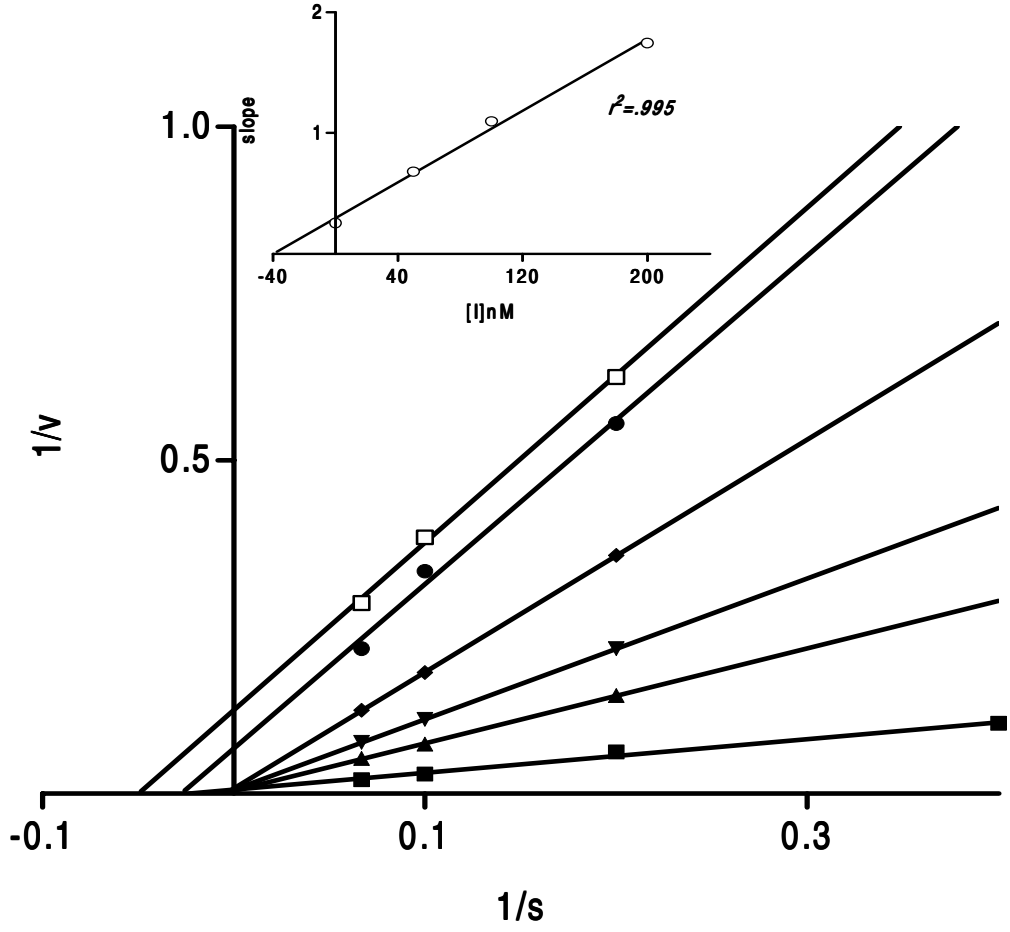

Figure 4
Inhibition of rat hepatic kynureninase by 3-hydroxydesaminokynurenine (4) Primary Lineweaver Burk (L/B) plot of kinetic data for inhibition of rat hepatic kynureninase by 3-hydroxydesaminokynurenine (**4**) (I = 0 (■); I = 50 nM (▲) I = 100 nM (▼); I = 200 nM (◄ ►); I = 400 nM (●); I = 600 nM (□)) depicting mixed inhibition. s = substrate (3-hydroxykynurenine) and v = specific activity. The inset is a secondary plot of slope against [I] to determine the K_i (40 nM). The slopes were calculated from a L/B plot (n = 3).

