

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/244058272>

# Derivatives of kynurenine as inhibitors of rat brain kynurenine aminotransferase

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · DECEMBER 1996

Impact Factor: 3.45 · DOI: 10.1016/S0223-5234(96)80002-X

---

CITATIONS

18

READS

43

## 7 AUTHORS, INCLUDING:



Mario Varasi

IEO - Istituto Europeo di Oncologia

113 PUBLICATIONS 1,959 CITATIONS

[SEE PROFILE](#)



Paolo Pevarello

Axxam SpA

59 PUBLICATIONS 1,361 CITATIONS

[SEE PROFILE](#)

## Derivatives of kynurenine as inhibitors of rat brain kynurenine aminotransferase

M Varasi<sup>1</sup>, A Della Torre<sup>1</sup>, F Heidempergher<sup>1</sup>, P Pevarello<sup>1</sup>, C Speciale<sup>1</sup>,  
P Guidetti<sup>2</sup>, DR Wells<sup>2</sup>, R Schwarcz<sup>2</sup>

<sup>1</sup>Pharmacia, R&D, CNS, Via Per Pogliano, I-20014 Nerviano, MI, Italy;

<sup>2</sup>Maryland Psychiatric Research Center, University of Maryland School of Medicine, Baltimore, MD, USA

(Received 13 June 1995; accepted 12 September 1995)

**Summary** — The structural requirements of the catalytic site of kynurenine aminotransferase (KAT), the enzyme responsible for the conversion of L-kynurenine (KYN) to kynurenic acid (KYNA), were examined using analogs and derivatives of KYN. KYNA production from KYN was monitored in rat brain homogenates and brain tissue slices. Modification of KYN's acylalanine side chain or its ring amino group resulted in compounds which did not substantially affect KYNA synthesis. Ring chlorination in positions 3, 4, 5 and 6 yielded KYN analogs which interfered with KYNA production. L-5-Cl-KYN was the most active of the chlorinated kynurenines, and one of the most potent of several other 5-substituted kynurenines. L-5-Cl-KYN was an excellent substrate of KAT, yielding 6-Cl-KYNA. Finally, in kinetic studies, L-5-Cl-KYN ( $K_i = 5.4 \mu\text{M}$ ) was found to have an approximately five times higher affinity to the enzyme than the natural substrate KYN ( $K_m = 28 \mu\text{M}$ ).

enzyme inhibition / kynurenone / kynurenic acid/ neuroprotection

### Introduction

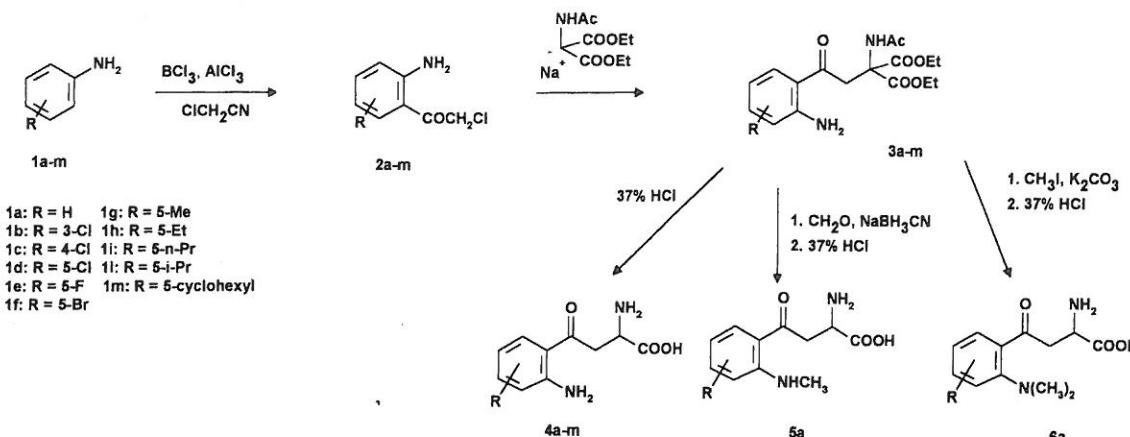
Kynurenic acid (KYNA) is a neuroactive brain metabolite with anticonvulsant and neuroprotective properties [1, 2]. Because of the ability of KYNA to serve as an antagonist at cerebral ionotropic excitatory amino acid receptors and because of its potential involvement in human brain diseases [3], the metabolism and function of KYNA in the brain have been the subject of several studies during recent years. In the brain, as in the periphery, KYNA is biosynthesized through enzymatic transamination from L-kynurenone (KYN) [4]. In the rat brain, a single kynurenone aminotransferase (KAT) appears to be responsible for the production of KYNA under physiological conditions [5]. KAT has been immunocytochemically localized to astrocytes in close apposition to glutamatergic synapses [6]. This provides further indirect evidence for an important role of its product, KYNA, in excitatory neurotransmission.

In the present study, modifications to the KYN molecule were made to examine the substrate specificity of KAT and, possibly, to develop compounds which could be used as tools for the further exploration of KYNA neurobiology.

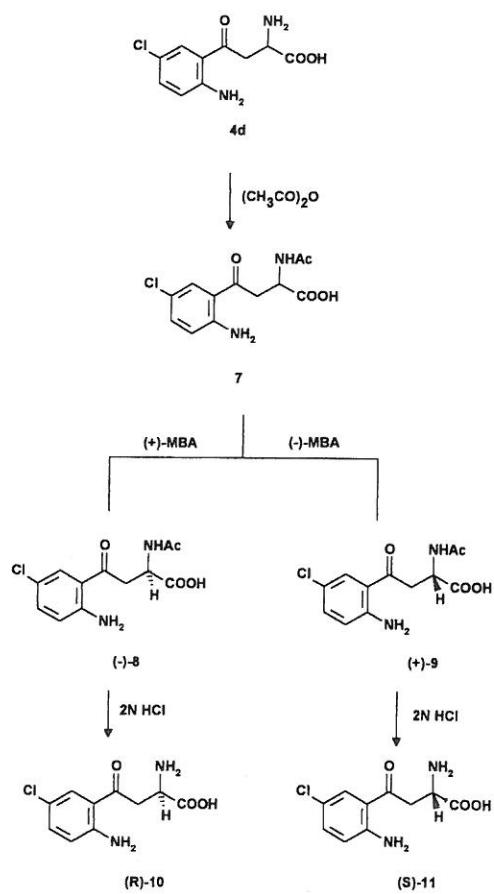
### Chemistry

The synthesis of KYN derivatives **4–6** (scheme 1) was performed starting from the corresponding substituted anilines (**1a–m**). Their conversion under Houben-Hoesch conditions into  $\alpha$ -chloroacetophenones (**2a–m**) was carried out following the procedure introduced by Susagawa *et al* [7]. Whereas the production of compounds (**2a–d**) was described in the cited paper [7], the synthesis of (**2e–m**) generalizes this useful synthetic procedure. For practical reasons, it was decided to employ the classical acetamidomalonate addition/decarboxylative hydrolysis for the generation of racemic kynurenines (**4a–m**, **5a**, **6a**) from  $\alpha$ -chloroacetophenone intermediates (**2a–m**). As shown in scheme 1, when mono- or dimethylation of the aromatic amino group was desired (**5a**, **6a**), the hydrolytic step was preceded by treatment with formaldehyde/sodium cyanoborohydride (**5a**) or methyl iodide/potassium carbonate (**6a**).

Scheme 2 outlines the diastereomeric salt resolution of racemic 5-chlorokynurenone (**4d**): after amino acidic *N*-acetylation, the salt obtained from acid **7** and (*R*)-(+)- $\alpha$ -methylbenzylamine was crystallized four times from ethyl acetate and hydrolyzed to give (*R*)-(–)-5-



Scheme 1. Synthesis of racemic kynurenines **4a–m**, **5a** and **6a**.

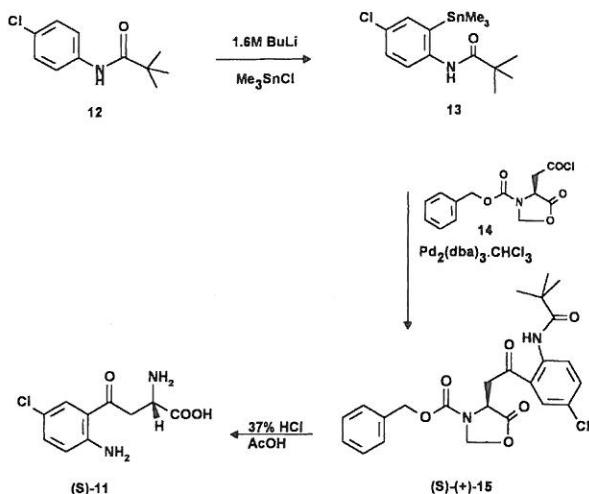


Scheme 2. Synthesis of *(R*)-5-chlorokynurenine (**10**; D-5-Cl-KYN) and *(S*)-5-chlorokynurenine (**11**; L-5-Cl-KYN) via diastereomeric salt resolution. The absolute configuration was assigned after enantiospecific synthesis of (**11**), as depicted in scheme 3. MBA:  $\alpha$ -methylbenzylamine.

