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A Comparative Study on the Inhibition of Human and Bacterial Kynureninase by Novel Bicyclic Kynurenine Analogues

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Abstract—A series of novel bicyclic analogues of kynurenine were synthesised as inhibitors of kynureninase. The tryptophan-induced bacterial enzyme from *Pseudomonas fluorescens* was compared to the constitutive recombinant human enzyme expressed in a baculovirus/insect cell system, with regard to their inhibition by these compounds. All the compounds studied were found to be simple competitive, reversible inhibitors of kynureninase. It was found that altering the size of the second ring of the inhibitor affected the observed K_i values for both enzymes. The addition of an oxygen atom into the second ring had little effect on binding to the bacterial enzyme but gave a more potent inhibitor of human kynureninase. Of the compounds tested, a naphthyl analogue of desaminokynurenine was found to be the most potent inhibitor for both enzymes with K_i values of 5 and 22 μM for bacterial and human enzyme respectively. This report also describes an alternative system for the expression of recombinant human kynureninase which is more convenient for expression in mammalian cells and produces a relatively greater quantity of enzyme. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Kynureninase [L-kynurenine hydroxylase E.C. 3.7.1.1] is a pyridoxal 5′-phosphate dependent enzyme which catalyses the hydrolytic cleavage of L-kynurenine (**1**, R=H) to anthranilic acid (**2**, R=H) and L-alanine (**3**).[1] The conversion is an important step in the tryptophan metabolic pathway of *Pseudomonas fluorescens* and some other bacteria. In mammals, a similar enzyme [E.C.3.7.1.3], preferentially catalyses the conversion of 3-hydroxykynurenine (**1**, R=OH) to 3-hydroxyanthranilic acid (**2**, R=OH) and L-alanine (Scheme 1).

Further metabolism of hydroxyanthranilic acid on this pathway,[2] ultimately produces nicotinamide adenine dinucleotide (NAD), an important co-factor for many enzymatic conversions. Deficiency of vitamin B_6 and hence PLP, results in reduced kynureninase activity and increased formation of xanthurenic acid, from 3-hydroxykynurenine transamination, which is excreted in the urine.[3] More interestingly, quinolinic acid, another metabolite on the pathway, is an agonist of the *N*-methyl-D-aspartate (NMDA) population of glutamate receptors. Its excitotoxic effect at the NMDA receptor may be a key factor in the aetiology of neurodegenerative diseases such as Huntington's disease, epilepsy, AIDS-related dementia and septicaemia. The design of potent and selective inhibitors of kynureninase may be useful in the treatment of these disorders. Furthermore, examination of the binding of such compounds to the enzyme has assisted in probing the active site of the enzyme and in investigating the mechanism of the enzymatic reaction, which is still poorly understood. To date there are relatively few reported inhibitors for kynureninase and many lack specificity.[4,5] A number of inhibitors have been developed that mimic the transition state for the kynureninase catalysed reaction including (4*S*) and (4*R*)-dihydro-L-kynurenine,[6] a series of *S*-aryl-L-cysteine *S,S*-dioxides[7] and a phosphinic acid analogue of kynurenine,[8] which are all competitive inhibitors of bacterial kynureninase with varying potency. More recently Drysdale and Reinhard[9] have also examined *S*-aryl-L-cysteine *S,S*-dioxides as inhibitors of mammalian kynureninase from rat liver and also observed good inhibition.

In this laboratory, we have synthesised a series of bicyclic analogues of kynurenine as inhibitors of the enzyme. We report here, the results of our studies using these compounds as inhibitors of both the bacterial enzyme (from *Pseudomonas fluorescens*) and recombinant human enzyme, expressed in a baculovirus/insect

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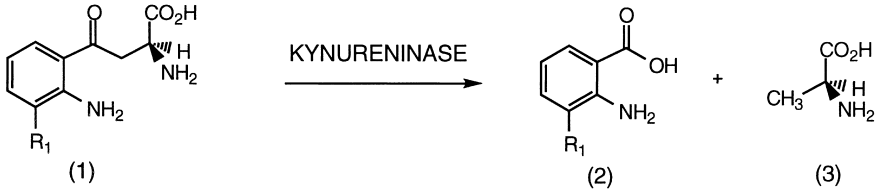


Scheme 1.

cell system. It is known that the bacterial and mammalian enzyme differ in their substrate-selectivity and therefore it is possible that the potency of inhibitory compounds is species dependent.

Results

Synthesis of inhibitors

The inhibitors were all synthesised in racemic form using the synthetic method[5,10] as shown in Scheme 2, which has also been used recently for the synthesis of 3-hydroxykynurenine and its *O*-β-D-glucopyranoside.[11] For example the tetralone derivative was prepared from the aromatic ketone, tetralone (**4**). This was selectively brominated on the carbon α to the carbonyl group using copper (II) bromide in a mixture of ethyl acetate and chloroform in 90% yield. The bromide (**5**) was then used without further purification and reacted with the carbanion derived from treatment of diethyl acetamidomalonate with sodium hydride. This produced the protected amino acid (**6**) derivative in 44% yield. Finally the protecting groups were all removed in a single step by heating the compound to reflux in 6N HCl. The desired amino acid (**7**) was then isolated as the free base and purified giving 15% of the final product. All the compounds had the expected spectral data and were shown to be pure by microanalysis.