icant, 3000-fold, decrease in inhibitor binding. Such a decrease would seem to imply that the hydroxyl group is acting as a hydrogen bond donor, which is not possible once it has been methylated. Indeed the observed value for the K_i for the 3-methoxy derivative (15 μM) (table 1) is similar to that for kynurenine (D-enantiomer = 12 μM, L-enantiomer = 20 μM) [10], which has no 3-substituent at all. This proposed binding role for the 3-hydroxyl group is further supported by the observation that another mixed inhibitor of the bacterial enzyme, desami-

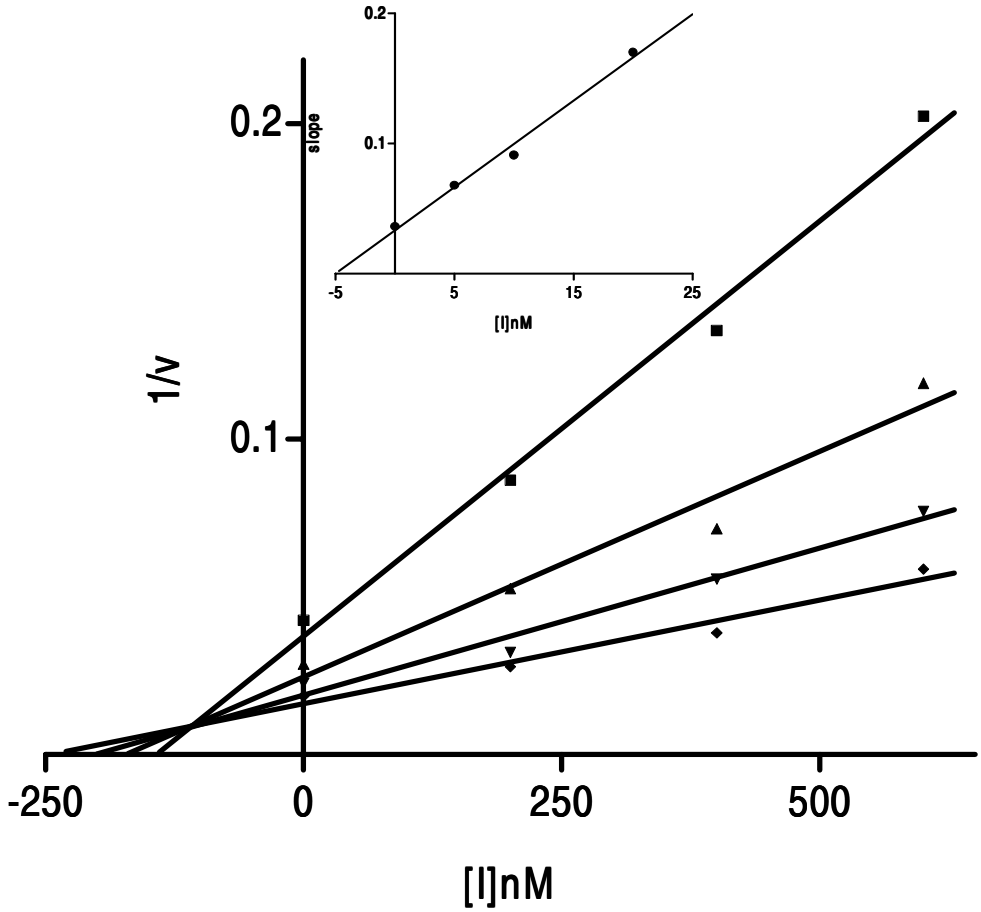

Figure 5
Inhibition of bacterial kynureninase by 3-hydroxydesaminokynurenine (4) Dixon plot illustrating inhibition of bacterial kynureninase by 3-hydroxydesaminokynurenine (**4**) (I = 0 (◆); I = 200 nM (▼); I = 400 nM (▲); I = 600 nM (■). The inhibition is mixed and gives a K_I = 100 nM and the graph is a replot of the data (n = 3) used to construct a Michael's–Menten plot. The inset is a secondary plot of slope (from L/B) against inhibitor concentration for recombinant human kynureninase (n= 3 and r^2 = 0.99) to calculate K_I (5 nM). The concentration of substrate was varied between 2.5 μM – 20 μM. Lines were fitted to a straight line equation for linear regression.

nokynurenine, also had a K_I of 12 ± 3.1 μM (unpublished results). The data also imply, as we have previously noted, that the 2-amino group does not have an important role in active site binding but appears to be required for activity and that interactions with the aromatic ring are necessary for both binding and activity. This conclusion is supported by the observation that cyclohexyl substrate analogue derivatives, when tested on the bacterial enzyme, manifested exceptionally reduced affinities for the active site with a K_I of 422 ± 47 μM (unpublished).