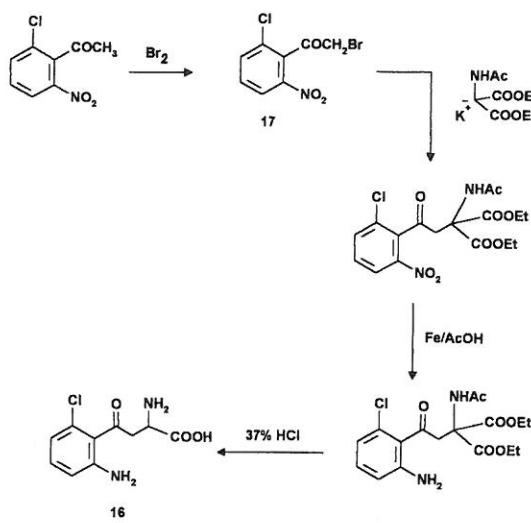
chloro-*N*-acetylkynurenine (**8**). The same procedure was applied to obtain *(S)*-*(+)*-5-chloro-*N*-acetylkynurenine (**9**), in this case using *(S)*-*(−)*- $\alpha$ -methylbenzylamine as a resolution partner. Mild acidic hydrolysis gave *(R)*-5-chlorokynurenine (**10**; D-5-Cl-KYN) and *(S)*-5-chlorokynurenine (**11**; L-5-Cl-KYN), respectively, with 35% and 30% overall yields starting from the racemic precursor (**4d**).

Since extension of the diastereomeric salt resolution to similar compounds was deemed unpredictable, in order to assign the proper absolute configuration, an enantiospecific approach to homochiral kynurenines was pursued (scheme 3), taking advantage of the palladium-catalyzed coupling of arylstannanes [8] with an aspartate-derived enantiopure building block [9, 10]. The procedure described by Salituro and McDonald [9] was then chosen for the synthesis of L-5-Cl-KYN (**11**). In comparison to previous work, some modifications were introduced that allowed us to shorten the synthesis and improve the total yield. 4-Chloro-*N*-pivaloylalanine (**12**), obtained in 97% yield from 4-chloroaniline, was *ortho*-metalated with *n*-butyllithium, and the resultant lithium salt was quenched with trimethylstannyll chloride to afford **13** in 83% yield after flash-chromatography as a white solid. Coupling of **13** with *(S)*-3-(benzyloxycarbonyl)-5-oxo-4-oxazolidineacetyl chloride (**14**) [9] was catalyzed by Pd<sub>2</sub>(dba)<sub>3</sub>·CHCl<sub>3</sub> [11] (dba: dibenzylidene acetone) and furnished the protected kynurenine (**15**) in 53% yield after purification. Deprotection of **15** to **11** was carried out with 37% HCl/AcOH at 70°C for 5.5 h to give L-5-Cl-KYN in 51% yield, the whole process consisting of four steps with a 22% total yield.

6-Chlorokynurenine (**16**) was prepared from the corresponding 6-chloro-2-nitro- $\alpha$ -bromoacetophenone (**17**) after acetamidomalonate substitution followed by Fe/AcOH reduction of the nitro function and hydrolysis (scheme 4).



**Scheme 3.** Enantiospecific synthesis of (S)-5-chlorokynurenine (11; L-5-Cl-KYN).



**Scheme 4.** Synthesis of 6-chlorokynurenine.

Compounds **20** [12], **21** [13], **22** [14] and **23** [15] (table I) were prepared according to the literature; D- and L-4-chlorokynurenine were kindly provided by Dr F Salituro (Vertex, Boston, MA).

## Results

### *Modifications of the acylalanine moiety and the aromatic amino group of KYN*

The first set of experiments was designed to examine the structure–activity relationships of compounds

**Table I.** Effect of chemically modified KYN analogs on KYNA production.

Compound	% of control KAT activity	% of KYNA production in slices
<b>20</b>	91 ± 7	—
<b>21</b>	100 ± 6	109 ± 19
<b>22</b>	105 ± 2	55 ± 3
<b>23</b>	105 ± 9	99 ± 3
<b>5a</b>	85 ± 8	55 ± 7
<b>6a</b>	102 ± 1	58 ± 10

Data are the mean ± SEM of three separate experiments. Test compounds were used at a final concentration of 1 mM. Control KAT activity was 0.38 ± 0.04 pmol/h/mg tissue. Control KYNA production in slices was 33.6 ± 3.9 pmol/h/mg protein. Experiments were performed as described in the text. —: Not tested.

where the acylalanine side chain or the aromatic amino group of KYN was modified. The data shown in table I revealed that KYN chain modification (reduction or elimination of the keto group or decarboxylation of the amino acidic moiety) resulted in

compounds (tested at a final concentration of 1 mM) which were unable to interfere with KYNA production in cell-free tissue homogenate. Similarly, elimination or partial and full methylation of the aromatic amino group produced compounds with low inhibitory effects. Three of the test compounds showed slight inhibition of KYNA production in slices though they were devoid of activity in tissue homogenate.

#### *Effect of chlorine substitution of KYN's aromatic ring*

In the next set of experiments, we examined if chlorination in different positions of the benzene ring would affect the production of KYNA in tissue homogenate or slices (table II). All racemic compounds in this group showed some degree of inhibitory activity. Chlorination in positions 3 and 4 produced compounds with a relatively low inhibitory effect. Interference with KYNA production increased when chlorine substitution was made in positions 5 and 6, with DL-5-Cl-KYN emerging as the most potent compound of the group. Overall, the same rank order of potency

was obtained using tissue homogenates and tissue slices, though differences in uptake characteristics appear to have influenced the relative efficacy of test compounds somewhat in the whole cell preparation.

#### *Stereospecificity of 4-Cl-KYN and 5-Cl-KYN*

The stereospecificity of the inhibitory effect was studied in tissue homogenates using the D- and L-enantiomers of 4-Cl-KYN, the bioprecursor of the preferential N-methyl-D-aspartate (NMDA) receptor antagonist 7-Cl-KYNA [16], and of 5-Cl-KYN, the most potent chlorinated KYN derivative obtained in the studies with racemic chlorinated compounds. At concentrations up to 1 mM, D-4-Cl-KYN and D-5-Cl-KYN showed very little effect on KYNA production (table III). In contrast, the two L-enantiomers showed approximately double the potency compared with their respective racemic parent compounds (*cf* table II), yielding IC<sub>50</sub> values of 540 and 63 μM for 4-Cl-KYN and 5-Cl-KYN, respectively.

**Table II.** Effect of ring chlorination of KYN on KYNA production in the rat brain.

	(D,L)-3-Cl-KYN		(D,L)-4-Cl-KYN		(D,L)-5-Cl-KYN		(D,L)-6-Cl-KYN	
	Hg	Slice	Hg	Slice	Hg	Slice	Hg	Slice
1000 μM	63 ± 7.8	35 ± 2.1	59 ± 3.6	35 ± 3.1	19 ± 1.7	16 ± 3.9	18 ± 2.3	32 ± 4.6
300 μM	82 ± 7.9	57 ± 4.1	78 ± 5.6	62 ± 2.4	34 ± 3.6	23 ± 5.7	35 ± 0.7	41 ± 5.9
100 μM	—	—	93 ± 4.6	99 ± 4.6	55 ± 11.3	37 ± 11.3	68 ± 4.1	76 ± 5.9
30 μM	—	—	—	—	79 ± 5.5	68 ± 11.8	86 ± 4.1	90 ± 14.5
10 μM	—	—	—	—	95 ± 5.5	72 ± 2.1	98 ± 5.0	125 ± 13.1

Test compounds were used at the final concentrations indicated. Experiments were performed as described in the text. Data are expressed as a percentage of control KAT activity (0.42 ± 0.05 pmol/h/mg tissue) or control KYNA production in slices (42.9 ± 4.0 pmol/h/mg protein), and are the mean ± SEM of three separate experiments. Hg: tissue homogenate. —: Not tested.

**Table III.** Effect of enantiomeric forms of 4-Cl-KYN and 5-Cl-KYN on KYNA production in cell-free homogenates.

	D-4-Cl-KYN	L-4-Cl-KYN	D-5-Cl-KYN	L-5-Cl-KYN
1000 μM	88 ± 1.6	37 ± 5.9	69 ± 1.9	11 ± 1.3
300 μM	—	62 ± 9.9	—	24 ± 3.9
100 μM	—	81 ± 11.1	—	39 ± 1.3
30 μM	—	94 ± 8.8	—	69 ± 8.2
10 μM	—	—	—	84 ± 9.3
3 μM	—	—	—	93 ± 7.3

Test compounds were used at the final concentrations indicated. Experiments were performed as described in the text. Data are expressed as a percentage of control KAT activity (0.42 ± 0.04 pmol/h/mg tissue), and are the mean ± SEM of three separate experiments. —: Not tested.

*Effect of different substitutions in position 5 of KYN's aromatic ring*

Seven racemic ring-5-substituted KYN derivatives were compared regarding their ability to influence KYNA production in both tissue homogenate and slices. As with other KYN analogs (see above), there was a reasonably good agreement between the results obtained in the two test systems. As shown in table IV, all compounds were capable of inhibiting KYNA synthesis, with 5-n-propyl-KYN and 5-Br-KYN being the most active. The potencies of the two latter substituted kynurenines were similar to each other and to DL-5-Cl-KYN.

*Kinetic analysis of the effects of L-4-Cl-KYN and L-5-Cl-KYN on KAT activity*

The inhibitory effects of the L-enantiomers of 4- and 5-Cl-KYN were further investigated using partially purified KAT. Variation of the concentration of KYN between 2 and 100  $\mu\text{M}$ , and the use of three different concentrations of each KYN analog, yielded saturation curves which were indicative of competitive enzyme inhibition by the two substituted kynurenines (fig 1). These data gave a  $K_m$  value of  $27.6 \pm 3.2 \mu\text{M}$  for KYN and  $K_i$  values of  $84.4 \pm 4.3$  and  $5.4 \pm 0.6 \mu\text{M}$  for L-4-Cl-KYN and L-5-Cl-KYN, respectively.