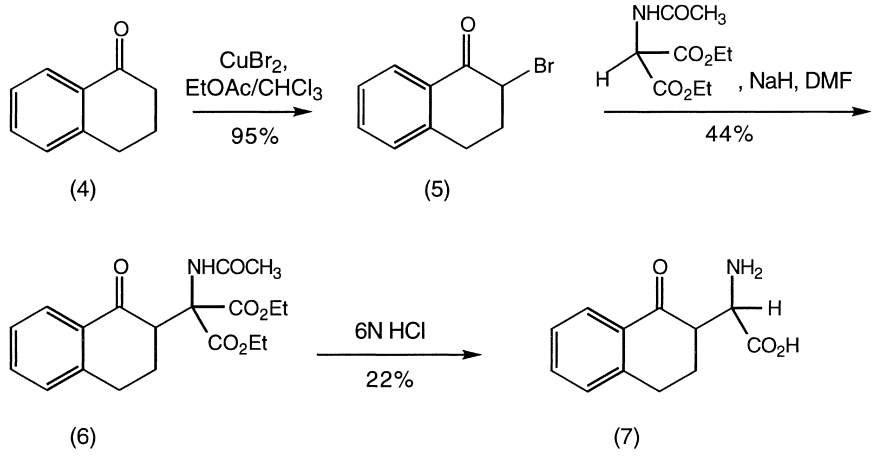
Enzyme isolation

The bacterial enzyme was isolated from *Pseudomonas fluorescens* using a modification of the method of Hayaishi and Stanier.[12] The activity of the enzyme activity was determined fluorimetrically at 37 °C, according to the method of Shetty and Gaertner.[13]

The cDNA clone encoding human liver kynureninase, which was a kind gift from Dr. Andrea Cesura, Hoffman la Roche Ltd., Basel, Switzerland, was isolated by the method of Alberati-Giani et al.[14] and cloned into the eukaryotic expression vector pBC/CMV.[15] The 1600 base pair (bp) kynureninase clone was inserted into the Sma site of the multiple cloning site and was supplied, in this form, to this laboratory. The Sma site is flanked on either side by an ECoR1 site and a BamH1 site. The 1600 bp cDNA encoding kynureninase was isolated from an agarose gel of a double digest, with these two restriction enzymes.

The 'Bac-to-Bac' Baculovirus Expression System (GibcoBRL) was then used to express kynureninase in SF9 insect cells. The expression of the kynureninase was monitored by SDS–PAGE analysis and by measuring enzyme activity in the resulting cell sonicates. The total enzyme activity from a 500 mL suspension culture, under these conditions was 1–2 μmol/min (specific activity 8–15 nmol/mg/min).

The K_m and V_{max} values of the bacterial and human kynureninase preparations were determined at 37 °C and pH 7.5. For bacterial kynureninase K_m was found to be 44.2±1.1 μM and V_{max} 1.45±0.09 μmol/min/mg for L-kynurenine. Values obtained for human kynureninase were K_m 44.2±1.1 μM and V_{max} 1.45±0.09 μmol/min/mg for L-3-hydroxykynurenine. The kinetic parameters determined for the human recombinant enzyme, expressed in the baculovirus/insect cell system are comparable to those determined[14] for the enzyme



Scheme 2.

expressed in a human embryonic kidney fibroblast cell line, HEK-293.

Inhibition studies

For the determination of K_i values, a substrate range was used, which spanned the K_m value. Representative Lineweaver–Burk plots, for a range of inhibitor concentrations, are shown for the interaction of the indanone derivative (**8**) with the human enzyme (Fig. 1). This graph demonstrates the competitive nature of the inhibition. Similar plots were obtained for each of the compounds in the series. For each compound, the slopes of these graphs were plotted against inhibitor concentration, to determine the value of K_i. The K_i values obtained for both the bacterial and human enzymes, are shown in Table 1. All the compounds studied showed reversibility of inhibition by dilution. Enzyme activity was restored to 100% on dilution, following inhibition with each compound, but was not restored when a known irreversible inhibitor, β-chloro-L-alanine was used.[4] A representative graph showing the reversibility of the indanone derivative (**8**) is shown (Fig. 2).

Discussion

Kynureninase represents an important target for drug action in some disorders affecting the brain. However, until recently the human enzyme was not available in sufficient quantities to conduct thorough mechanistic and kinetic investigations. Therefore, most of our present understanding of the chemical mechanism and properties of kynureninase has come from studies of the bacterial enzyme.[16] The cloning of human kynureninase and the ability to express the activity efficiently as reported herein has now allowed us to begin to study this enzyme further and will be important in the design of drugs to treat diseases involving impairment of the kynurenine pathway.

