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Pyrrolo[3,2-*c*]quinoline derivatives: a new class of kynurenine-3-hydroxylase inhibitors

Franco Heidempergher, Paolo Pevarello *, Antonio Pillan, Vittorio Pinciroli, Arturo Della Torre, Carmela Speciale, Marina Marconi, Massimo Cini, Salvatore Toma, Felicita Greco, Mario Varasi

Pharmacia & Upjohn, Chemistry Department, Viale Pasteur 10, I-20014 Nerviano, Milan, Italy

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Abstract

A series of pyrrolo[3,2-*c*]quinoline derivatives were synthesised and evaluated as inhibitors of selected enzymes of the kynurenine pathway. 7-Chloro-3-methyl-1*H*-pyrrolo[3,2-*c*]quinoline-4-carboxylic acid (**7a**) was found to be a relatively potent and selective inhibitor of kynurenine-3-hydroxylase (KYN-3-OHase). A molecular modelling study showed a good superimposition of **7a** with PNU-156561 and kynurenine the natural substrate of KYN-3-OHase.

Keywords: Kynurenine; Hydroxylase; Enzyme inhibition

1. Introduction

It is well known that through the kynurenine (KYN) pathway tryptophan metabolism gives rise to the formation of both quinolinic acid (QUIN) and kynurenic acid (KYNA) [1].

Kynurenine aminotransferase (KAT) is the enzyme responsible for the synthesis of KYNA from KYN. Kynurenine-3-hydroxylase (KYN-3-OHase) provides 3-hydroxy-kynurenine (3-OH-KYN), which is further metabolized to QUIN [2] (Fig. 1).

KYNA is endowed with neuroprotective properties [3], whereas QUIN is a relatively potent neurotoxin [4] which has been implicated in the pathogenesis of a variety of neurological disorders [5]. The enzymes responsible for the synthesis of both KYNA and QUIN are attractive pharmacological targets for shifting kynurenine metabolism towards enhanced KYNA synthesis.

Inhibitors of KYN-3-OHase, if devoid of KAT inhibitory activity, may play a role in the neuronal protection by increasing KYNA levels in the brain [6–8]. The strategy aimed at favourably altering KYNA/QUIN balance may also be a useful tool in Parkinson's disease and other neurodegenerative diseases where excitotoxic mechanisms have been implicated [9].

With the aim of obtaining new KYN-3-OHase inhibitors we prepared and evaluated in vitro a series of pyrrolo[3,2-*c*]quinoline derivatives (**7a–c**, **8**, **9**, **10**) and a correlated tetrahydroquinoline analogue **11**. A molecular modelling study was performed in order to identify pharmacophoric features of these compounds by comparing the representative compound of this series (**7a**) with PNU-156561, a potent benzoylalanine analogue inhibitor [10] and with KYN, the natural substrate of KYN-3-OHase.

2. Results

2.1. Chemistry

The synthesis of the pyrrolo[3,2-*c*]quinoline derivatives (**7a–c**, **8**) was performed according to the route shown (Scheme 1). The Reissert reaction [11] on the acetylated intermediates **3a–c** and **4** and subsequent acid hydrolysis of **5a–c** and **6** with 48% HBr afforded the tricyclic compounds **7a–c** and **8** (Scheme 1).

Compound **9** was obtained by alkylating **7a** with MeI in the presence of NaOH and by acid hydrolysis of the

* Corresponding author.