*L-4-Cl-KYN and L-5-Cl-KYN as substrates of KAT*

Using a partially purified enzyme preparation, a final set of experiments was designed to examine the ability of L-4-Cl-KYN and L-5-Cl-KYN to serve as substrates for KAT. As depicted in figure 2, L-4-Cl-KYN and L-5-Cl-KYN were readily converted to 7-Cl-KYNA and 6-Cl-KYNA, respectively. Since the two KYN derivatives (at a concentration of 2  $\mu\text{M}$

each) were incubated simultaneously with 2  $\mu\text{M}$  KYN, it was possible to evaluate quantitatively the relative affinity of the three substrates to the enzyme. In agreement with the low  $K_i$  value of L-5-Cl-KYN (cf above), 6-Cl-KYNA production was found to be several times in excess of 7-Cl-KYNA synthesis (fig 2B). Moreover, neither of the two D-enantiomers yielded a chlorinated KYNA analog (fig 2C).

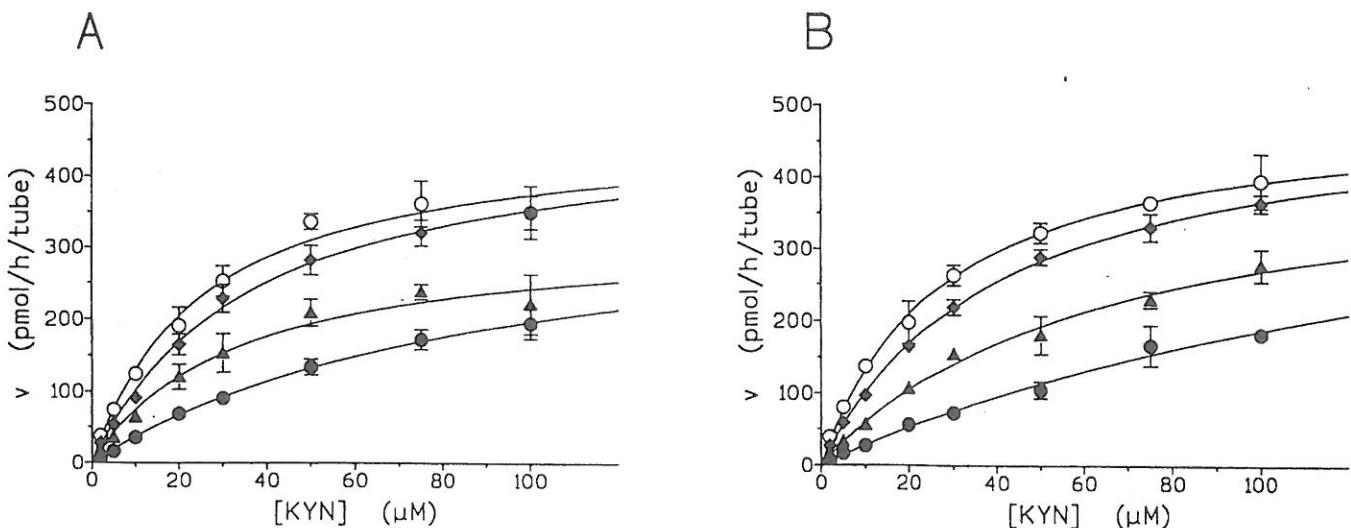
## Discussion

Although it is not possible to fit all data into a simple rational scheme, several conclusions can be drawn from the present study. Thus, a preserved carbonyl residue is clearly essential for KAT activity since partial or complete reduction of this group yielded compounds which were completely inactive as enzyme inhibitors. This could mean that a H-bond acceptor residue (*ie* the keto group) in the enzyme's active site is important. Moreover, the results demonstrate unequivocally that the carboxy function in the acylalanine moiety of KYN is necessary for catalytic activity. The presence of the *ortho* amino group in the aromatic ring of KYN is clearly also mandatory for activity since its deletion and either monomethyl or dimethyl substitution resulted in loss of enzyme inhibition. The effect of varying the substitution pattern on KYN's aromatic ring shows that substitution *meta* to the acylalanine side chain (ring position 5) yields compounds with good inhibitory activity against KAT. A certain degree of steric hindrance was clearly tolerated among a series of 5-alkylated KYN derivatives (*n*-propyl > *i*-propyl > cyclohexyl > ethyl > methyl). Taken together, the existence of a lipophilic pocket in the active site of the enzyme, corresponding to position 5 of KYN's aromatic ring, is likely to play a role in enzyme inhibition. The importance of the

**Table IV.** Effect of different KYN substituents in position 5 on KYNA production in the rat brain.

	5-F-KYN		5-Br-KYN		5-Me-KYN		5-Et-KYN		5-n-Pr-KYN		5-i-Pr-KYN		5-Cyclohexyl-KYN	
	Hg	Slice	Hg	Slice	Hg	Slice	Hg	Slice	Hg	Slice	Hg	Slice	Hg	Slice
1000 $\mu\text{M}$	26 $\pm$ 2.1	35 $\pm$ 5.9	17 $\pm$ 1.1	29 $\pm$ 4.9	48 $\pm$ 3.6	36 $\pm$ 8.9	22 $\pm$ 1.6	36 $\pm$ 6.9	28 $\pm$ 3.1	30 $\pm$ 3.5	28 $\pm$ 1.8	46 $\pm$ 1.8	24 $\pm$ 1.2	55 $\pm$ 3.9
300 $\mu\text{M}$	45 $\pm$ 3.7	49 $\pm$ 3.0	30 $\pm$ 0.7	35 $\pm$ 2.6	70 $\pm$ 2.7	50 $\pm$ 6.5	48 $\pm$ 4.2	49 $\pm$ 6.7	46 $\pm$ 1.0	38 $\pm$ 0.6	39 $\pm$ 0.9	74 $\pm$ 6.6	29 $\pm$ 0.5	58 $\pm$ 5.1
100 $\mu\text{M}$	70 $\pm$ 5.0	60 $\pm$ 4.2	48 $\pm$ 0.8	41 $\pm$ 5.8	86 $\pm$ 6.1	71 $\pm$ 11.5	70 $\pm$ 3.2	69 $\pm$ 6.1	50 $\pm$ 2.4	73 $\pm$ 12.3	55 $\pm$ 2.0	95 $\pm$ 9.1	63 $\pm$ 4.3	72 $\pm$ 6.7
30 $\mu\text{M}$	-	-	73 $\pm$ 3.7	66 $\pm$ 4.4	-	-	-	-	70 $\pm$ 2.5	81 $\pm$ 11.4	-	-	-	-

Test compounds were used at the final concentrations indicated. Experiments were performed as described in the text. Data are expressed as a percentage of control KAT activity (0.44  $\pm$  0.03 pmol/h/mg tissue) or control KYNA production in slices (39.7  $\pm$  3.8 pmol/h/mg protein), and are the mean  $\pm$  SEM of three separate experiments. Hg: tissue homogenate. -: Not tested.



**Fig 1.** Kinetic analysis of the effect of L-4-Cl-KYN (A) and L-5-Cl-KYN (B) on partially purified rat KAT. KAT activity was determined in 10  $\mu$ l of the enzyme preparation (activity per tube at 2  $\mu$ M KYN: 20 pmol/h). The concentrations used were: 30 ( $\blacklozenge$ ), 100 ( $\blacktriangle$ ) and 300 ( $\bullet$ )  $\mu$ M in A and 3, ( $\blacklozenge$ ), 10 ( $\blacktriangle$ ) and 30 ( $\bullet$ )  $\mu$ M in B. (O): Control activity in the absence of test compounds. Data are the mean  $\pm$ SEM of three separate experiments.

proper orientation of the KYN analogs in the enzyme's active site is underscored by the dependency of the inhibition on the stereochemistry of the amino-acidic residue. In fact, as judged from the use of the enantiomers of 4- and 5-Cl-KYN, the L-isomers are the eutomers for KAT inhibition. Finally, both kinetic analysis and the enzymatic conversion of L-4-Cl-KYN and L-5-Cl-KYN to their expected products (7-Cl-KYNA and 6-Cl-KYNA, respectively) show that KAT inhibition by KYN analogs, at least with these two model compounds, is due to competition with the natural substrate, KYN.

The demonstration that 5-substituted KYN derivatives are relatively potent competitive inhibitors (and substrates) of KAT suggests their possible use in neurobiological studies, should it become desirable to modulate the concentrations of KYN or KYNA experimentally *in vitro* or *in vivo*. In contrast to 4-Cl-KYN, which is a moderately active KAT inhibitor and is metabolically transformed to the potent and highly selective excitatory amino acid receptor antagonist 7-Cl-KYNA [10], compounds such as the prototypical 5-Cl-KYN could be used to *decrease* endogenous KYNA levels and do not appear to give rise to powerful excitatory amino acid receptor antagonists [16, 17]. 5-Substituted KYN derivatives could therefore conceivably serve as tools to test the hypothesis that removal of endogenous KYNA from excitatory synapses in the brain results in overexcitation with (neuro)pathological consequences [3]. It will also be interesting, however, to examine the effects of these

compounds on other KYN-metabolizing enzymes, such as kynurenine-3-hydroxylase and kynureinase [1, 18]. Interaction with any of these enzymes is not unlikely and would substantially impact the use of substituted kynureines as biological probes.