In the case of the rat and *Pseudomonas* enzymes, activity has been reported with both 3-hydroxykynurenine and L-kynurenine. The rat enzyme gives K_m values of 5 μM and 500 μM respectively, with values of 200 μM [8] and 44.2 μM [11] determined for the *Pseudomonas* enzyme. There

does appear to be a correlation between the K_i and K_m values in terms of modifications to the ring for both the bacterial and rat enzymes. The observed pattern is the same as with the human enzyme. In the case of the bacterial enzyme, removal of the 2-amino group and adding a 3-hydroxy to the ring profoundly increases the affinity (Table 1; K_i = 100 nM) but with a concomitant loss of activity. Addition of a 3-methoxy group decreases the affinity 200 fold and hence it can again be inferred that the 2-amino group is crucial for activity whilst the 3-hydroxy group plays an important role in binding at the active site. The findings with the rat enzyme follow the same trend with the exception being that there is a greater increase in the K_i for 3-methoxydesaminokynurenine when compared to the value obtained for the bacterial enzyme.

Conclusions

In conclusion thus it can be stated that for the three species the 2-amino group is crucial for activity whilst the 3-hydroxyl group plays a fundamental role in binding at the active site presumably *via* hydrogen bonding. The potency of the various structural analogue inhibitors does indeed appear to be species specific depending on the substrate specificity of the enzyme. In general the 3-hydroxylated inhibitor had a greater affinity for the human enzyme whilst the methoxylated version yielded no significant difference between bacterial and human kynureninase.

Methods

All chemicals were of reagent grade and purchased from Sigma-Aldrich unless otherwise indicated.

Overexpression of recombinant human kynureninase

This was achieved with the 'Bac-to-Bac' baculovirus expression system [11]. Enzyme activity was determined using a fluorescence based assay as previously described [11].

Enzyme purification

The bacterial enzyme was partially purified from *Pseudomonas fluorescens* using a modification of a literature procedure [10]. Rat hepatic kynureninase was also purified using a modification of the protocol of Takeuchi *et al*. [12].

Recombinant human kynureninase was purified in the following manner: Harvested insect cells were Dounce homogenised in 90 mM L-His /containing 0.25 M sucrose, 1 mM DTT, 0.5 mM EGTA, 10 μM PLP, 100 μM PMSF, 2 μg/mL aprotinin plus 1 μg/mL pepstatin and leupeptin respectively at pH 7.5 and 0°C. The resultant homogenate was then centrifuged at 40 K for 2 hrs @ 0°C in a Beckman 42.1 rotor. The supernatant was retained after assaying positive for activity and the pellet was discarded. This solution was then syringe filtered twice, initially with a 0.45 μM unit which was then followed by a 0.20 μM filter (Millipore).

Step 1 (S-Cation)

This column and the 0.2 μM syringe filtered fraction were equilibrated with 15 mM L-His /pH 6.0 with subsequent sample application to the strong cationic column (Biorad (20 μM particle size)). Bound enzyme was eluted with 2 M NaCl /10 mM Tricine over 260 mL. The enzyme was released at 10% NaCl and dialysed over 18–24 hrs in 2 × 5 L of 10 mM Tricine/pH 7.7, containing the various additives as mentioned earlier.

Step 2 (Q-Anion)

This dialysed enzyme was applied to a strong anion exchange column (Biorad (20 μM particle size)) previously equilibrated with 10 mM Tricine pH 8.8 and the bound enzyme eluted with 7% 2N NaCl in 5 mM KH_2PO_4 after extensive washing with 100 mL gradient of 0 – 5% 2 M NaCl in 5 mM of the same buffer. The eluted enzyme was dialysed as above in 5 mM KH_2PO_4 again with the necessary additives at pH 6.8.