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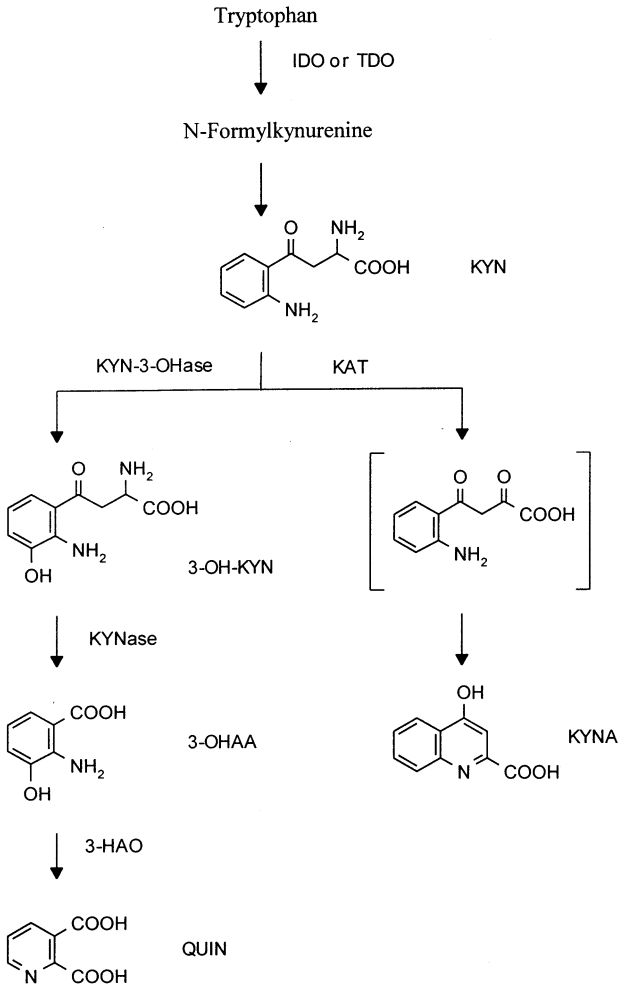


Fig. 1. Kynurenine pathway. IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan pyrrolase; KYN-3-OHase, kynurenine-3-hydroxylase; KAT, kynurenine aminotransferase; KYNase, kynureninase/3-hydroxykynureninase; 3-HAO, 3-hydroxy anthranilic acid oxygenase; QUIN, quinolinic acid; KYNA, kynurenic acid.

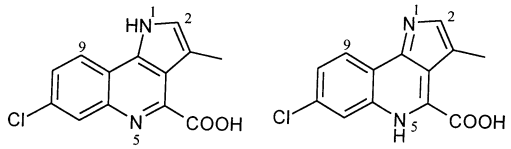


Fig. 2. 1-NH vs. 5-NH tautomerism in compound **7a**.

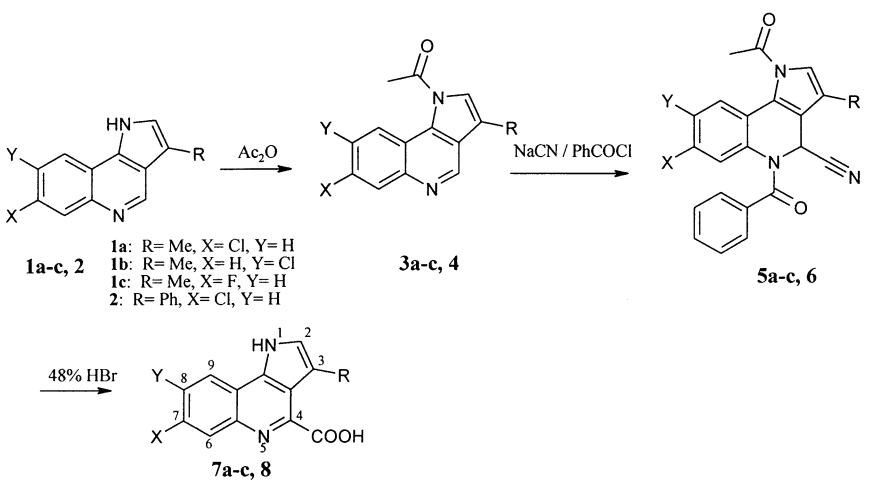
intermediate methyl ester with 23% HCl in AcOH (Scheme 2).

Compound **10** was obtained by dehalogenation of **7a** under reductive conditions with ammonium formate and 10% Pd/C (Scheme 3). Starting from **7a**, an attempt to reduce the double bond of the pyrrole ring with H_2/PtO_2 failed, and compound **11** was obtained as the product of partial reduction and dehalogenation of the quinoline moiety (Scheme 3).

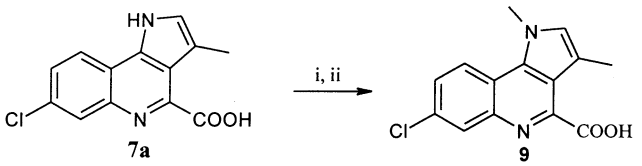
7-Chloro-quinolinic acid (**12**) was synthesized following a known procedure [12], and compound **13** was prepared starting from **14** [13] through *N*-alkylation of the *N*-tosyl intermediate following a procedure previously described for analogues [14] (Scheme 4).

