Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2907–2912

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

4-Phenyl-4-oxo-butanoic Acid Derivatives Inhibitors of Kynurenine 3-Hydroxylase

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Received 19 June 1998; accepted 8 September 1998

Abstract: Kynurenine 3-hydroxylase (KYN 3-OHase) is a key enzyme in the kynurenine pathway of tryptophan degradation and its inhibition may be an effective mechanism for counteracting neuronal excitotoxic damage. We present here a new class of inhibitors derived from a structure-activity relationship (SAR) study of the benzoylalanine side-chain of **1**. 2-hydroxy-4-(3,4-dichlorophenyl)-4-oxo-butanoic acid (**8**) and 2-benzyl-4-(3,4-dichlorophenyl)-4-oxo-butanoic acid (**10**) emerged as the most interesting derivatives. Enantiospecific synthesis for both enantiomers of **8** and diastereomeric salt resolution for those of **10** were successfully applied.

Keywords: enzyme inhibition; kynurenine; hydroxylase; neuroprotection; stereoselectivity

Excitotoxic processes are likely to play a significant role in the pathogenesis of a number of neurological and psychiatric diseases.[1-3] Pharmacological blockade of cerebral excitatory amino acid receptors provides an attractive mean to prevent or arrest excitotoxic neuronal death[4] but since excitatory amino acid (EAA) receptors have defined and important functions in brain physiology their unmitigated blockade, particularly when maintained over an extended period, carries substantial clinical hazards.[5]

An alternative approach to direct blockade of EAA receptors may be that of influencing EAA receptor function indirectly. In this regard, modulation of the balance between two endogenous neuroactive kynurenines, the neuroprotective broad spectrum EAA receptor antagonist kynurenic acid (KYNA) and the excitotoxic N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid (QUIN), may result in a decrease in receptor mediated pathologies. KYNA and QUIN are two metabolites of the kynurenine pathway of L-tryptophan degradation in the periphery and in the brain.[6]

In the kynurenine pathway, kynurenine aminotransferase (KAT) is the enzyme responsible for the synthesis of KYNA from kynurenine. Kynurenine 3-hydroxylase (KYN 3-OHase) provides 3-hydroxykynurenine (3-OH-KYN) and kynureninase converts 3-OH-KYN to 3-hydroxyanthranilic acid (3-OH-AA), which is further metabolised to QUIN. Potent inhibitors of KYN 3-OHase, if devoid of KAT inhibitory activity, could therefore be useful tools to increase KYNA levels in the brain.

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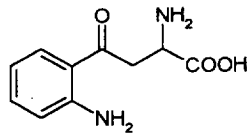
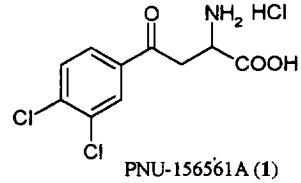
0960-894X/98/$ - see front matter
PII: S0960-894X(98)00517-4



Kynurenine

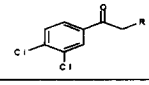


PNU-156561A (1)

From a natural substrate analogue approach[7] we discovered PNU-156561A (1) as a potent KYN-3-OHase inhibitor[8] endowed with neuroprotective effects in animal models of ischemia.[9] The SAR of the aromatic part of 1 was previously examined and a 3,4-dichloro substitution of the phenyl ring was found as the most effective.[10] Recently N-hetaryl-phenylsulfonamides were reported as very potent competitive KYN 3-OHase inhibitors.[11] Here we wish to report the exploration of the SAR in the benzoylalanine side chain of 1.

We first defined the role of the carboxy and amino functions in the aminoacidic part of 1 (Table 1). Compounds were initially tested in the rat liver KYN 3-OHase assay[12] and for interesting inhibitors, confirmation was done in the rat brain KYN 3-OHase test.[13] Preservation of the acid function was mandatory for inhibiton of KYN 3-OHase (Table 1).

Table 1. Variations in the aminoacidic group of 1



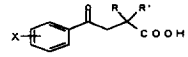
Entry	R	Rat Liver KYN 3-OHas. (IC_{50} ± SEM; μM) or (%inhibition@100μM)[12]	Rat Brain KYN 3-OHase (IC_{50} ± SEM; μM)[13]]
1	$CH(NH_2)COOH$	0.33±0.03	0.20±0.02
2	CH_2COOH	3.9±0.2	0.9±0.1
3	CH_2NH_2	2@100μM	nt

The effect of selected substitutions at position-2 of 2 are reported in Table 2. The presence of a hydrogen bond donor such as in 8 gave a compound roughly equipotent to 1 in the rat liver and brain assays, while alkyl, alkoxy or halogen substituents were less effective.