## Experimental protocols

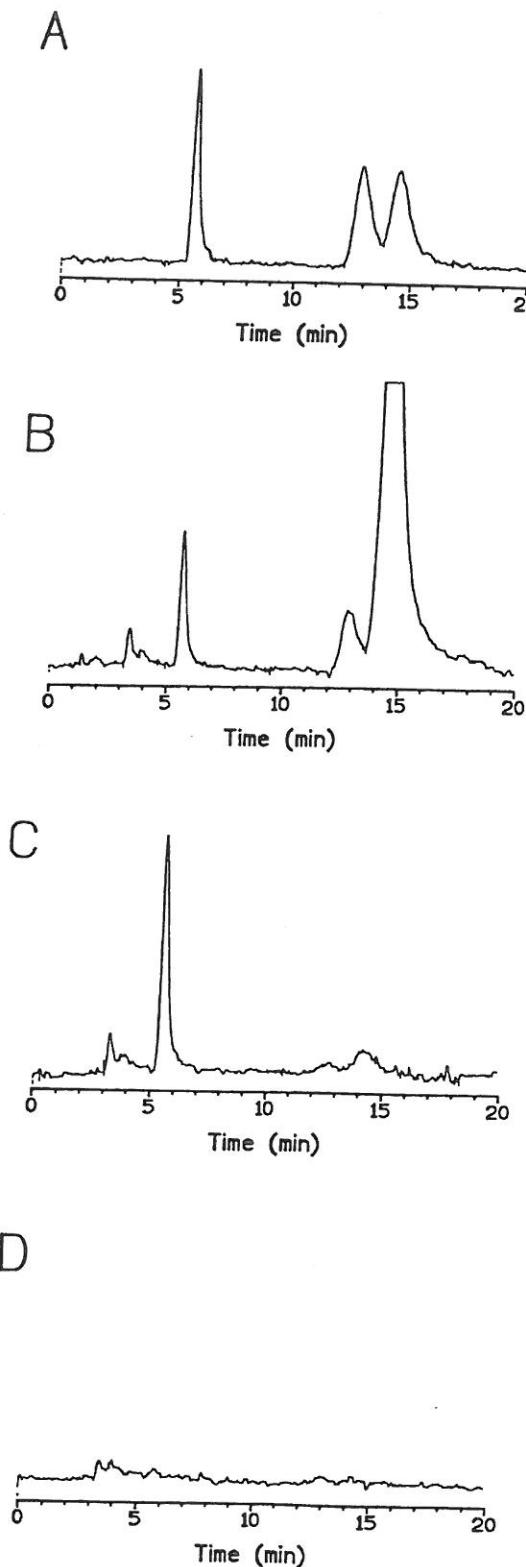
### Chemistry

#### General procedures

Melting points were determined in open capillaries using a Büchi 512 apparatus and are uncorrected. Proton-NMR spectra were recorded on a Varian VXR-200 instrument. Chemical shifts are reported as values in parts per million relative to tetramethylsilane as an internal standard. Mass spectra were recorded on a Varian MAT 311/A instrument. Optical rotations were determined on a Perkin-Elmer automatic polarimeter. Elemental analyses were performed by the analytical laboratories of Pharmacia and agreed with theoretical values within  $\pm 0.4\%$ . Common reagent-grade chemicals and starting materials were purchased from commercial sources and were used as received. Drying of solvents was performed by storage on 3 or 4  $\text{\AA}$  molecular sieves. Evaporations were made *in vacuo* (rotating evaporator) and were preceded by drying over sodium sulfate. Flash-chromatography was carried out using silica gel 60, 230–400 mesh (Carlo-Erba) and the solvent mixture reported within parentheses was used as eluent.

#### Chloroacetophenones 2e–m

To a stirred solution of boron trichloride in dichloromethane (0.1 mol), a solution of 4-substituted aniline (1e–m) (0.095 mol) in dry benzene (120 ml) was added dropwise under



nitrogen at a temperature ranging from 5 to 10°C. To the resulting mixture, chloroacetonitrile (0.115 mol) and aluminum trichloride (0.1 mol) were added successively. The mixture was refluxed for 20 h. After cooling, ice-cold 2 N hydrochloric acid (100 ml) was added and a yellow precipitate formed. The mixture was warmed at 80°C under stirring until the precipitate had dissolved (45 min). The cooled solution was adjusted to pH 2 by addition of 2 N NaOH and extracted with dichloromethane (three times). The organic layer was washed with water, dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated. The residue was ground with hexane (120 ml) to obtain the desired products 2e–m.

**2e:** (41%); mp 121–123°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 4.61 (s, 2H,  $\text{COCH}_2$ ); 6.15 (bs, 2H,  $\text{NH}_2$ ); 6.6–7.4 (m, 3H, arom). **2f:** (31%); mp 127–130°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 4.65 (s, 2H,  $\text{COCH}_2$ ); 6.30 (bs, 2H,  $\text{NH}_2$ ); 6.55–7.75 (m, 3H, arom). **2g:** (24%); mp 127–129°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 2.25 (s, 3H,  $\text{CH}_3$ ); 4.70 (s, 2H,  $\text{COCH}_2$ ); 6.15 (bs, 2H,  $\text{NH}_2$ ); 6.55–7.40 (m, 3H, arom). **2h:** (41%); mp 118–120°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.2 (t, 3H,  $\text{CH}_3$ ); 2.55 (q, 2H,  $\text{CH}_2\text{CH}_3$ ); 4.70 (s, 2H,  $\text{COCH}_2$ ); 6.15 (bs, 2H,  $\text{NH}_2$ ); 6.60–7.45 (m, 3H, arom). **2i:** (32%); mp 120–122°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 0.95 (t, 3H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 1.35–1.80 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 2.50 (t, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 4.70 (s, 2H,  $\text{COCH}_2$ ); 6.00 (bs, 2H,  $\text{NH}_2$ ); 6.60–7.40 (m, 3H, arom). **2l:** (31%); mp 66–70°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.22 (d, 6H,  $\text{CH}(\text{CH}_3)_3$ ); 2.60–3.00 (m, 1H,  $\text{CH}(\text{CH}_3)_2$ ); 4.70 (s, 2H,  $\text{COCH}_2$ ); 5.7 (bs, 2H,  $\text{NH}_2$ ); 6.60–7.50 (m, 3H, arom). **2m:** (33%); mp 88–91°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.00–2.00 (m, 10H,  $\text{CH}(\text{CH}_2)_5$ ); 2.20–2.60 (m, 1H,  $\text{CH}(\text{CH}_2)_5$ ); 4.70 (s, 2H,  $\text{COCH}_2$ ); 6.15 (bs, 2H,  $\text{NH}_2$ ); 6.55–7.45 (m, 3H, arom).

#### 2-Acetylamino-2-[2-(phenyl-substituted)-2-oxoethyl]malonic acid diethyl esters 3a–m

Sodium (0.0453 mol) was dissolved in ethanol (80 ml) and diethylacetamido malonate (diethyl formamidomalonate in the case of **2b**) (0.0453 mol) in ethanol (120 ml) was added. The solution was warmed at 45°C for 1 h. After cooling at room temperature, a solution of 5-substituted-2-amino- $\alpha$ -chloroacetophenone (**2a–m**) (0.0453 mol) in ethanol (150 ml) was added dropwise, while stirring under nitrogen. The reaction was stirred for 20 h at room temperature, and the mixture was evaporated to dryness and the residue was taken up with water (300 ml) and 1 N NaOH (20 ml), and extracted with diethyl ether (3 × 80 ml). The organic phase was washed with water, dried and evaporated to dryness to give a solid which was recrystallized from isopropyl alcohol or purified by flash-chromatography (*n*-hexane/ethyl acetate 3:2) to obtain the product (**3a–m**) as a white solid.

**3a:** (54%); mp 139–142°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 4.22 (s, 2H,

**Fig 2.** Chromatograms illustrating the enzymatic conversion of L-4-Cl-KYN and L-5-Cl-KYN by KAT. Assays were performed as described in the text using partially purified KAT. A: Standards (retention time): KYNA (5.6 min), 7-Cl-KYNA (12.9 min), 6-Cl-KYNA (14.6 min); B: Incubation with 2  $\mu\text{M}$  each of KYN, L-4-Cl-KYN and L-5-Cl-KYN; C: Incubation with 2  $\mu\text{M}$  each of KYN, D-4-Cl-KYN and D-5-Cl-KYN; D: Heat-inactivated enzyme incubated with 2  $\mu\text{M}$  each of KYN, L-4-Cl-KYN and L-5-Cl-KYN.