Step 3 (Hydroxyapatite (CHT 1)): The active dialysed fraction was collected and applied to a hydroxyappatite column (Biorad prepacked (10 μM particle size)) that has been equilibrated with 5 mM KH_2PO_4 at pH 6.8. The column with bound kynureninase was washed with 3 volumes of the start buffer and eluted with a 5 – 500 mM KH_2PO_4over 250 mL. Kynureninase eluted at approximately 60 mM. This highly active fraction was then concentrated with a vivaspin 20 mL concentrator incorporating a 30 kDa exclusion limit polyethersulfone membrane, saturated with PLP and stored in a 0.05% NaN_3 at -80°C until future use. This enzyme had a specific activity of 300 nmoles/mg protein/min and a $K_m \approx 3.0$ μM

The various purification steps were followed with 10% SDS-PAGE utilising Invitrogen gel kits [13]. A tryptic mass fingerprint obtained by MALDI-TOF mass spectrometry of a band of the expected molecular weight confirmed its identity as kynureninase. The protein concentrations were determined with the Bradford assay [14]. Recombinant human kynureninase from the final hydroxyapatite step was assayed for purity using SDS-PAGE (fig 3). The purification was executed at 4°C and performed with the aid of a BIOCAD 700E perfusion chromatography workstation that is coupled to an ADVANTEC SF-2120 super fraction collector all supplied by Perceptive Biosystems.

Synthesis of novel inhibitors

The two inhibitors were synthesised using adaptations of previously reported methods [11]. The 3-hydroxydesaminokynurenine (4) was prepared from 3-hydroxyacetophe-

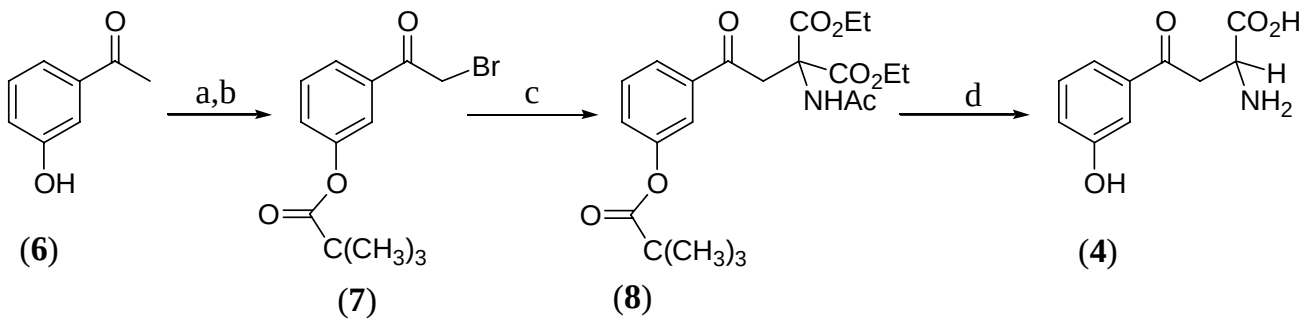

Figure 6
Synthesis of inhibitors a) $(CH_3)_3COCl$, pyridine, 0°C (79%); b) CuBr, ethyl acetate, $CHCl_3$, reflux, 4 hr (63%); c) NaH, $AcNHCH(CO_2Et)_2$, DMF, 0°C (57%); d) HCl, diethyl ether, reflux, 6 hr (76%)