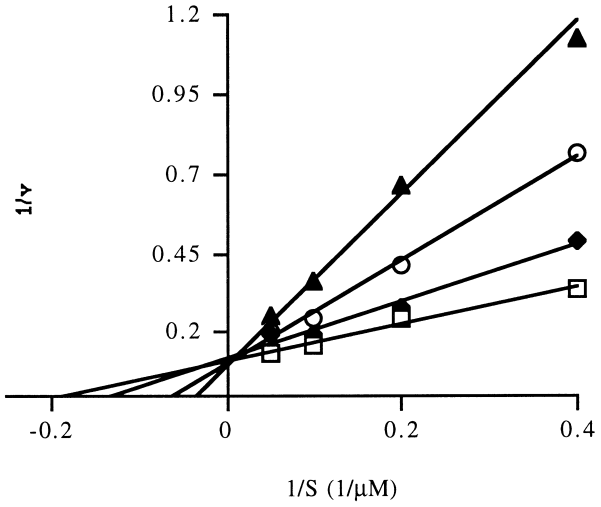
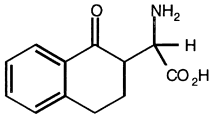
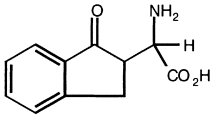
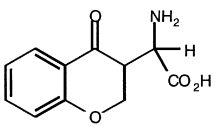
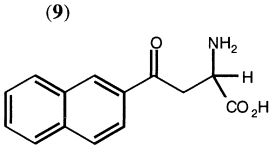


Figure 1. Inhibition of human recombinant kynureninase by (**8**). The inhibition of human recombinant kynureninase by (**8**) with respect to hydroxykynurenine. Data are presented as Lineweaver–Burk plots for the following inhibitor concentrations, □ 0 mM, ◆ 0.05 mM, ○, 0.1 mM, ▲ 0.2 mM. Initial rates of reaction (v) were assayed as described in the text . The concentration of substrate was varied between 2.5–20 μM. The graph shown is representative. The final K_i values quoted are the mean±standard error from three separate determinations.

It was already known that bacterial and mammalian kynureninase differ in their substrate preference. The bacterial enzyme is more active towards kynurenine as a substrate, while the mammalian enzyme preferentially hydrolyses 3-hydroxykynurenine. All four of the novel inhibitors tested were indeed found to competitively inhibit the kynureninase reaction for both bacterial and human enzymes, Table 1 gives the full set of K_i values. Note that the synthetic methods used to prepare the inhibitors were not stereospecific. The bicyclic inhibitors were produced as mixtures of diastereomers (**7**–**9**) (due to chiral centres at C-2 and C-3), while the naphthyl analogue (**10**) was a simple racemic mixture. The intention was that any very promising inhibitors would be prepared in an enantiomerically pure form at a later date. The 2S enantiomers might be expected to bind most tightly as kynureninase only reacts with the 2*S*-kynurenine, however the inhibitory effect of 2*R*-kynurenine has not been studied in detail.

The basis of the rationale for the design of the bicyclic inhibitors was that they should be capable of undergoing the early steps of the kynureninase catalysed reaction, as far as cleavage of the β,γ-carbon bond. However the presence of the new carbon chain linking the alanine fragment to the 2 position of the benzene ring means that following this cleavage step the two products remain covalently attached together. Normally the anthranilic acid debinds from the enzyme prior to

Table 1. K_i values for the inhibition of induced kynureninase from *Pseudomonas fluorescens* and recombinant human kynureninase expressed in SF9 cells, by bicyclic kynurenine analogues. Data are the mean and standard error of at least three separate experiments

Compound	Bacterial	Human
 (**7**)	170 ± 24 μM	227 ± 47 μM
 (**8**)	34.9 ± 10 μM	45 ± 12 μM
 (**9**)	162 ± 31 μM	77 ± 23 μM
 (**10**)	5 ± 2 μM	22 ± 6 μM

the alanine, but in this case the anthranilic acid type fragment cannot be released from the active site. It was thought that this may give tight binding inhibitors which may even show some irreversible inhibition.

From the results in Table 1 it can be seen that all the compounds inhibited to some degree, with all the K_i values in the μM range and of similar magnitude to the K_m values for the substrate. With the bicyclic inhibitors, for both the bacterial and human enzymes, altering the size of the new ring from a 6- to a 5-membered one, reduced the observed K_i by ca. 5-fold. Such a structural change would be expected to effect the spatial 'fit' of the compound in the active site. This could occur by changing the relative positions of the carbonyl group and benzene ring. However, it is very likely that the positions of these two groups will be fixed by interactions with binding groups at the active site. The most important factor may thus be the length and bulk of the linker. For the 5-membered ring a single CH_2 group has been inserted and in this case the new 5-membered ring is close to planar. With the 6-membered ring there are two CH_2 groups and the geometry of the new ring is such that it is puckered with one of the CH_2 groups forced out of the plane. This CH_2 group may then clash with amino acid side chains in the active site increasing K_i. However the difference in binding is rather small to be the result of a steric clash. The 6-membered ring will also have more conformational flexibility and if only one of the conformers were to fit appropriately into the active site, this would provide a more likely explanation for the decreased binding.