A nuclear Overhauser effect (NOE) NMR study was performed on **7a** (Fig. 2), the representative compound of this series. Theoretically, two tautomers that differ in the position of the mobile proton (either on N-1 or on N-5) are possible for this molecule.

Only one is observed in DMSO-d_6 solution and NOE data are in agreement with a structure bearing the exchangeable proton on N-1. In fact, upon irradiation of the NH proton NOE enhancements are observed for H-2 and H-9 signals. Similarly, irradiation of H-9 and H-2 enhances the NH-1 signal.



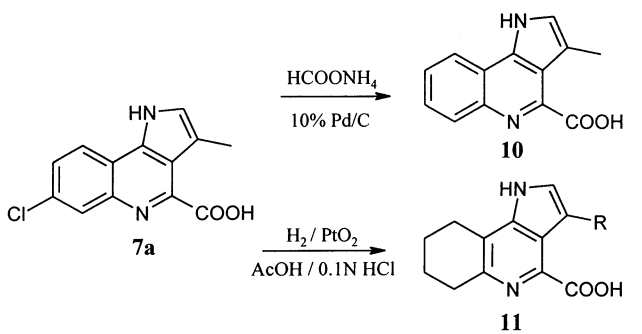
Scheme 1.



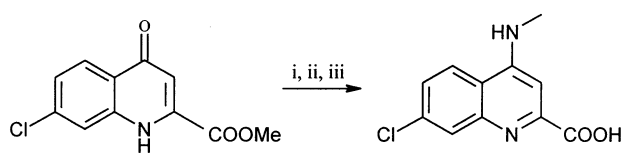
Scheme 2. (i) MeI, KOH, DMSO; (ii) 23% HCl/AcOH.

3. Discussion

In vitro inhibitory activity on KYN-3-OHase of the compounds was evaluated in rat liver tissue (Table 1). Among the compounds tested, 7-chloro-3-methyl-1*H*-pyrrolo[3,2-*c*]quinoline-4-carboxylic acid (**7a**) was found to be a micromolar inhibitor of this enzyme. The importance of the carboxy group is apparent from the complete inactivity of **1a** (Scheme 1). All the chemical modifications performed on the **7a** scaffold did not afford any improvement in potency (**7b–11**). The nature of the substitution at position 3 (Me versus Ph; **7a** versus **8**) did not dramatically influence the inhibitory potency (24.0 versus 34.5 μM), thus indicating that a quite large lipophilic pocket may exist in that region. The influence of the substitution pattern on the aromatic ring was not completely aligned with that observed for the benzoylalanine class [10]: while a chlorine in the formal 4′ position of the benzoylalanine scaffold (corresponding to position 7 in **7a**) was clearly beneficial to activity, a fluorine replacement in the same position led to an inactive compound (**7c**). In the benzoylalanine series and in another closely related series [15] a fluorine and a chlorine on the aromatic ring were, by and large, interchangeable. This may reflect a subtle difference in orientation of the phenyl rings in the catalytic site between the series. It might however be anticipated that a chlorine substitution at position 6 would increase activity in this series. Furthermore, either the complete deletion of the pyrrole moiety (**12**) or its deletion while maintaining the amino functionality (**13**), led to completely inactive compounds.



Scheme 3.



Scheme 4. (i) *p*-TsNCO, CH_3CN; (ii) MeI, K_2CO_3; (iii) 37% HCl/AcOH.

Compound **7a** showed a remarkable selectivity towards other enzymes (KAT and KYNase). The affinity of **7a** for a related microbial enzyme, *para*-hydroxybenzoate-hydroxylase (PHBH), was negligible thus reinforcing its specificity for KYN-3-OHase.

Although **7a** is considerably less active (micromolar versus low micromolar [10] or nanomolar [16] range) than previously reported inhibitors, its rigid structure makes it an interesting tool, particularly useful for modelling studies.