$\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.15 (bs, 2H,  $\text{NH}_2$ ); 6.55–7.85 (m, 5H, arom +  $\text{NHCO}$ ). **3b:** (39%); mp 110–115°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 4.25 (s, 2H,  $\text{COCH}_2$ ); 4.30 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.60 (bs, 2H,  $\text{NH}_2$ ); 6.50–8.10 (m, 5H, arom +  $\text{NHCHO}$ ). **3c:** (46%); mp 134–137°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 4.20 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.25 (bs, 2H,  $\text{NH}_2$ ); 6.55–7.75 (m, 4H, arom +  $\text{NHCO}$ ). **3d:** (34%); mp 156–158°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 4.20 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.15 (bs, 2H,  $\text{NH}_2$ ); 6.5–7.75 (m, 4H, arom +  $\text{NHCO}$ ). **3e:** (34%); mp 143–145°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 4.20 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.05 (bs, 2H,  $\text{NH}_2$ ); 6.50–7.55 (m, 4H, arom +  $\text{NHCO}$ ). **3f:** (35%); mp 151–154°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 4.20 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.20 (bs, 2H,  $\text{NH}_2$ ); 6.45–7.90 (m, 4H, arom +  $\text{NHCO}$ ). **3g:** (49%); mp 179–181°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 2.25 (s, 3H,  $\text{ArCH}_3$ ); 4.22 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.00 (bs, 2H,  $\text{NH}_2$ ); 6.50–7.60 (m, 4H, arom +  $\text{NHCO}$ ). **3h:** (42%); mp 121–123°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.20 (t, 3H,  $\text{CH}_2\text{CH}_3$ ); 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 2.55 (q, 2H,  $\text{CH}_2\text{CH}_3$ ); 4.22 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.00 (bs, 2H,  $\text{NH}_2$ ); 6.52–7.60 (m, 4H, arom +  $\text{NHCO}$ ). **3i:** (50%); mp 115–117°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 0.95 (t, 3H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.35–1.80 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 2.50 (t, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 4.22 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.05 (bs, 2H,  $\text{NH}_2$ ); 6.50–7.60 (m, 4H, arom +  $\text{NHCO}$ ). **3l:** (37%); mp 110–115°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.22 (d, 6H,  $\text{CH}(\text{CH}_3)_2$ ); 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 2.60–3.00 (m, 1H,  $\text{CH}(\text{CH}_3)_2$ ); 4.22 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.00 (bs, 2H,  $\text{NH}_2$ ); 6.55–7.65 (m, 4H, arom +  $\text{NHCO}$ ). **3m:** (43%); mp 89–93°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.00–2.00 (m, 10H,  $\text{CH}(\text{CH}_3)_2$ ); 1.25 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.98 (s, 3H,  $\text{COCH}_3$ ); 2.15–2.60 (m, 1H,  $\text{CH}(\text{CH}_3)_2$ ); 4.22 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.05 (bs, 2H,  $\text{NH}_2$ ); 6.50–7.60 (m, 4H, arom +  $\text{NHCO}$ ).

#### Preparation of the acetamidomalonate precursors of **5a** and **6a** by mono- and dimethylation of **4a**

**Procedure for monomethylation.** To a solution of 2-acetyl-amino-2-[2-(2-amino-phenyl)-2-oxo-ethyl]-malonic acid diethyl ester (**3a**) (2.7 g; 0.0077 mol) in acetonitrile (60 ml), 40% aq formaldehyde (11.6 ml; 0.154 mol) and sodium cyanoborohydride (2.9 g; 0.046 mol) were added. The pH was adjusted to 6 by addition of acetic acid and the reaction mixture was stirred at room temperature for 8 h. After evaporation, the residue was taken up with water, basified with 2 N NaOH and extracted with diethyl ether. The organic layer was washed with water, dried and evaporated to dryness. The crude residue was purified by flash-chromatography (cyclohexane/ethyl acetate 3:1) to yield the acetamidomalonate precursor of **5a** as an amorphous solid (1.8 g; 64%).  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.22 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.95 (s, 3H,  $\text{COCH}_3$ ); 2.85 (d, 3H,  $\text{NHCH}_3$ ); 4.20 (s, 2H,  $\text{COCH}_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 6.45–7.85 (m, 5H, arom +  $\text{NHCO}$ ); 8.45–8.70 (m, 1H,  $\text{NHCH}_3$ ).

**Procedure for dimethylation.** To a solution of 2-acetyl-amino-2-[2-(2-amino-phenyl)-2-oxo-ethyl]-malonic acid diethyl ester (**3a**) (2.45 g; 0.0070 mol) in anhydrous DMF (30 ml), methyl iodide (3.5 ml; 0.056 mol) and  $\text{K}_2\text{CO}_3$  (2.13 g; 0.0154 mol) were added. The mixture was heated at 80°C for 24 h. After

cooling to room temperature, the reaction mixture was poured into water and extracted twice with diethyl ether. The organic layer was washed with water, dried and concentrated to dryness. The crude product was ground with diisopropyl ether to give the acetamidomalonate precursor of **6a** as a beige solid (2.2 g; 85%); mp 119–121°C;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ ): 1.22 (t, 6H, 2  $\text{COOCH}_2\text{CH}_3$ ); 1.93 (s, 3H,  $\text{COCH}_3$ ); 2.75 (s, 6H,  $\text{N}(\text{CH}_3)_2$ ); 4.25 (q, 4H, 2  $\text{COOCH}_2\text{CH}_3$ ); 4.30 (s, 2H,  $\text{COCH}_2$ ); 6.78–7.45 (m, 5H, arom +  $\text{NHCO}$ ).

#### 2-Amino-4-(2-phenyl-substituted)-4-oxobutyric acids (*D,L*-5-substituted kynurenines) **4b–m**, **5a** and **6a**

A solution of the appropriate 2-acetylaminoo-2-[2-(2-amino-5-substituted phenyl)-2-oxoethyl]malonic acid diethyl esters **3a–m** (0.0138 mol) in 37% HCl (60 ml) was refluxed for 6 h under stirring. The reaction was evaporated to dryness under vacuum and the residue was taken up with water and washed with methylene chloride. The aqueous layer was concentrated, the residue dissolved in water (100 ml) and the resulting solution was adjusted to pH 6.5 by addition of 2 N NaOH to yield the desired product as a yellow precipitate. The solid was filtered and washed with water and if necessary transformed into its hydrochloride salt to obtain the desired product **4b–m**, **5a**, **6a**.

**4b·HCl:** (62%); mp 210–213°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 3.70 (d, 2H,  $\text{CH}_2\text{CH}$ ); 4.20–4.35 (m, 1H,  $\text{CHCH}_2$ ); 6.60–7.80 (m, 3H, arom); 7.30 (bs, 2H,  $\text{NH}_2$ ); 8.30–8.52 (m, 3H,  $\text{NH}_3^+$ ). MS ( $m/z$ ): 242 (15,  $[\text{M}]^{++}$ ); 225 (35.6,  $[\text{M} - \text{NH}_3]^{+}$ ); 180 (95.4,  $[\text{M} - \text{NH}_3 - \cdot\text{COOH}]^+$ ); 154 (100,  $[\text{C}_7\text{H}_5\text{ClNO}]^+$ ); 126 (37.9,  $[\text{C}_6\text{H}_5\text{ClIN}]^+$ ). Anal  $\text{C}_{10}\text{H}_{11}\text{ClN}_2\text{O}_3\cdot\text{HCl}$  (C, H, Cl, N). **4c:** (90%); mp 227°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 3.08–3.52 (m, 2H,  $\text{CH}_2\text{CH}$ ); 3.53–3.62 (m, 1H,  $\text{CHCH}_2$ ); 6.50–7.75 (m, 3H, arom); 7.35 (bs, 2H,  $\text{NH}_2$ ). MS ( $m/z$ ): 243 (100,  $[\text{M} + \text{H}]^+$ ); 226 (16,  $[\text{M} - \text{NH}_3 + \text{H}]^+$ ); 154 (12,  $[\text{C}_7\text{H}_5\text{ClNO}]^+$ ). Anal  $\text{C}_{10}\text{H}_{11}\text{ClN}_2\text{O}_3$  (C, H, Cl, N). **4d:** (64%); mp 236–238°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 3.15–3.60 (m, 3H,  $\text{CH}_2\text{CH}$ ); 6.75–7.65 (m, 3H, arom); 7.25 (bs, 2H,  $\text{NH}_2$ ). MS ( $m/z$ ): 242 (20.7,  $[\text{M}]^{++}$ ); 225 (44.3,  $[\text{M} - \text{NH}_3]^{+}$ ); 180 (68.9,  $[\text{M} - \text{NH}_3 - \cdot\text{COOH}]^+$ ); 154 (100,  $[\text{C}_7\text{H}_5\text{ClNO}]^+$ ); 126 (39.6,  $[\text{C}_6\text{H}_5\text{ClIN}]^+$ ). Anal  $\text{C}_{10}\text{H}_{11}\text{ClN}_2\text{O}_3$  (C, H, Cl, N). **4e:** (64%); mp 220°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 3.15–3.52 (m, 2H,  $\text{CH}_2\text{CH}$ ); 3.54–3.61 (m, 1H,  $\text{CHCH}_2$ ); 6.75–7.50 (m, 3H, arom); 7.10 (bs, 2H,  $\text{NH}_2$ ). MS ( $m/z$ ): 226 (12.3,  $[\text{M}]^{++}$ ); 209 (52.8,  $[\text{M} - \text{NH}_3]^{+}$ ); 164 (100,  $[\text{M} - \text{NH}_3 - \cdot\text{COOH}]^+$ ); 138 (94.3,  $[\text{C}_7\text{H}_5\text{FNO}]^+$ ); 110 (52.8,  $[\text{C}_6\text{H}_5\text{FN}]^+$ ). Anal  $\text{C}_{10}\text{H}_{11}\text{FN}_2\text{O}_3$  (C, H, F, N). **4f:** (92%); mp 233°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 3.15–3.50 (m, 2H,  $\text{CH}_2\text{CH}$ ); 3.58–3.61 (m, 1H,  $\text{CHCH}_2$ ); 6.75–7.80 (m, 3H, arom); 7.22 (bs, 2H,  $\text{NH}_2$ ). MS ( $m/z$ ): 287 (100,  $[\text{M} + \text{H}]^+$ ); 270 (24.4,  $[\text{M} - \text{NH}_3 + \text{H}]^+$ ); 198 (20,  $[\text{C}_7\text{H}_5\text{BrNO}]^+$ ). Anal  $\text{C}_{10}\text{H}_{11}\text{BrN}_2\text{O}_3$  (C, H, Br, N). **4g:** (46%); mp 240°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 2.18 (s, 3H,  $\text{ArCH}_3$ ); 3.10–3.60 (m, 3H,  $\text{CH}_2\text{CH}$ ); 6.62–7.48 (m, 3H, arom); 7.00 (bs, 2H,  $\text{NH}_2$ ). MS ( $m/z$ ): 222 (18.8,  $[\text{M}]^{++}$ ); 205 (30.2,  $[\text{M} - \text{NH}_3]^{+}$ ); 160 (50,  $[\text{M} - \text{NH}_3 - \cdot\text{COOH}]^+$ ); 134 (100,  $[\text{C}_8\text{H}_8\text{NO}]^+$ ); 106 (50,  $[\text{C}_8\text{H}_8\text{N}]^+$ ). Anal  $\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_3$  (C, H, N). **4h:** (95%); mp 228–232°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 1.13 (t, 3H,  $\text{CH}_2\text{CH}_3$ ); 2.46 (q, 2H,  $\text{CH}_2\text{CH}_3$ ); 3.20–3.60 (m, 3H,  $\text{CH}_2\text{CH}$ ); 6.68–7.48 (m, 3H, arom); 7.00 (bs, 2H,  $\text{NH}_2$ ). MS ( $m/z$ ): 236 (20.7,  $[\text{M}]^{++}$ ); 219 (57.5,  $[\text{M} - \text{NH}_3]^{+}$ ); 204 (23.6,  $[\text{M} - \text{NH}_3 - \cdot\text{CH}_3]^{+}$ ); 174 (79.2,  $[\text{M} - \text{NH}_3 - \cdot\text{COOH}]^+$ ); 148 (100,  $[\text{C}_9\text{H}_{10}\text{NO}]^+$ ). Anal  $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_3$  (C, H, N). **4i:** (97%); mp 234°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}/\text{CF}_3\text{COOH}$ ): 0.86 (t, 3H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 1.40–1.50 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 2.22 (t, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ); 3.45–3.70 (m, 2H,  $\text{COCH}_2\text{CH}$ ); 4.18–4.35 (m, 1H,  $\text{CHCH}_2\text{CO}$ ); 6.68–7.55 (m, 3H, arom). MS ( $m/z$ ): 251 (52.3,  $[\text{M} + \text{H}]^+$ ); 234 ( $[\text{M} - \text{NH}_3 + \text{H}]^+$ ); 188 (62.3,  $[\text{M} - \text{NH}_3 -$