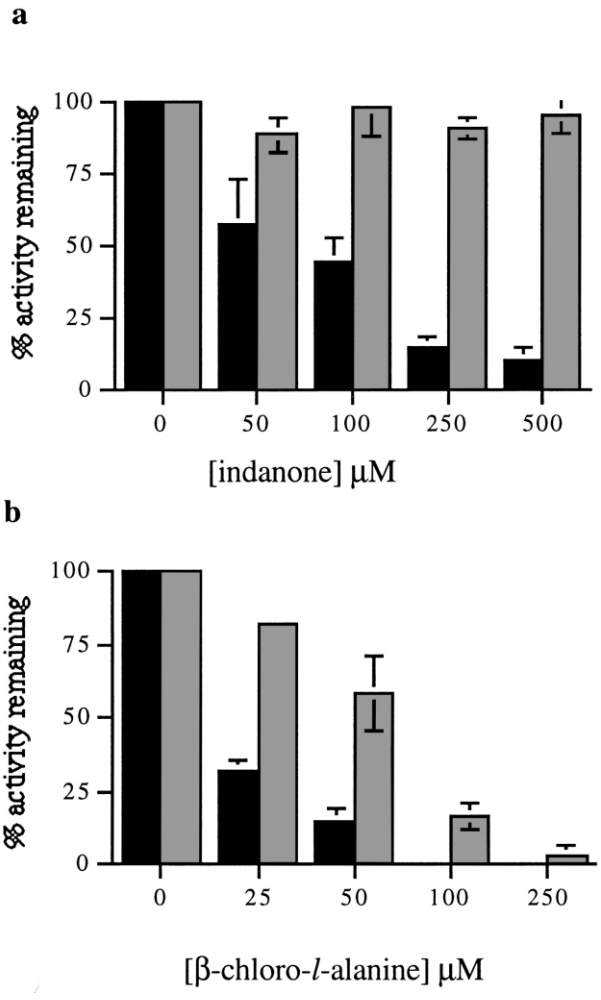
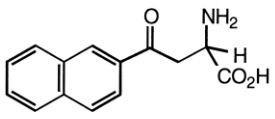


Figure 2. Bacterial kynureninase was assayed as described in Experimental, using L-kynurenine as substrate. The reversibility of the indanone derivative (**8**) is shown above (a) and is compared to a known irreversible inhibitor, β-chloro-L-alanine (b). In each case, (▭) indicates assays in which the final assay concentration of inhibitor, in a 3 mL cuvette, was as indicated and (■) indicates reactions in which the enzyme was incubated for 10 min with the same concentration of inhibitor and then diluted into the assay. Data shown are the mean ±s.e.m. of three determinations assayed in duplicate.

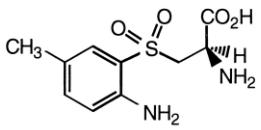
When an oxygen is introduced into the linker in compound (**9**), it has a similar K_i for the bacterial enzyme when compared with the all carbon ring (**6**). With the human enzyme, however, it is 3-fold more potent, although still not as good as the 5-membered ring. This may be a result of additional binding interactions between the ring oxygen and groups at the active site which usually bind the hydroxyl group of 3-hydroxykynurenine, the preferred substrate for the human enzyme.

With all the bicyclic inhibitors experiments were carried out to see whether the inhibition was irreversible, or apparently irreversible due to tight binding of the cleaved inhibitor. However, no evidence could be obtained for this and the compounds all appeared to be simple competitive reversible inhibitors.

The final inhibitor is a naphthyl analogue of desaminokynurenine. This appears to be the most potent inhibitor for both enzymes with K_i values of 5 and 22 μM. In this case it appears that extending the benzene ring of kynurenine to give a larger planar aromatic system produces an increase in binding. Presumably the new aromatic system is filling a hydrophobic pocket at the enzyme active site. It also appeared that there was some, albeit slow, substrate activity as TLC examination of incubations of the naphthyl analogue with kynureninase did show the presence of small amounts of alanine.[17] Most previous studies on the inhibition of kynureninase have included more polar functional groups on the kynurenine benzene ring, such as methoxy and nitro groups.[6] However, recent work on the inhibition of rat liver kynureninase by *S*-aryl-L-cysteine *S,S*-dioxides[9] showed that the most potent derivative (**11**), was that with a methyl group in the 5-position, which gave an IC_{50} of 11 μM. This increase in binding would also be consistent with the methyl group fitting into a hydrophobic pocket. These results imply that a fruitful approach may be to exploit this apparent hydrophobic binding site to produce more potent inhibitors.