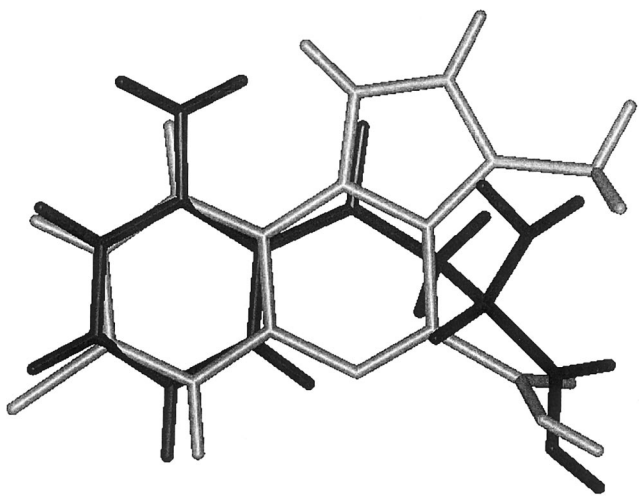


Fig. 3. Superimposition of compound **7a** (grey) and KYN (black).

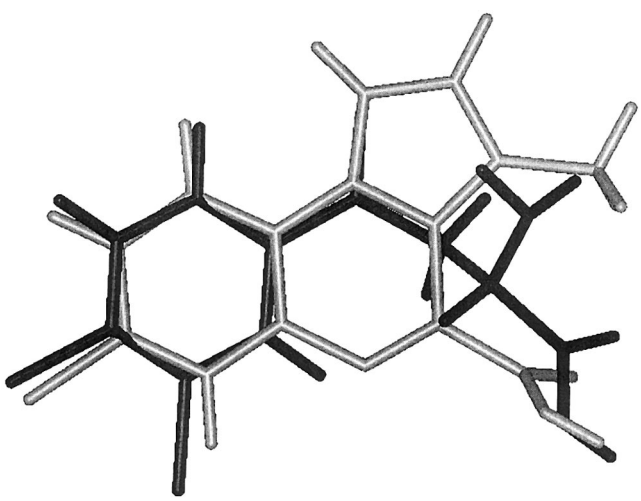
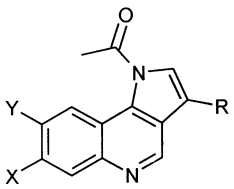
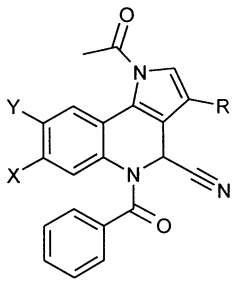


Fig. 4. Superimposition of compound **7a** (grey) and PNU-156561 (black).

Table 1
Physicochemical data of intermediates **3a–c**, **4** and **5a–c**, **6**



3a-c, 4



5a-c, 6

Comp.	X	Y	R	Yield (%)	M.p. (°C)	Formula
3a	Cl	H	Me	66	159–160.5	$C_{14}H_{11}ClN_2O$
3b	H	Cl	Me	63	210–213	$C_{14}H_{11}ClN_2O$
3c	F	H	Me	65	150–152	$C_{14}H_{11}FN_2O$
4	Cl	H	Ph	83	149–150	$C_{19}H_{13}ClN_2O$
5a	Cl	H	Me	60	196–201	$C_{22}H_{16}ClN_3O_2$
5b	H	Cl	Me	77	190 dec.	$C_{22}H_{16}ClN_3O_2$
5c	F	H	Me	63	85 dec. (amorphous)	$C_{22}H_{16}FN_3O_2$
6	Cl	H	Ph	64	90 dec. (amorphous)	$C_{27}H_{18}ClN_3O_2$

A molecular modelling study was then performed to mimic a mechanism for KYN-3-OHase inhibition by **7a**. In this study PNU-156561, a potent KYN-3-OHase inhibitor, and KYN, the natural substrate of KYN-3-OHase, were superimposed on the rigid compound **7a**. Although a substrate and an inhibitor do not necessarily share the same interactions at a catalytic site, in this case, due to the remarkable similarity of PNU-156561 and KYN structures, it is reasonable that the inhibitor and the substrate act by the same mechanism (see Table 2 for KYN-3-OHase inhibition of compounds **7–13**).