$\text{COOH}]^+)$ ; 162 (89.2,  $[\text{C}_{10}\text{H}_{12}\text{NO}]^+$ ); 136 (100,  $[\text{C}_9\text{H}_{11}\text{O}]^+$ ). Anal.  $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_3$  (C, H, N). 4*I*: (81%); mp 235°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}/\text{CF}_3\text{COOH}$ ): 1.15 (d, 6H,  $\text{CH}(\text{CH}_3)_2$ ); 2.70–2.90 (m, 1H,  $\text{CH}(\text{CH}_3)_2$ ); 3.47–3.72 (m, 2H,  $\text{COCH}_2\text{CH}$ ); 4.20–4.35 (m, 1H,  $\text{CHCH}_2\text{CO}$ ); 6.72–7.50 (m, 3H, arom). MS ( $m/z$ ): 251 (80,  $[\text{M} + \text{H}]^+$ ); 234 (40.7,  $[\text{M} - \text{NH}_3 + \text{H}]^+$ ); 188 (28.4,  $[\text{M} - \text{NH}_3 - \text{COOH}]^+$ ); 162 (63.8,  $[\text{C}_{10}\text{H}_{12}\text{NO}]^+$ ); 136 (100,  $[\text{C}_9\text{H}_{11}\text{O}]^+$ ). Anal.  $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_3$  (C, H, N). 4*M*: (70%); mp 245°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 1.10–1.90 (m, 10H,  $\text{CH}(\text{CH}_3)_5$ ); 2.25–2.65 (m, 1H,  $\text{CH}(\text{CH}_2)_5$ ); 3.20–3.60 (m, 3H,  $\text{COCH}_2\text{CH}$ ); 6.66–7.50 (m, 3H, arom); 6.93 (bs, 2H,  $\text{ArNH}_2$ ). MS ( $m/z$ ): 291 (100,  $[\text{M} + \text{H}]^+$ ); 274 (81,  $[\text{M} - \text{NH}_3 + \text{H}]^+$ ); 228 (16,  $[\text{M} - \text{NH}_3 - \text{COOH}]^+$ ); 202 (23.2,  $[\text{C}_{13}\text{H}_{16}\text{NO}]^+$ ); 176 (35.2,  $[\text{C}_{12}\text{H}_{15}\text{O}]^+$ ). Anal.  $\text{C}_{16}\text{H}_{22}\text{N}_2\text{O}_3$  (C, H, N).

**5*a*·2HCl:** (85%); mp 200°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 2.85 (s, 3H,  $\text{NCH}_3$ ); 3.70 (d, 2H,  $\text{COCH}_2\text{CH}$ ); 4.15–4.30 (m, 1H,  $\text{CHCH}_2$ ); 6.55–7.82 (m, 4H, arom), 8.30–8.85 (m, 3H,  $\text{NH}_3^+$ ). MS ( $m/z$ ): 223 (100,  $[\text{M} + \text{H}]^+$ ); 206 (60.8,  $[\text{M} - \text{NH}_3 + \text{H}]^+$ ); 160 (10.4,  $[\text{M} - \text{NH}_3 - \text{COOH}]^+$ ). Anal.  $\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_3\cdot2\text{HCl}$  (C, H, Cl, N). **6*a*·2HCl:** (95%); mp 130°C (dec);  $^1\text{H-NMR}$  ( $\delta$ ,  $(\text{CD}_3)_2\text{SO}$ ): 3.02 (s, 6H,  $\text{N}(\text{CH}_3)_2$ ); 3.80 (d, 2H,  $\text{COCH}_2\text{CH}$ ); 4.25–4.40 (m, 1H,  $\text{CHCH}_2$ ); 7.25–7.85 (m, 4H, arom) 8.40–8.85 (m, 3H,  $\text{NH}_3^+$ ). MS ( $m/z$ ): 237 (100,  $[\text{M} + \text{H}]^+$ ); 220 (100,  $[\text{M} - \text{NH}_3 + \text{H}]^+$ ). Anal.  $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_3\cdot2\text{HCl} \cdot \text{H}_2\text{O}$  (C, H, Cl, N,  $\text{H}_2\text{O}$ ).

#### Preparation of (*R*)-2-amino-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid (*D*-5-chlorokynurenone) **10** and (*S*)-2-amino-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid (*L*-5-chlorokynurenone) **11** by diastereomeric salt resolution

To a stirred solution of 2-amino-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **4d** (5.23 g, 0.0215 mol),  $\text{NaHCO}_3$  (3.98 g, 0.0474 mol) and  $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$  (6.16 g, 0.0215 mol) in 300 ml of water, acetic anhydride (2.24 ml, 0.0237 mol) was added dropwise at room temperature. The solution was stirred for 1 h, acidified to pH 5 with 2 N HCl, extracted with ethyl acetate (four times), washed with water, dried and evaporated to give a light yellow solid. The crude product was ground with hexane to obtain 2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **7** (4.20 g; 68%; mp 187–190°C).

To a stirred solution of 2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **7** (4.20 g, 0.0147 mol) in 500 ml of 95% ethanol, *R*-(+)- $\alpha$ -methylbenzylamine (1.88 ml, 0.0147 mol) was added dropwise. After 20 min, the solvent was evaporated; the residue was ground with diethyl ether to obtain a solid (5.10 g; mp 130–150°C;  $[\alpha]_D^{20} = +2.5^\circ$  (1%,  $\text{CH}_3\text{OH}$ )). Four further recrystallizations from ethyl acetate yielded 1.12 g (enantiomeric yield 37%) of the (*–*)- $\alpha$ -methylbenzylamine salt of 2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid (mp 154.5–156°C);  $[\alpha]_D^{20} = -60.8^\circ$  (1%,  $\text{CH}_3\text{OH}$ ).

To a vigorously stirred solution of (*–*)- $\alpha$ -methylbenzylamine salt (1.12 g, 0.00276 mol) in ethyl acetate (110 ml), water (20 ml) and then 1 N HCl (2.9 ml, 0.0029 mol) were added. After 30 min, the organic phase was separated and the aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with water, dried and evaporated to give (*–*)-2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid (0.76 g; 97%) **8** as a light brown solid (mp 182–183.5°C; (dec));  $[\alpha]_D^{20} = -88.7^\circ$  (1%,  $\text{CH}_3\text{OH}$ ).

A solution of (*–*)-2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **8** (0.76 g, 0.00267 mol) in 2 N HCl (90 ml) was heated at reflux for 3 h. The aqueous solution was evaporated to dryness, the residue was dissolved in water, washed with methylene chloride and evaporated again. The residue was dissolved in water (30 ml) and adjusted to pH 6.5

with 1 N NaOH; a light yellow solid separated. The solid was filtered and washed with cold water to obtain (*R*)-2-amino-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **10** (0.40 g; 59.7%); mp 210–211°C (dec);  $[\alpha]_D^{20} = +11.5^\circ$  (1%,  $\text{CH}_3\text{COOH}$ ); ee = 96%).