(**10**)



(**11**)

Experimental

Pseudomonas fluorescens was obtained from NCIMB, Aberdeen. DH5α cells were obtained from Promega. The components of the 'Bac-to-Bac' Expression system (pFastBac donor plasmid, MAX efficiency DH10Bac competent cells, CellFECTIN reagent, and protocols), were obtained from GibcoBRL. BamH1, ECoR1, T4 DNA ligase and RNase 1, were obtained from Promega. Ampicillin, gentamycin, kanamycin, tetracycline, X-Gal and dithiothreiton (DTT) were obtained from Melford Laboratories. The cDNA clone encoding human kynureninase was a kind gift from Dr. Andrea Cesura, Hoffman la Roche Ltd., Basel, Switzerland. All other compounds were obtained from Sigma Chemical Co.

Assay of kynureninase. Enzyme activity was determined fluorimetrically at 37 °C, according to the method of Shetty and Gaertner.[13] The rate of formation of product was measured at wavelengths of excitation and emission of 310 nm and 417 nm (anthranilic acid) and 330 nm and 410 nm (hydroxyanthranilic acid) using a Perkin Elmer luminescence spectrometer (MODEL LS50B) connected to a GRANT circulating water bath. The reaction mixture contained 20 μM pyridoxal-5-phosphate (PLP), 100 mM Tris–HCl buffer or 40 mM potassium phosphate buffer, pH 7.5 and an appropriate amount of enzyme in a final volume of 3 mL. The reaction was initiated by the addition of L-kynurenine or DL-hydroxykynurenine. Preliminary assays were performed to confirm the linear relationship between product formation and both time and protein concentration under these conditions. The amount of product formed was measured by reference to standard curves of fluorescence intensity against anthranilate/hydroxyanthranilate concentration.

Kinetic parameters were calculated using the MacCurveFit computer program (version 1.2) using non-linear regression analysis of the experimental data.

Inhibitor studies. Preliminary studies involved screening compounds for their ability to reduce the rate of reaction at 1 mM inhibitor. K_i values were then determined by assaying the activity of the enzyme as above, but in the presence of appropriate concentrations of inhibitor and at a range of substrate concentrations spanning the K_m value. For inhibitor studies, the reaction was initiated with enzyme rather than substrate.

The values of 1/V were plotted against the reciprocal concentration of substrate, for each of the inhibitor concentrations studied. Intersection of the plots on the X-axis indicates competitive inhibition. A graph of the slopes of these plots versus inhibitor concentration gives a straight line with an intercept on the X axis equal to $-K_i$

Reversibility studies. Reversibility of inhibition was examined by dilution techniques. Enzyme, at 300-fold its normal assay concentration was incubated for 10 min with inhibitor, at a range of concentrations, based on the K_i value determined. The enzyme/inhibitor reaction solution was then diluted 300-fold into the assay buffer and the activity measured by the addition of substrate. A control series was also set up in which enzyme was incubated alone for 10 min and diluted into the assay before adding inhibitor to give the diluted concentration. If inhibition was reversible, then the activity was restored following dilution, when compared to an undiluted series, in which enzyme was assayed in the presence of the same concentrations of inhibitor.

Preparation of bacterial enzyme. The bacterial enzyme was isolated from *Pseudomonas fluorescens* using a modification of the method of Hayaishi and Stanier.[13] The growth of *Pseudomonas fluorescens* was initiated in 'Lab-Lemco' nutrient broth, in an overnight culture at 28 °C. This culture (50 mL) was then used to inoculate 500 mL of the tryptophan-rich growth medium which was incubated for at least 8 h at 28 °C. The culture was then used to inoculate 6×1 L flasks of the tryptophan-rich medium which were incubated for at least 18 h at 28 °C. Tryptophan-rich medium contained 0.3% L-tryptophan, 0.2% peptone, 0.1% glycerin, 0.05% yeast extract, 0.1% KH_2PO_4, 0.2% K_2HPO_4 and 0.01% $MgSO_4$, pH 7.0.

The total enzyme activity obtained from a 6 L culture was about 40 μmol/min. Kynureninase was purified from the sonicated fraction using modifications of previously described methods.[18–20] The buffer used throughout the purification was 20 mM potassium phosphate, pH 7.5 containing 100 μM PLP, 0.01% mercaptoethanol and 1 mM EDTA.