The conformational analysis of KYN showed that, among the several possible conformations, those leading to an intramolecular hydrogen bond between the carbonyl moiety and the aromatic amino group were preferred. This conclusion was supported by a conformational search and by a dynamic simulation study performed either in vacuum or in water, and by the semi-empirical quantum mechanics AM1 method [17]. The same conformation was found for the X-ray crystallographic structure of *N*-acetylkynurenine (ref. code ACKYNU0001) reported in the Cambridge Structural Database (CSD) (release 5.12). Such an intramolecular hydrogen bond is energetically and geometrically so favourable that it is reasonable to assume it to be maintained in the active site of the enzyme. The stable conformations of PNU-156561 and KYN were superimposed on compound **7a** using the carboxylic group and the phenyl ring as common pharmacophore features. The most stable conformation of PNU-156561 and KYN showed the best overlapping (see Figs. 3 and 4). The analysis of the superimposed compounds suggested that the aromatic NH_2 of kynurenine and the pyrrolo NH of **7a**, pointing in the same direction, may give a hydrogen bond with the same residue of the enzyme. However, this hydrogen bond does not seem essential for a strong inhibitory activity since it is not present in PNU-156561.

In conclusion, the synthesis and biological in vitro evaluation of a series of pyrrolo[3,2-*c*]quinolines were presented. The parent compound structure (**7a**) was attributed by NOE analysis. Pharmacophoric features of this series have been defined by a molecular modelling study of the parent compound (see Table 3 for biological data for **7a**).

4. Experimental

4.1. Chemistry

Melting points were determined in open glass capillaries on a Buchi apparatus and are not corrected. ^1H NMR spectra were registered on a VXR-200 or VXR-400S instrument at 27°C. The reference signal was the solvent (DMSO-d_6, 2.49 ppm). Pre-saturation delay was set at 7.0 s in the stationary 1D NOE experiment. Positive ion fast bombardment (FAB), field desorption (FD) and electronic impact (EI) mass spectra (MS) were obtained on a Varian MAT 311-A and on a Finnigan

TSQ 70 instrument. Elemental analyses were performed for new compounds by a Carlo Erba 1106 instrument. Where elemental analyses are indicated, the results were within 0.4% of the theoretical values (see Table 4). Yields refer to the purified products and are not optimized. Column chromatography was carried out using silica gel 60, 230–400 mesh (Carlo Erba). Starting materials that were not commercially available were prepared according to procedures already published. In particular, intermediates **1c** and **2** were prepared following the procedure described for **1a** and **1b** [18].

*4.1.1. General procedure for the synthesis of intermediates **3a–c**, **4***

A solution of the appropriate pyrrolo[3,2-*c*]quinoline **1a–c**, **2** (31 mmol) in an excess of acetic anhydride (35 ml) was refluxed for 4 h under nitrogen atmosphere. The cooled mixture was cautiously poured into crushed ice and allowed to stand overnight. The crude solid so obtained was collected by filtration and crystallized from anhydrous ethanol.

Yields and analytical data of intermediates **3a–c**, **4** are reported in Table 1.

*4.1.2. General procedure for the preparation of carbonitrile intermediates **5a–c**, **6***

The procedure is reported for the preparation of 1-acetyl-5-benzoyl-7-chloro-3-phenyl-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-4-carbonitrile (**6**). Benzoyl chloride (5.64 ml, 48.6 mmol) was dropped at room temperature (r.t.) into a vigorously stirred mixture of **4** (7.79 g, 24.3 mmol) and potassium cyanide (4.73 g, 72.9 mmol) in methylene chloride (80 ml) and water (30 ml) and left for 2 h. The mixture was stirred for 20 h, then diluted with water. The aqueous layer was extracted with methylene chloride; the combined organic layers were washed with brine, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by silica gel flash-chromatography (diisopropyl ether as eluant) to give 5.55 g of the desired product as a beige solid.

^{1}H NMR (200 MHz, DMSO): δ 2.79 (s, 3H, CH_3CO), 6.65 (s, 1H, H-4), 6.86 (d, $J = 2.2$ Hz, 1H, H-6), 7.27 (dd, $J = 8.8$, 2.2 Hz, 1H, H-8), 7.3–7.7 (m, 10H, Ph × 2), 7.79 (d, $J = 8.8$ Hz, 1H, H-9), 8.06 (s, 1H, H-2). MS (EI) m/z (rel. int.) 451 ($M^{\bullet+}$, 4.5), 346 (7.3), 304 (16.4), 278 (17.3), 105 (100).

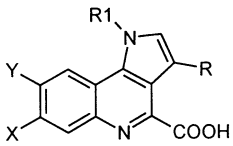
Intermediates **5a–c**, **6** were prepared similarly. Yields and analytical data are reported in Table 1. The ^{1}H NMR spectra and MS data are consistent with the assigned structures.