Substitution by a phenyl group led to diminished activity but a benzyl group (10) featured again low micromolar activity. Double substitution at position 2 was detrimental to activity as exemplified in Table 2 (entries 11-14).

Since 8 displayed an interesting activity we sought for this series confirmation of the SAR for the aromatic ring substitution pattern, in analogy with the work done on benzoylalanines.[10] Again, substitution on the aromatic ring by halogens and nitro groups (Table 2; entries 20-24) favoured activity. In particular, as in the case of 1, disubstitution in 3,4-position by two halogens (chlorine or fluorine) gave the most potent compounds (8 and 23).

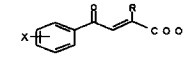
Table 2. Variations in the 4-phenyl-4-oxo-butanoic backbone



Entry	R	R'	X	Rat Liver KYN 3-OHase (IC_{50} ± SEM; μM)[12]	Rat Brain KYN 3-OHase (IC_{50} ± SEM; μM)[13]
2	H	H	3,4-diCl	3.9±0.2	0.9±0.1
4	CH_3	H	3,4-diCl	6.9±1.2	3.5±0.2
5	OCH_3	H	3,4-diCl	6.9±3.7	1.2±0.1
6	$=CH_2$		3,4-diCl	36.0±8.3	nt
7	Cl	H	3,4-diCl	14.8±2.4	2.2±0.2
8	OH	H	3,4-diCl	1.4±0.3	0.30±0.06
9	Ph	H	3,4-diCl	19.5±1.8	nt
10	CH_2Ph	H	3,4-diCl	2.9±1.2	0.18±0.01
11	CH_3	CH_3	3,4-diCl	21@100μM	nt
12	CH_3	NH_2	3,4-diCl	35@100μM	nt
13	CH_3	OH	3,4-diCl	43@100μM	nt
14	Cyclopropyl		3,4-diCl	20@100μM	nt
20	OH	H	3-Cl	1.10±0.3	0.48±0.02
21	OH	H	3-F	9.1±1.1	5.6±0.9
22	OH	H	3-NO_2	11.5±4.0	1.95±0.15
23	OH	H	3,4-diF	3±0.2	1.45±0.08
24	OH	H	OCH_3	7@100μM	nt

Introduction of a E-double bond across the side-chain of **2** gave a compound (**15**) that still retained part of the inhibitory effect (Table 3). The presence of an hydroxy substituent in 2-position of **15** enhanced the potency of this class of compounds in parallel with the saturated series (see Table 2)

Table 3. Variations in the 4-oxo-butenoic acid backbone



Entry	R	X	Rat Liver KYN 3-OHase (IC_{50} ± SEM; μM)[12]	Rat Brain KYN 3-OHase (IC_{50} ± SEM; μM)[13]
15	H	3,4-diCl	10.4±1.4	0.9±0.1
16	H	3,4-diF	21.1±1.9	1.1±0.1
17	CH_3	3,4-diCl	11.3±1.2	2.2±0.2
18	OH	3,4-diCl	6.4±1.3	0.95±0.08
19	OH	3,4-diF	1.2±0.2	0.44±0.02

At this stage **8** and **10** were the compounds we deemed as the most interesting in this series based upon their activity in the primary assays and their potential for further manipulation. Table 4 reports the activities of **8** and **10** against KYN 3-OHase from different sources (rat liver, rat brain and human recombinant), other enzymes in the kynurenine pathway (KYNase and KAT) and other monoxygenases such as para-hydroxy-benzoate hydoxylase (PHBH) and liver flavin-dependent monoamine oxidase (FMO). All compounds demonstrated selectivity towards other enzyme wich uses kynurenine as a substrate (KYNase

and KAT) and specificity with respect to an aromatic hydroxylase such as PHBH or a general monooxygenase like FMO.

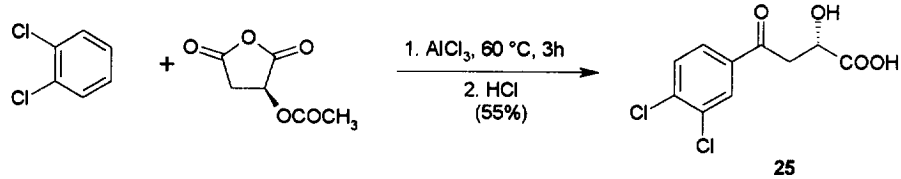
If one compares the structures and activities of **8** and **10** it is apparent that the 2-hydroxy (**8**) and the 2-benzyl (**10**) functions play a role at increasing the potency with respect to the parent compound **2**. In the case of **8** the hydroxy group is probably involved in an accessory hydrogen bond with an enzyme's amino acid residue, as it does in the benzoylalanine **1**. This assumption is corroborated by the fact that **18** and **19** (still active compounds) tautomerism favors the enol over the keto form. By the other hand, the effect of the phenyl moiety of **10**, which due to the rotation of the methylene bond occupies a different spatial region from the hydroxy group of **8**, may reasonably be ascribed to a hydrophobic interaction with a suitable amino acid residue.