none (**6**) (Figure 6). The hydroxy group was first protected as the pivalate ester derivative and then the methyl group brominated to give (**7**). The bromide (**7**) was then coupled to anion of diethyl acetamidomalonate in DMF to give the fully protected amino acid (**8**). Deprotection under acidic conditions provided the target compound. In the case of the 3-methoxydesaminokynurenine (**5**), the starting material was 3-methoxyacetophenone which could be used without necessity for an initial protection step. The spectral data for the two final products are as follows; **3-Hydroxydesaminokynurenine** (**4**): m.p. 188°C (dec.); (Found: C, 48.61; H, 5.00; N, 5.63 $C_{10}H_{12}ClNO_4$ requires C, 48.89; H, 4.92; N, 5.69%); ν_{max} (nujol)/cm^{-1} 3383 (NH), 1739 (CO, acid), 1660 (CO); δ_H (300 MHz, ^2H_2O) 3.73 (2H, d, $J_{3,2}$ 5.0 Hz, 3-C H_2), 4.39 (1H, t, $J_{2,3}$ 5.0 Hz, 2-C<u>H</u>), 7.06 (1H, dd, J 2.4, 1.0 Hz, 4'-<u>H</u>), 7.26 (1H, m, 5'-<u>H</u>), 7.30 (1H, m, 2'-<u>H</u>), 7.41 (1H, dd, J 7.8, 1.0 Hz, 6'-<u>H</u>); δ_C(75.4 MHz, ^2H_2O) 38.21 (s, 3-<u>C</u>H_2), 48.88 (s, 2-<u>C</u>H), 114.47 120.78 (s, 4'-<u>C</u>), 121.90 (s, 2'-<u>C</u>), 121.90 (s, 6'-<u>C</u>), 130.51 (s, 5'-<u>C</u>), 136.56 (1'-<u>C</u>), 156.10 (s, 3'-<u>C</u>), 171.77 (s, O<u>C</u>OH), 199.07 (s, 4-<u>C</u>O); *m/z* (CI) 210 ([M + H]$^+$, 13), 195 (100, ([M+H-NH]$^+$), 177 (18, [$C_{10}H_8O_3$ +H]$^+$), 164 (17, [M-(CO_2H)$^+$]), 149 (35, [$C_9H_9O_2$]$^+$), 43 (95, [CH_2CNH_2]$^+$). **3-Methoxydesaminokynurenine** (**5**): m.p. 164°C (dec.); ν_{max}(nujol)/cm^{-1} 3378 (NH), 1738 (CO, acid), 1681 (CO); δ_H (200 MHz, ^2H_2O) 3.77 (3H, s, O<u>C</u>H_3), 3.80 (2H, d, $J_{3,2}$ 5 Hz, 3-C<u>H</u>$_2$), 4.47 (1H, t, $J_{2,3}$ 5 Hz, 2-C<u>H</u>), 7.17 (1H, dt, J 8, 2.8 Hz, 4'-H), 7.39 (2H, m, 2', 5'-H), 7.53 (1H, m, 6'-H); δ_C (50.31 MHz, ^2H_2O), 41.26 (s, 3-<u>C</u>H_2), 51.82 (s, 2-<u>C</u>H), 58.48 (s, O<u>C</u>H_3), 115.72 (s, 2'-C), 123.64 (s, 4'-C), 124.35 (s, 6'-C), 133.22 (s, 5'-C), 139.06 (s, 1'-C), 162.06 (s, 3'-C), 174.46 (s, <u>C</u>O_2H), 201.66 (s, 4-<u>C</u>O); *m/z* (CI) 224.0913 (MH+) $C_{11}H_{14}NO_4$requires 224.0922, 209 (100, ([M+H-CH_3]$^+$), 191 (49, [HO-$C_6H_4COCH_2CHCO_2HNH_2$-H_2O]$^+$), 178 (73, [M+H-$C_2H_2O_2$]$^+$), 135 (18, [$CH_3OC_6H_4CO$]$^+$). Analysis by HPLC (C_{18}, reverse phase silica, 3 μ, flow rate 3 mL/min, 1% acetic acid, 20% methanol) confirmed the purity of both inhibitors.

Graphs were plotted using the GraphPad Prism3 software package and the kinetic parameters K_m and V_{max} were obtained using non linear regression. Lineweaver Burk and Dixon [9] plots allowed characterisation of the type of inhibition.

Authors' contributions

HAW carried out the protein expression, purification and inhibition studies. KCO was responsible for the synthesis of the inhibitors. NPB conceived of the study, and participated in its design and coordination. NPB and HAW prepared and approved the final manuscript.

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