The mother liquors obtained during the preparation of the (*–*)- $\alpha$ -methylbenzylamine salt were mixed and evaporated to give 4.9 g (0.012 mol) of a solid. This mixture of (*–*) and (+) salt was vigorously stirred with ethyl acetate (400 ml), water (80 ml) and 1 N HCl (12.6 ml, 0.0126 mol). After 30 min, the organic phase was separated and the aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with water, dried and evaporated to give 2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **7** (light brown solid) enriched in the (+)-enantiomer (3.41 g; 100%; mp 172–178°C).

To a stirred solution of 2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid (3.41 g, 0.012 mol) in 400 ml of 95% ethanol, *S*-(–)- $\alpha$ -methylbenzylamine (1.52 ml, 0.012 mol) was added dropwise. After 20 min, the solvent was evaporated. Three recrystallizations from ethyl acetate afforded 1.49 g (61%) of the (+)- $\alpha$ -methylbenzylamine salt of 2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid, (mp 154.5–155.5°C;  $[\alpha]_D^{20} = +60.9^\circ$  (1%,  $\text{CH}_3\text{OH}$ )). This salt (1.49 g) was processed as previously described for the (*–*)-enantiomer and afforded 1.03 g (98.5%) of (+)-2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **9** (mp 179–180°C; (dec));  $[\alpha]_D^{20} = +89.0^\circ$  (1%,  $\text{CH}_3\text{OH}$ ).

Starting from 1.03 g of (+)-2-acetamido-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid as described above for the preparation of (*R*)-enantiomer, 0.65 g (71%) of (*S*)-2-amino-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **11** was obtained (mp 208–209°C; (dec));  $[\alpha]_D^{20} = -12.6^\circ$  (1%,  $\text{CH}_3\text{COOH}$ ); ee = 96%).

#### Enantiospecific synthesis of (*S*)-2-amino-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid (*L*-5-chlorokynurenone) **11**

A solution of 4-chloro-*N*-pivaloylaniline **12** (2.11 g, 0.01 mol) in 30 ml of dry THF was cooled in an ice bath under an atmosphere of nitrogen. A hexane solution of 1.6 M *n*-BuLi (15.625 ml, 0.025 mol) was then added dropwise. After stirring at 0°C for 2.5 h, trimethyltin chloride (2.19 g, 0.011 mol) dissolved in dry THF (8 ml) was added within 20 min at 0°C. The reaction was then stirred for 2 h at 0°C and for 16 h at room temperature, quenched with water and extracted with ethyl acetate. The organic layer was washed with saturated NaCl, dried and concentrated to dryness. The residue was purified by flash chromatography on silica gel (diisopropylether/petroleum ether 40°/70°C = 10:23) to yield 4-chloro-2-trimethylstannyl-*N*-pivaloylaniline **13** as a white solid (3.1 g; 83%; mp 169–172°C).

4-Chloro-2-trimethylstannyl-*N*-pivaloylaniline **13** (1.6 g, 0.0042 mol) and (*S*)-3-benzylloxycarbonyl-5-oxo-4-oxazolidine-acetyl chloride [9] (1.25 g, 0.0042 mol) were dissolved in anhydrous toluene (50 ml). To this solution  $\text{Pd}(\text{dba})_3\text{CHCl}_3$  (40.5 mg) was added and the mixture was heated at 70°C for 8 h. The mixture was cooled, and the catalyst was removed by filtration over celite. The filtrate was concentrated and then diluted with ethyl acetate; the resulting solution was washed successively with saturated bicarbonate, water and saturated NaCl, then dried and evaporated. The residue was purified by flash chromatography on silica gel (ethyl acetate/hexane = 8:30) to obtain (*S*)-(+)*N*-pivaloyl-2-[1-oxo-2-[3-(benzyloxy-carbonyl)-5-oxo-oxazolidin-4-yl]-ethyl]-4-chloroaniline **15** as colorless crystals 1.06 g (53%); mp 113–116°C;  $[\alpha]_D^{20} = -114.5^\circ$  (1%; ethyl acetate).

A stirred mixture of (*S*)-(+)-*N*-pivaloyl-2-[1-oxo-2-[3-(benzyloxycarbonyl)-5-oxo-oxazolidin-4-yl]ethyl]-4-chloroaniline **15** (1.06 g, 0.00224 mol), 37% HCl (20 ml), H<sub>2</sub>O (20 ml) and CH<sub>3</sub>COOH (28 ml), was heated at 70°C for 5.5 h. The solution was evaporated to dryness. The residue was dissolved in water (30 ml), washed with methylene chloride and evaporated again. The crude product was purified by flash chromatography on silica gel (chloroform/methanol/30% NH<sub>4</sub>OH = 140:60:6) to yield (*S*)-2-amino-4-(2-amino-5-chlorophenyl)-4-oxobutyric acid **11** as yellow crystals (0.29 g; 51%; mp 206–208°C; [α]<sub>D</sub><sup>20</sup> = -12.0° (1%; CH<sub>3</sub>COOH); ee 96%.

#### 2-Amino-4-(2-amino-6-chlorophenyl)-4-oxobutyric acid (DL-6-chlorokynurenone) **16**

To a stirred solution of 2-chloro-6-nitroacetophenone [19] (13.7 g; 0.0686 mol) in diethylether (400 ml), bromine (3.86 ml; 0.075 mol) was added dropwise at 30–32°C. The solution was stirred for 2 h at mild reflux; after cooling at 5°C, a saturated solution of NaHCO<sub>3</sub> was added. The organic phase was washed with water, dried and evaporated to dryness. The crude product was ground with *n*-hexane to give 2-chloro-6-nitro- $\alpha$ -bromoacetophenone **17** (16.1 g; 84.3%) as a light yellow solid (mp 101–105°C) which was used in the next step without further purification. <sup>1</sup>H-NMR (δ, CDCl<sub>3</sub>): 4.42 (s, 2H, COCH<sub>2</sub>); 7.45–8.25 (m, 4H, arom).

To a stirred solution of 97% potassium *t*-butoxide (7.6 g; 0.066 mol) in 200 ml of anhydrous THF, at room temperature and under nitrogen, a solution of diethyl acetamidomalonate (14.33 g; 0.066 mol) in anhydrous THF (150 ml) was added dropwise. The solution was heated at 45°C for 1 h, cooled at 32–33°C and 2-chloro-6-nitro- $\alpha$ -bromoacetophenone (16.0 g; 0.0574 mol) in 250 ml of anhydrous THF was added dropwise within 2 h. The mixture was stirred for 20 h at room temperature and evaporated to dryness. The residue was taken up with water and extracted with ethyl acetate. The organic phase was washed with water, dried with anhydrous sodium sulfate and evaporated to dryness. The residue was purified by flash-chromatography (cyclohexane/ethyl acetate = 23:15) to give 2.80 g (10.2%) of 2-acetylaminoo-2-[2-(6-chloro-2-nitrophenyl)-2-oxoethyl]malonic acid diethyl ester as brown crystals (mp 165–170°C). <sup>1</sup>H-NMR (δ, CDCl<sub>3</sub>): 1.25 (t, 6H, 2 COOCH<sub>2</sub>CH<sub>3</sub>); 2.05 (s, 3H, COCH<sub>3</sub>); 4.20 (s, 2H, COCH<sub>2</sub>); 4.30 (q, 4H, 2 COOCH<sub>2</sub>CH<sub>3</sub>); 7.15 (bs, H, NHCOCH<sub>3</sub>); 7.45–8.25 (m, 3H, arom).

To a stirred solution of 2-acetylaminoo-2-[2-(6-chloro-2-nitrophenyl)-2-oxoethyl]malonic acid diethyl ester (2.64 g; 0.00636 mol) in glacial acetic acid (26 ml) at 95°C, 97% iron powder (1.83 g; 0.0318 g atom) was added in small portions during 1 h. The mixture was stirred throughout and water (6.6 ml portions) was added 0, 20, 40 and 60 min after the start of the reaction while maintaining the temperature at 90–95°C. After 30 min, water (100 ml) was added and the product was extracted with ethyl acetate. The extract was washed with water, diluted aqueous NaHCO<sub>3</sub>, and water again, and dried over anhydrous sodium sulfate and evaporated. The crude residue was purified by flash-chromatography (hexane/ethyl acetate = 20:15) to obtain 2.05 g (84%) of 2-acetylaminoo-2-[2-(2-amino-6-chlorophenyl)-2-oxoethyl]malonic acid diethyl ester as a pink solid (mp 134–136°C). <sup>1</sup>H-NMR (δ, CDCl<sub>3</sub>): 1.25 (t, 6H, 2 COOCH<sub>2</sub>CH<sub>3</sub>); 1.98 (s, 3H, COCH<sub>3</sub>); 4.25 (s, 2H, COCH<sub>2</sub>); 4.30 (q, 4H, 2 COOCH<sub>2</sub>CH<sub>3</sub>); 4.55 (bs, 2H, NH<sub>2</sub>); 6.45–7.25 (m, 4H, arom + NHCO).