Table 4. Activity of selected compounds against KYN 3-OHase from different sources, selectivity towards kynurenine pathway enzymes and specificity against representative monooxygenases.

Entry	Rat Liver KYN 3-OHase. (IC_{50} ± SEM; μM)[12]	Rat Brain KYN 3-OHase (IC_{50} ± SEM; μM)[13]	Human recombinant KYN 3-OHase (IC_{50} ± SEM; μM)[13]	Rat liver KYNase (% of inhibition at concentration of inhibitor)[14]	Rat liver KAT (% of inhibition at concentration of inhibitor)[15]	PHBH (% of inhibition at concentration of inhibitor)[16]	FMO (% of inhibition at concentration of inhibitor)[17]
1	0.33±0.03	0.20±0.02	0.20±0.01	10@100μM	14@1000μM	2@100μM	8.5@100μM
8	1.4±0.3	0.30±0.06	3.3±0.9[19]	0@100μM	23@100μM	29@100μM	nt
10	2.9±0.2	0.18±0.01	0.18±0.01	0@100μM	8@100μM	8@100μM	16@100μM

Therefore we developed an enantiospecific synthesis for obtaining both enantiomers of **8** (here reported for the S-enantiomer) on the gram-scale, while for the 2-benzylderivatives we recurred to fractional crystallization of the ephedrine salts of **10**. In the latter case we could not ascribe the absolute configuration of **27** and **28**, but based upon comparison of optical and pharmacological properties with **1** and **8** enantiomers we can *bona fide* assign the S-configuration to the (-)-enantiomer (**27**) and the R-configuration to the (+)-enantiomer (**28**). Schemes 1 and 2 describe the synthetic routes and experimental data for previously unknown **25** and **27** are reported.[19]

Scheme 1. Enantioselective Synthesis of **25**.



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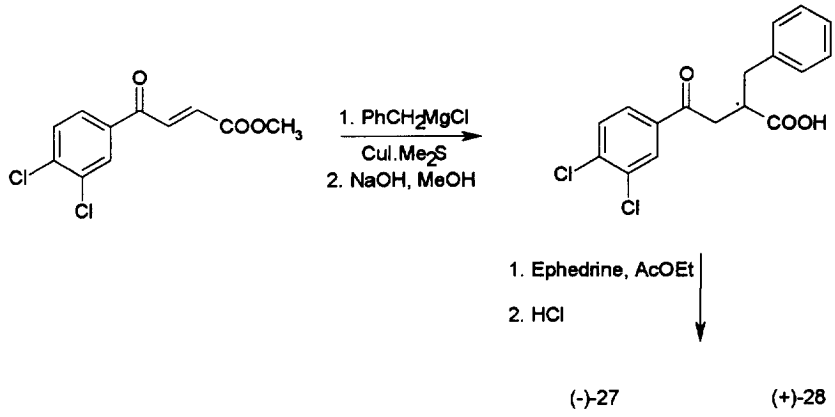
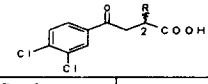
Scheme 2.Synthesis of **10** and diastereomeric salt resolution to **27** and **28.**



In parallel with what we found in the benzoylalanine series, the S-enantiomers of both derivatives (**25** and **27**) were the eutomers (Table 5).

Table 5. Stereoselectivity in the effect of the enantiomers of **8** and **10.**



Entry	R	Conf	Rat liver KYN 3-OHase inhib. (IC_{50} ± SEM; μM)[12]
8	OH	R,S	1.4±0.3
25	OH	S-(-)	0.42±0.03
26	OH	R-(+)	14.4±3.3
10	CH_2Ph	R,S	2.9±1.2
27	CH_2Ph	S-(-)	2.3±0.5
28	CH_2Ph	R-(+)	10.7±1.6

In summary, we reported on the generation and testing of a new class of KYN-3-OHase inhibitors as a continuation of a natural substrate analogue study we started with benzoylalanines. Compound **8** and **10** (their eutomers being **25** and **27**) showed robust enzymatic inhibition, selectivity towards other kynurenine pathway enzymes and specificity against other monooxygenases, and due to their lack of metabolic liability with respect to **1** they represent promising compounds for further *in vivo* studies.