*4.1.3. General procedure for the synthesis of compounds **7a–c**, **8***

This procedure is illustrated for the preparation of 7-chloro-3-methyl-1*H*-pyrrolo[3,2-*c*]quinoline-4-carboxylic acid (**7a**). A solution of **5a** (4.9 g, 12.6 mmol) in acetic acid (35 ml) and 48% hydrobromic acid (35 ml) was heated at 100°C for 1 h. The cooled solution was evaporated to dryness and the residue crystallized from ethanol/water 1:1 to obtain a beige solid (2.06 g, 63% yield), m.p. 300°C dec.

Anal. ($C_{13}H_9ClN_2O_2$) C, H, Cl, N. ^{1}H NMR (400 MHz, DMSO): δ 2.35 (d, $J = 0.8$ Hz, 3H, CH_3), 7.48 (m, 1H, H-2), 7.72 (dd, $J = 8.8$, 2.1 Hz, 1H, H-8), 8.12

Table 2
KYN-3-OHase inhibition of compounds **7–13**



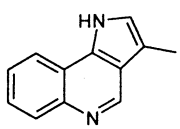
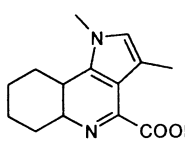
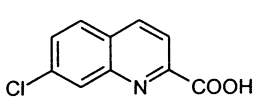
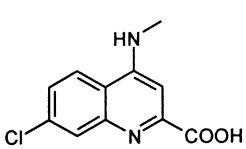
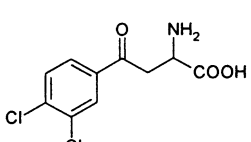
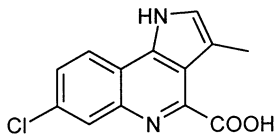
Comp.	X	Y	R	R^1	KYN-3-OH-inhibition IC_{50} (μM)/C or % enzyme inhibition (100 μM) rat liver tissue
7a	Cl	H	Me	H	24.0 ± 3.1
7b	H	Cl	Me	H	61.4 ± 6.5
7c	F	H	Me	H	23@100
8	Cl	H	Ph	H	34.5 ± 1.8
9	Cl	H	Me	Me	53.3 ± 3.2
10	H	H	Me	H	97 ± 11
1a					5@100
11					100 ± 12
12					14.4@100
13					1.5@100
PNU-156561					0.33 ± 0.03 0.20 ± 0.02 (rat brain tissue)

Table 3
Biological data for compound **7a**



KYN 3-OHase inhibition IC_{50} (μM)		KAT enzyme inhibition (%)	KYNase enzyme inhibition (%)	PHBH IC_{50} (μM)
Liver	Brain			
24.0 ± 3.1	8.1 ± 1.2	24@100 μM	25@100 μM	>1000

(d, $J = 2.1$ Hz, 1H, H-6), 8.44 (d, $J = 8.8$ Hz, 1H, H-9), 12.7 (bs, 1H, H-1). FAB-MS m/z 259 $(M-H)^-$.

The following compounds were also prepared by the general procedure described above.

8-Chloro-3-methyl-1*H*-pyrrolo[3,2-*c*]quinoline-4-carboxylic acid (**7b**): dark yellow solid, 53% yield, m.p. 230°C dec. *Anal.* ($C_{13}H_9ClN_2O_2$) C, H, Cl, N. ^{1}H NMR (200 MHz, DMSO): δ 2.31 (d, $J = 1.0$ Hz, 3H, CH_3), 7.44 (m, 1H, H-2), 7.62 (dd, $J = 8.9$, 2.3 Hz, 1H, H-7), 8.05 (d, $J = 8.9$ Hz, 1H, H-6), 8.50 (d, $J = 2.3$ Hz, 1H, H-9), 12.5 (bs, 1H, H-1). MS (EI) m/z (rel. int.) 260 ($M^{\bullet+}$, 10), 216 (84.5), 215 (100).