Following sonication, a protamine sulphate precipitation step and a 20–60% ammonium sulphate step, the dialysed enzyme was purified by chromatography on a Fast-Flow DEAE-Sepharose 6B (Pharmacia) column, using a linear gradient of KCl (0–0.5 M) in 20 mM potassium phosphate buffer, pH 7.5 and a volume of 500 mL. PLP was omitted from the buffers during this step. Following concentration of the active fractions to $<$ 10 mL (amicon ultrafiltration unit-YM10 membrane), the dialysed fraction was chromatographed on a Sephadex G-100 (Pharmacia) column. The active fractions were pooled and injected onto a mono-Q HR 5/5 FPLC column and the enzyme was eluted using a linear gradient of KCl (0–0.5 M) in 20 mM potassium phosphate buffer, pH 7.5. This protocol results in a 50–80-fold purification in a yield of 30–60% and the final specific activity of the preparation varied between 1.2–2.2 μmol/mg/min.

Cloning and expression of recombinant human kynureninase in baculovirus/insect cell system. The cDNA clone encoding human liver kynureninase was isolated by the method of Alberati-Giani et al.[14] and cloned into the eukaryotic expression vector pBC/CMV.[15] The 1600 base pair (bp) kynureninase clone was inserted into the Sma site of the multiple cloning site and was supplied, in this form, to this laboratory. The Sma site is flanked on either side by an ECoR1 site and a BamH1 site. The 1600 bp cDNA encoding kynureninase was isolated

from an agarose gel of a double digest, with these two restriction enzymes.

The 'Bac-to-Bac' Baculovirus Expression System (GibcoBRL) was used to express kynureninase in SF9 insect cells. The methods used were those described in the manufacturer's manual. Kynureninase cDNA was cloned into pFastBac donor plasmid, which was then transformed into DH10Bac cells for transposition to the bacmid. SF9 cells were then transfected with the recombinant bacmid DNA To determine the optimal conditions for expression of kynureninase, preliminary studies were conducted in SF9 cells grown on 6-well plates, where both the duration of infection and the concentration of baculovirus were varied Expression of the kynureninase was monitored by SDS–PAGE analysis and by measuring enzyme activity in the resulting cell sonicates. Subsequently, cells were grown in suspension cultures in 1L-stirred bottles, containing 500 mL total volume and the duration of baculovirus infection was 96 h.

The harvested cells were resuspended in 20 mM Tris–HCl, pH 7.5, containing 0.25 M sucrose, 100 μM PLP, 1mM dithiothreitol (DTT), 0.5 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/mL leupeptin, 1 μg/mL pepstatin and 2 μg/mL aprotonin. The suspension was sonicated (10×30 s bursts) and centrifuged at 16,400 *g*. The supernatant was decanted, aliquoted and stored at −20 °C until required for assay. The total enzyme activity from a 500 mL suspension culture, under these conditions was 1–2 μmol/min (specific activity 8–15 nmol/mg/min).

Synthesis of inhibitors

Full experimental details are given below for the reaction sequence in Scheme 2, followed by full spectroscopic data for the other three inhibitors synthesised using similar methods.

2-Bromo-3,4-dihydro-1-(2H)-naphthalenone (5). Cupric bromide (6.126 g, 27.4 mmol, 2 equiv) was heated at reflux in ethyl acetate (10 mL) with stirring. To this was added 3,4-dihydro-1-(2*H*)-naphthalenone (2.091 g, 14.3 mmol) in chloroform (10 mL). The reaction was heated at reflux for a further 5 h and then cooled. Copper bromide and cupric bromide residues were filtered off, the filtrate decolourised with activated charcoal and filtered through a bed of Celite and washed with ethyl acetate (4×50 mL). The solvent was removed under reduced pressure to give an orange oil which was dried further under vacuum (2.9 g, 90%); δ_H (200 MHz, $CDCl_3$) 2.49 (2H, t, $J_{3,4}$ 4, 4-C$\underline{H}_2$), 2.93 (1H, dt, $J_{2,3}$ 17.1, $J_{3,4}$ 4, H_A, 3-C$\underline{H}_2$), 3.26–3.41 (1H, m, H_B, 3-C$\underline{H}_2$), 4.74 (1H, s, C$\underline{H}$Br), 7.29 (1H, d, *J* 7.7, 5-C$\underline{H}$), 7.38 (1H, t, *J* 7.7, 7-C$\underline{H}$), 7.54 (1H, t, *J* 7.7, 6-C$\underline{H}$), 8.10 (1H, d, *J* 7.7, 8-C$\underline{H}$); δ_C (50.31 MHz, $CDCl_3$) 26.6 (4-$\underline{C}H_2$), 32.5 (3-$\underline{C}H_2$), 51.1 (2-$\underline{C}$H), 112.8 (7-$\underline{C}$), 127.7 (5-$\underline{C}$), 129.2 (8-$\underline{C}$), 134.7 (6-$\underline{C}$), 137.5 (8a-$\underline{C}$), 143.2 (4a-$\underline{C}$), 191.1 ($\underline{C}$=O); *m*/*z* (CI) 227 & 225 ([*M*+H], 98% and 100%), 147 (83, $[C_{10}H_{10}O]^+$), 118 (12, $[C(O)ArCH_3+H]^+$).