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12. Modification of Erickson, J. B.; Flanagan, E. M.; Russo, S.; Rheinhard, J. F., Jr. *Analyt. Biochem.* **1992**, 205, 257-262. Briefly, the efficacy of the compounds was evaluated in the radiometric KYN 3-OHase inhibitory assay using rat liver mitochondrial extracts as an enzyme source. This assay is based on the enzymatic synthesis of tritiated water during the hydroxylation reaction. Radiolabeled water was quantified following selective adsorption of the isotopic substrate and its metabolite with activated charcoal. The reaction mixture of a total volume of 30 µl was constituted of 44 µg of suspended extract, 100 mM Tris/Cl$^-$ buffer pH 8.1, 10 mM EDTA, 100 mM KCl, 0.8 mM NADPH, 0.025 mM L-KYN, 0.3 µCi L-(3,5-^{3}H)-KYN (10 Ci/mmol) and 3 µl of different concentration of inhibitor solutions. After the incubation, the reaction was terminated by addition of 300 µl of 7.5% (w/v) activated charcoal, and centrifuged for 7 min. A 75µl aliquot of supernatant was transferred to optiplate and liquid scintillation added. The optiplates were vortexed and the radioactivity counted in a microplate scintillation counter. The apparent discrepancies in potency as compared to the rat brain enzyme seem to be due to the lesser sensitivity of the radiometric detection rather than to different tissue specificity.
13. KYN-3-OHase activity was measured in a crude mitochondrial preparation of whole rat forebrain (Carpenedo, R. A. Chiarugi, P.; Russi, G.; Lombardi, V.; Carlá, R.; Pellicciari, R.; Mattoli, L.; Moroni, F. *Neuroscience* **1994**, 61, 237-243) and in human liver recombinant enzyme expressed in trasformed E. Coli (Magagnin, S.; Covini, N.; Bormetti, R.; Cini, M.; Speciale, C.; Benatti, L. Society for Neuroscience, New Orleans, LA, Oct 25-30,1997;P903.6) according to the method described by Carpenedo, R. A. et al.
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16. PHBH was measured in Pseudomonas Fluorescens purified enzyme by the conversion of p-hydroxybenzoic acid to 3,4 dihydroxybenzoic acid. Briefly, the enzyme was incubated in the presence of 3 µM FAD, 150 µM NADPH, 25µM of substrate and of increasing concentrations (final volume 200µl) of inhibitor at 30 °C. After 15 min the reaction was terminated by adding of 200 µl 1M $HClO_4$. After centifugation, supernatants were directly used for the measurement of 3,4 dihydroxybenzoic acid. The concentration of 3,4 dihydroxybenzoic acid was measured by HPLC with coulometric detection (det. 1 +0.05V, det 2 +0.2V) using a 10 cm C18 reversed phase (3µm) column with a mobile phase containing water / acetonitrile / trifluoroacetic acid (95 / 5 / 0.05). The flow rate was 1 ml/min.
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18. KYN-3-OHase activity was measured in human liver recombinant enzyme expressed in COS-1 cells according to Breton J. et al., manuscript in preparation, and following the method described in 12].
19. Selected analytical data for representative compounds: **25**: $[\alpha]_D^{25}$=-8.5 (c = 1.0; abs. EtOH); mp 148-149.5°C; ^{1}H NMR (200 MHz, DMSO) δ: 3.29 (2H, d, CH_2, J=6.1Hz), 4.40 (1H, t, CH, J = 6.1Hz), 7.75 (1H, d, H5' arom., J=8.5Hz), 7.88 (1H, dd, H6' arom., J=8.5Hz; J=2.0Hz), 8.10 (1H, d, H2' arom., J=2.0Hz); MS (FAB; m/z) : 263 $[M+H]^+$; 261[M-H]; **27**: $[\alpha]_D^{25}$=-15.4; mp 162-163°C; ^{1}H NMR (200 MHz, DMSO) δ: 2.6-3.4 (5H,m, CH_2CHCH_2Ph,), 7.21 (5H, m, Ph), 7.30 (1H, d, H5' arom., J=8.2Hz), 7.51 (1H, dd, H6' arom., J=8.2Hz; J=2.0Hz), 7.95 (1H, d, H2' arom., J=2.0Hz); MS (FAB; m/z) : 337 $[M+H]^+$; 335 $[M-H]^-$