7-Fluoro-3-methyl-1*H*-pyrrolo[3,2-*c*]quinoline-4-carboxylic acid hydrate (**7c**): yellow solid, 72% yield, m.p. >300°C dec. *Anal.* ($C_{13}H_9FN_2O_2 \cdot H_2O$) C, H, N. ^{1}H NMR (200 MHz, DMSO): δ 2.31 (d, $J = 0.9$ Hz, 3H, CH_3), 7.41 (m, 1H, H-2), 7.58 (ddd, $J = 8.8$, 8.8, 2.6 Hz, 1H, H-8), 7.80 (dd, $J = 10.5$, 2.6 Hz, 1H, H-6), 8.45 (dd, $J = 8.8$, 6.0 Hz, 1H, H-9), 12.6 (bs, 1H, H-1). FAB-MS m/z 243 $(M-H)^-$.

7-Chloro-3-phenyl-1*H*-pyrrolo[3,2-*c*]quinoline-4-carboxylic acid (**8**): yellow solid, 57% yield, m.p. >300°C. *Anal.* ($C_{18}H_{11}ClN_2O_2$) C, H, Cl, N. ^{1}H NMR (200 MHz, DMSO): δ 7.2–7.5 (m, 5H, Ph), 7.73 (dd, $J = 8.8$, 2.0 Hz, 1H, H-8), 7.75 (s, 1H, H-2), 8.12 (d, $J = 2.0$ Hz, 1H, H-6), 8.50 (d, $J = 8.8$ Hz, 1H, H-9), 13.0 (bs, 1H, H-1), 13.4 (bs, 1H, COOH). FD MS m/z 322 ($M^{\bullet+}$).

*4.1.4. 7-Chloro-1,3-dimethyl-1H-pyrrolo[3,2-c]-quinoline-4-carboxylic acid (**9**)*

Potassium hydroxide (0.47 g, 8.4 mmol) was added to a solution of **7a** (0.37 g, 1.4 mmol) in anhydrous dimethylsulfoxide (6 ml). The mixture was stirred at r.t. for 1 h, then methyl iodide (0.35 ml, 5.6 mmol) was added dropwise and the mixture stirred at r.t. for 3 h. The solution was poured into cold water and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The residue was purified by silica gel flash-chromatography (ethyl acetate as eluant) to give 7-chloro-1,3-dimethyl-1*H*-pyrrolo[3,2-*c*]quinoline-4-carboxylic acid methyl ester as a beige solid (0.32 g, 78% yield), m.p. 165°C dec. *Anal.* ($C_{15}H_{13}ClN_2O_2$) C, H, Cl, N.

The solution of 7-chloro-1,3-dimethyl-1*H*-pyrrolo-[3,2-*c*]quinoline-4-carboxylic acid methyl ester (0.32 g, 1.1 mmol) in acetic acid (5 ml) and 23% HCl (5 ml) was refluxed for 16 h. The mixture was evaporated to dryness, taken up with water and basified with 2 N sodium hydroxide. The basic solution was extracted with ethyl acetate, acidified with 2 N hydrochloric acid and the light yellow solid collected by filtration (0.27 g, 90% yield), m.p. 195°C dec. *Anal.* ($C_{14}H_{11}ClN_2O_2$) C, H, Cl, N. ^{1}H NMR (200 MHz, DMSO): δ 2.30 (d, $J = 0.9$ Hz, 3H, CH_3), 4.23 (s, 3H, NCH_3), 7.39 (m, 1H, H-2), 7.69 (dd, $J = 9.0$, 2.2 Hz, 1H, H-8), 8.14 (d, $J = 2.3$ Hz, 1H, H-6), 8.57 (d, $J = 9.0$ Hz, 1H, H-9). FAB-MS m/z 275 $(M+H)^{\bullet}$.

*4.1.5. 3-Methyl-1H-pyrrolo[3,2-c]quinoline-4-carboxylic acid hydrate (**10**)*

Ammonium formate (2.3 g, 36 mmol) and 10% Pd/C (0.2 g) were added to a solution of **7a** (0.8 g, 3 mmol) in ethanol (30 ml) and the mixture was refluxed for 2 h, cooled, and filtered. After evaporation, the residue was taken up with water and 1 N sodium hydroxide was added until complete dissolution. The solution was acidified with 2 N hydrochloric acid, and the precipitate was collected by filtration and washed with water to give a yellow solid (0.57 g, 76% yield), m.p. 230°C dec. *Anal.* ($C_{13}H_{10}N_2O_2 \cdot H_2O$) C, H, N. ^{1}H NMR (200 MHz, DMSO): δ 2.38 (d, $J = 1.1$ Hz, 3H, CH_3), 7.48 (m, 1H, H-2), 7.70 (m, 2H, H-8 and H-7), 8.13 (m, 1H, H-6), 8.44 (m, 1H, H-9), 12.7 (bs, 1H, H-1). MS (EI) m/z (rel. int.) 226 ($M^{\bullet+}$,19), 182 (71.8), 181 (100).