Diethyl 2-(acetylamino)-2-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl) malonate (6). Sodium hydride (1.39 g, 34.9 mmol, 2.5 equiv) 60% in mineral oil was suspended in dry DMF (4 mL) and a solution of diethyl acetamidomalonate (4.565 g, 21 mmol, 1.5 equiv) in dry DMF (14 mL) added. The solution was stirred at 0 °C under a nitrogen atmosphere for 3 h until the anion had formed. A solution of 2-bromo-3,4-dihydro-1-(2*H*)-naphthalenone (3.148 g, 13.9 mmol) in dry DMF (10 mL) was added and the solution warmed to room temperature and stirred overnight under nitrogen. The mixture was poured into distilled water (100 mL), acidified to pH 3 with 1M hydrochloric acid in an ice-bath and extracted into diethyl ether (4×70 mL). The organic phases were washed with brine (2×50 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure to give an orange oil, which was purified by column chromatography (silica; diethyl ether) (1.86 g, 44%); (Acc. mass found 361.151867 calcd. for $C_{19}H_{23}NO_6$ 361.152538); δ_H (300 MHz, $CDCl_3$) 1.23 (6H, t, *J* 7.2, $CH_3CH_2CO_2$), 1.99 (3H, s, CH_3CO), 2.83–3.26 (4H, m, 3-C$\underline{H}_2$ & 4-C$\underline{H}_2$), 3.93 (1H, dd, J_1 13.9, J_2 3.75, 2-C$\underline{H}$), 4.19–4.30 (4H, m, $CH_3C\underline{H}_2CO_2$), 6.86 (1H, br s, N$\underline{H}$), 7.26–7.31 (2H, m, 6-C$\underline{H}$ & 5-C$\underline{H}$), 7.47 (1H, t, *J* 7.7, 7-C$\underline{H}$), 7.93 (1H, d, *J* 7.7, 8-C$\underline{H}$); δ_C (73.76 MHz, $CDCl_3$) 13.7 ($\underline{C}H_3CH_2CO_2$), 23.1 ($\underline{C}H_3CO$), 26.7 (4-$\underline{C}H_2$), 29.7 (3-$\underline{C}H_2$), 55.9 ($CH_3\underline{C}H_2CO_2$), 63.0 (2-CH), 66.1 (α-$\underline{C}$), 126.7 (7-$\underline{C}$), 127.4 (5-$\underline{C}$), 128.9 (8-$\underline{C}$), 132.3 (6-$\underline{C}$), 134.0 (8a-$\underline{C}$), 144.7 (4a-$\underline{C}$), 168.6 ($CH_3CH_2\underline{C}O_2$), 169.8 ($CH_3\underline{C}O$), 198.1 ($\underline{C}$=O); *m*/*z* (EI) 361 ([M^+], 19%), 316 (10, [M-$CH_3CH_2O]^+$), 288 (16, [M-$CO_2CH_2CH_3]^+$), 246 (100, [M-$AcHNCCO_2H]^+$), 217 (64, [M-$AcHNCCO_2Et+H]^+$), 171 (70, $[C_{11}H_9NO]^+$), 145 (55, $[C_{10}H_9O]^+$), 129 (30, $[AcHNCCO_2CH_2]^+$), 115 (24, $[AcHNCCO_2H]^+$).

Amino-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl)-acetic acid (7). Diethyl 2-(acetylamino)-2-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl)-malonate (1.63 g, 4.5 mmol) was dissolved in 1,4-dioxane (50 mL) and 6M hydrochloric acid (70 mL) added. The reaction was heated under reflux for 8 h until no starting material was visible by TLC (silica; pet. ether: ethyl acetate; 1:1). The solution was then cooled and washed with ethyl acetate (50 mL). The aqueous phase was concentrated under reduced pressure to give a brown liquid which was triturated with acetone to produce a grey solid. The solid was taken up in isopropyl alcohol (25 mL) and propylene oxide (10 mL) added, the solution was stirred overnight and the solvent removed under reduced pressure to give the product as an off-white solid. The product was obtained as a mixture of diastereomers in a 3:1 ratio and spectral data is given for the major isomer (0.147 g, 15%); mp 186–192 °C; (Found: C, 65.90; H, 6.09; N, 6.31. Calcd. for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.97; N, 6.38%); δ_H (200 MHz, D_2O) 2.04 (2H, t, *J* 6.25, 4-C$\underline{H}_2$), 3.01 (2H, d, *J* 6.25, 3-C$\underline{H}_2$), 3.13- 3.38 (1H, m, 2-C$\underline{H}$), 4.25- 4.37 (1H, m, α-C$\underline{H}$), 7.29 (2H, m, 7-C$\underline{H}$ & 5-C$\underline{H}$), 7.52 (1H, t, *J* 6.25, 6-C$\underline{H}$), 7.88 (1H, d, *J* 6.25, 8-C$\underline{H}$); δ_C (50.31 MHz, D_2O) 27.8 (4-$\underline{C}H_2$), 30.1 (3-$\underline{C}H_2$), 64.2 (2-CH), 66.5 (α-$\underline{C}$), 126.5 (7-$\underline{C}$), 127.1 (5-$\underline{C}$), 128.5 (8-$\underline{C}$), 132.5 (6-$\underline{C}$), 133.7 (8a-$\underline{C}$), 144.2 (4a-$\underline{C}$), 173.4 ($\underline{C}O_2H$), 197.0 ($\underline{C}$=O); *m*/*z* (CI) 220 ([*M*+H], 25%), 203 (100, [*M*H-OH]$^+$), 147 (22, $[C_{10}H_{10}O]^+$).