A solution of 2-acetylaminoo-2-[2-(2-amino-6-chlorophenyl)-2-oxoethyl]malonic acid diethyl ester (0.7 g; 0.00182 mol) in 37% HCl (10 ml) was refluxed for 5 h under stirring. The mixture was evaporated, the residue diluted with water and

concentrated twice. The crude product was taken up with acetone and filtered to yield 2-amino-4-(2-amino-6-chlorophenyl)-4-oxobutyric acid dihydrochloride hemihydrate **16** (0.512 g; 87%) as a light brown solid (mp 160–165°C). <sup>1</sup>H-NMR (δ, (CD<sub>3</sub>)<sub>2</sub>SO): 3.54 (d, 2H, CH<sub>2</sub>CH); 4.25–4.40 (m, 1H, CHCH<sub>2</sub>); 6.60–7.15 (m, 3H, arom); 8.40–8.60 (m, 3H, NH<sub>3</sub><sup>+</sup>). MS (m/z): 243 (100, [M + H]<sup>+</sup>); 226 (23, [M – NH<sub>3</sub> + H]<sup>+</sup>); 154 (27, [C<sub>7</sub>H<sub>5</sub>CINO]<sup>+</sup>). Anal C<sub>10</sub>H<sub>11</sub>ClN<sub>2</sub>O<sub>3</sub>·0.5 H<sub>2</sub>O (C, H, Cl, N, H<sub>2</sub>O).

#### Enzymology

##### Chemical reagents

L-KYN (sulfate salt), pyridoxal-5'-phosphate, 2-oxoglutarate, KYNA and 7-Cl-KYNA were obtained from Sigma Chemical Co (Saint Louis, MO, USA). 6-Cl-KYNA was synthesized as described in ref [20]. Custom-synthesized <sup>3</sup>H-KYN (specific activity: 1.85 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, USA). All other chemicals were obtained from different commercial suppliers and were of the highest available purity.

##### Animals

Young, adult male Sprague–Dawley rats (Charles River), kept on a 12 h/12 h light/dark cycle with free access to food and water, were used in all experiments.

##### Tissue preparation

Animals were sacrificed by decapitation, and their brains were immediately removed. The cortex was rapidly dissected on ice. For the determination of KAT activity, the tissue was homogenized using a glass-teflon homogenizer, followed by sonication (1:10, w/v) in 5 mM Tris-acetate buffer, pH 8, containing 10 mM mercaptoethanol and 50 μM pyridoxal-5'-phosphate. For the determination of KYNA production in intact cells, brain prisms (base: 1 × 1 mm) were prepared with a McIlwain chopper as described previously [21].

##### Determination of KAT activity

In principle, KAT activity was assayed by the method of Okuno *et al* [5], using 2 μM <sup>3</sup>H-KYN as the substrate. Test compounds, titrated to pH 7.6, were added in a volume of 20 μl when indicated. The reaction mixture and the enzyme preparation were incubated in a total volume of 0.2 ml at 37°C for 2 h. The reaction was stopped with trichloroacetic acid, 1 ml of 0.1 N HCl was added and <sup>3</sup>H-KYNA was eluted from a Dowex 50 W ion exchange column and quantitated by liquid scintillation spectrometry. All enzyme measurements were performed in triplicate samples. Blanks were obtained by using tissue that had been heat-deactivated for 10 min in a boiling water bath.

##### Determination of KYNA production in tissue slices

KYNA production in slices was measured as described by Turski *et al* [21], using 6–7 prisms per culture well. Test compounds were preincubated with the tissue for 10 min at 37°C. The reaction was initiated by the addition of KYN (final concentration = 50 μM), and the incubation continued for 2 h at 37°C. The reaction was stopped by the addition of 1 N HCl, and KYNA was determined in the incubation medium using HPLC with spectrophotometric detection at 340 nm.

##### Partial enzyme purification

Partial purification of KAT was carried out following the procedure described by Okuno *et al* [22]. Briefly, 100 g of rat kidney were homogenized in 5 volumes of 0.15 M KCl (w/v) containing 50 μM pyridoxal 5'-phosphate, centrifuged, and the super-

natant heated for 1 min at 60°C. The preparation was then quickly chilled on ice, and the precipitate removed by centrifugation (10 000 g, 10 min). Solid  $(\text{NH}_4)_2\text{SO}_4$  was added to the supernatant to achieve 40% saturation, and the precipitated protein was removed by centrifugation (10 000 g, 10 min). After dissolution in 5 mM phosphate buffer, pH 7.5, containing 10 mM mercaptoethanol and 50  $\mu\text{M}$  pyridoxal 5'-phosphate, the solution was desalted by gel filtration on a Sephadex G25 column. The enzyme solution was then applied to a DEAE Sepharose column, precipitated with  $(\text{NH}_4)_2\text{SO}_4$  (70% saturation), desalted by gel filtration on a Sepharose G25 column and applied to a hydroxylapatite column. The active fractions were combined, precipitated with  $(\text{NH}_4)_2\text{SO}_4$  (70% saturation) and again desalted by gel filtration on a Sephadex G25 column. The concentrated enzyme was stored in 50% glycerol at -20°C until assayed.

#### Kinetic analysis

Apparent  $K_m$  values for KYN were determined using partially purified KAT at different KYN concentrations (2–100  $\mu\text{M}$ ). Enzyme measurements were carried out as described above. In addition, apparent  $K_i$  values were obtained for L-4-Cl-KYN and L-5-Cl-KYN by varying the concentration of KYN in the presence of different concentrations of the inhibitor (30, 100 and 300  $\mu\text{M}$  for L-4-Cl-KYN and 3, 10 and 30  $\mu\text{M}$  for L-5-Cl-KYN).  $K_m$  and  $K_i$  values were obtained by fitting a rectangular hyperbola to the data according to the Michaelis–Menten equation using a computerized least squares fit method.

#### Evaluation of the enzymatic conversion of L-4-Cl-KYN and L-5-Cl-KYN

A separate set of experiments was designed to examine the ability of L-4-Cl-KYN and L-5-Cl-KYN to serve as substrates for KAT. To this end, partially purified KAT was incubated under routine assay conditions in the presence of 2  $\mu\text{M}$  of the D- or L-enantiomers of the two test compounds. After stopping the reaction with trichloroacetic acid and removal of the precipitated proteins, the supernatants were diluted (1:10, v/v) in 50 mM sodium acetate/250 mM zinc acetate buffer, pH 6.2, containing 7% acetonitrile, and a 200  $\mu\text{l}$  aliquot was directly subjected to HPLC analysis using the same buffer as the mobile phase [23]. KYNA, 7-Cl-KYNA and 6-Cl-KYNA were separated using a C<sub>18</sub> reverse phase column (LiChrosorb 250 x 4.5 mm, Alltech Assoc, Deerfield, IL, USA), and were determined fluorimetrically (excitation: 344 nm; emission: 398 nm).

#### Protein determination

Acid-precipitated protein from homogenized brain slices was measured according to the method of Lowry *et al* [24] using bovine serum albumin as a standard.

#### Acknowledgments

We thank A Rassoulpour for technical assistance.

#### References

- 1 Stone TW (1993) *Pharmacol Rev* 45, 309–379
- 2 Foster AC, Vezzani A, French ED, Schwarcz R (1984) *Neurosci Lett* 48, 273–278
- 3 Schwarcz R (1992) In: *Drug Research Related to Neuroactive Amino Acids* (Schoesboe A, Diemer NH, Kofoed H, eds) Munksgaard, Copenhagen, 287–301
- 4 Mason M (1959) *J Biol Chem* 235, 2770–2773
- 5 Okuno E, Schmidt W, Parks DA, Nakamura M, Schwarcz R (1991) *J Neurochem* 57, 533–540
- 6 Roberts RC, Du F, McCarthy K, Okuno E, Schwarcz R (1992) *J Comp Neurol* 326, 82–90
- 7 Susagawa T, Adachi M, Sakasura K, Kitagawa A (1979) *J Org Chem* 44, 578–586
- 8 Stille JK (1986) *Angew Chem Int Ed Engl* 25, 508–524
- 9 Salituro FG, McDonald IA (1988) *J Org Chem* 53, 6138–6139
- 10 Salituro FG, Tomlinson RC, Baron BM *et al* (1994) *J Med Chem* 37, 334–336
- 11 Ukai T, Kawazura H, Ishii Y (1974) *J Organomet Chem* 65, 253–266
- 12 Veronese FM, Bocci E, Benassi CA, Scuffone E (1969) *Z Naturforsch* 24B, 294–300
- 13 Phillips RS, Dua KR (1991) *J Am Chem Soc* 113, 7385–7388
- 14 Wattney JWF (Ciba-Geigy Corp) (1983) US patent 4,410,520; *Chem Abstr* 100 034427
- 15 Butenandt A, Renner U (1953) *Z Naturforsch* 8B, 454–462
- 16 Leeson PD, Iversen LL (1994) *J Med Chem* 37, 4053–4067
- 17 McNamara D, Smith ECR, Calligaro DO, O'Malley PJ, McQuaid LA, Dingledine R (1990) *Neurosci Lett* 120, 17–20
- 18 Bender DA (1989) In: *Quinolinic Acid and the Kynurenes* (Stone TW, ed) CRC Press, Boca Raton, 5–28
- 19 Chapman NS, Clarke K, Sawhney SN (1968) *J Chem Soc* 2747–2751
- 20 Baxter R, Bramhall RR (1972) *J Med Chem* 15, 230–233
- 21 Turski WA, Gramsbergen JBP, Trautler H, Schwarcz R (1989) *J Neurochem* 52, 1629–1636
- 22 Okuno E, Du F, Ishikawa T *et al* (1990) *Brain Res* 534, 37–44
- 23 Swartz KJ, Matson WR, MacGarvey UM, Ryan EA, Beal MF (1990) *Anal Biochem* 185, 363–376
- 24 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) *J Biol Chem* 193, 265–275