Amino-(1-oxo-2,3-dihydro-1H-inden-2-yl)-acetic acid (8). The product was obtained as a mixture of diastereomers in a 3:2 ratio and spectral data is given for the major isomer; mp 152–158 °C (decomp.); (Acc. mass found 206.082462 calcd. for $C_{11}H_{12}NO_3$, 206.081718); δ_H (200 MHz, D_2O) 3.1 (1H, dd, J_1 14, J_2 4.5, 2-CH), 3.77–3.93 (1H, d, J 4.5, α-CH), 4.14–4.3 (2H, m, 3-CH_2), 7.31 (2H, m, 4- and 6-CH), 7.51 (1H, t, $J_{4,5}=J_{5,6}$ 7.5, 5-CH), 7.67 (1H, d, $J_{6,7}$ 7.5, 7-CH); δ_C (50.31 MHz, D_2O) 44.1 (3-CH_2), 52.4(2-CH), 58.5 (α-CH), 126.7 (7-C), 129.7 (4-C), 130.6 (5-C), 131.6 (7a-C), 138.9 (6-C), 155.3 (3a-C), 175.1 (CO_2H), 190.5 (C=O); *m/z* (CI) 206 ([M+H], 15%), 191 (76, [M+H-NH]$^+$), 151 (45, [M+H-NH_2CHCO]$^+$), 76 (99, [M+H-Ar]$^+$).

Amino-(4-oxo-3,4-dihydro-2H-chromen-3-yl)-acetic acid (9). The product was obtained as a mixture of diastereomers in a 3:1 ratio and spectral data is given for the major isomer; mp 138–140 °C [decomp.]; (Acc. mass found 244.059856 calcd. for $C_{11}H_{11}NO_4Na$, 244.058578); δ_H (300 MHz, DCl/D_2O) 3.37 (1H, dt, $J_{2,3}$ 9.3, $J_{3,9}$ 2.6, 3-CH), 4.01 (1H, d, $J_{3,9}$ 2.6, α-CH), 4.31 (2H, d, $J_{2,3}$ 9.3, 2-CH_2), 6.60 (1H, d, $J_{7,8}$ 7.9, 8-CH), 6.66 (1H, t, $J_{5,6}$ 7.9, 5-CH), 7.17 (1H, dt, $J_{7,8}$ 7.9, $J_{6,7}$ 1.7, 7-CH), 7.35 (1H, dd, $J_{5,6}$7.9, $J_{6,7}$ 1.7, 6-CH); δ_C (300 MHz, DCl/D_2O) 43.9 (3-CH), 47.8 (α-CH), 66.3 (2-CH_2), 115.8 (8-C), 120.0 (6-C), 124.7 (5-C), 135.7 (7-C), 159.5 (4a-C), 167.7 (1a-C), 171.7 (CO_2H), 191.6 (C=O); *m/z* (CI) 244 ([M+H+Na], 19%), 205 (100, [M-NH_2]).

2-Amino-4-oxo-4-naphthyl-butyric acid (10). Mp 201 °C; (Found: C, 69.00; H, 5.55; N, 5.78. Calcd. for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.39; N, 5.76%); υ_{max} (nujol)/cm^{-1} 1690 (C=O), 1650 (C=O acid), 1580 (aromatic); δ_H (200 MHz; CD_3OD), 3.95 (2H, m, 3-CH_2), 4.50 (1H, m, α-CH), 7.65 (2H, m, 6′H, 7′H), 8.05 (4H, m, 3′H, 4′H, 5′H, 8′H), 8.68 (1H, s, 1′H); δ_C (74.76 MHz; $C^2H_3O^2H$) 39.42 (β-C), 50.01 (α-C), 124.44 (3′-C), 128.34 (7′-C), 128.96 (5′-C), 129.89 (4′-C), 130.33 (6′-C), 130.95 (8′-C), 131.87 (1′-C), 134.08 (10′-C), 134.31 (9′-C), 137.62 (2′-C), 171.45 (CO_2H), 197.82 (C=O); *m/z* (FAB) 266 ([M+Na]$^+$, 25%), 244 (65, [M+H]$^+$).

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