*4.1.6. 3-Methyl-6,7,8,9-tetrahydro-1H-pyrrolo-[3,2-c]quinoline-4-carboxylic acid hydrate (**11**)*

The solution of **7a** (0.1 g, 0.38 mmol) in acetic acid (20 ml) and 0.1 M HCl (3.8 ml, 0.38 mmol) added to 0.02 g PtO_2 was hydrogenated at 3 atm for 18 h at r.t. The mixture was filtered, evaporated and the crude product was purified by silica gel flash-chromatography

Table 4
Elemental analyses

Comp.	Formula	Elemental analysis [Calc. (found)]			
		C	H	N	Cl
3a	$C_{14}H_{11}ClN_2O$	64.99 (64.91)	4.29 (4.30)	10.83 (10.80)	13.71 (13.69)
3b	$C_{14}H_{11}ClN_2O$	64.99 (64.83)	4.29 (4.07)	10.83 (10.61)	13.71 (13.59)
3c	$C_{14}H_{11}FN_2O$	69.41 (69.21)	4.58 (4.62)	11.57 (11.54)	
4	$C_{19}H_{13}ClN_2O$	71.14 (71.04)	4.08 (4.11)	8.74 (8.70)	11.05 (11.06)
5a	$C_{22}H_{16}ClN_3O_2$	67.77 (67.48)	4.13 (4.48)	10.78 (10.59)	9.09 (9.24)
5b	$C_{22}H_{16}ClN_3O_2$	67.77 (67.53)	4.13 (4.49)	10.78 (10.49)	9.09 (8.82)
5c	$C_{22}H_{16}FN_3O_2$	70.77 (70.43)	4.32 (4.42)	11.25 (10.95)	
6	$C_{27}H_{18}ClN_3O_2$	71.76 (71.46)	4.01 (4.24)	9.30 (8.99)	7.85 (7.53)
7a	$C_{13}H_9ClN_2O_2$	59.89 (59.50)	3.48 (3.61)	10.74 (10.58)	13.60 (13.65)
7b	$C_{13}H_9ClN_2O_2$	59.89 (59.53)	3.48 (3.63)	10.74 (10.48)	13.60 (13.67)
7c	$C_{13}H_9FN_2O_2 \cdot H_2O$	59.54 (59.23)	4.23 (4.19)	10.68 (10.44)	
		H_2O (K.F.) Calc. 6.87% Found: 7.10%			
8	$C_{18}H_{11}ClN_2O_2$	66.98 (66.67)	3.44 (3.51)	8.68 (8.45)	10.99 (10.66)
9	$C_{14}H_{11}ClN_2O_2$	61.21 (60.89)	4.04 (4.03)	10.20 (10.04)	12.91 (13.15)
10	$C_{13}H_{10}N_2O_2 \cdot H_2O$	63.92 (63.58)	4.95 (4.92)	11.43 (11.23)	
		H_2O (K.F.) Calc. 7.38% Found: 7.42%			
11	$C_{13}H_{14}N_2O_2 \cdot H_2O$	62.88 (62.67)	6.49 (6.43)	11.28 (11.15)	
		H_2O (K.F.) Calc. 7.26% Found: 7.20%			
13	$C_{11}H_9ClN_2O_2 \cdot H_2O$	51.87 (52.13)	4.35 (4.36)	11.00 (10.89)	13.92 (13.67)
		H_2O (K.F.) Calc. 7.10% Found: 6.84%			

(chloroform/methanol/30% ammonium hydroxide 80:20:2 as eluant) to give a beige solid (0.042 g, 45% yield), m.p. 230°C dec. *Anal.* ($C_{13}H_{14}N_2O_2 \cdot H_2O$) C, H, N. ^{1}H NMR (200 MHz, DMSO): δ 1.7–2.0 (m, 4H, CH_2-7 and CH_2-8), 2.37 (d, $J = 1.0$ Hz, 3H, CH_3), 2.7–3.1 (m, 4H, CH_2-6 and CH_2-9), 7.40 (m, 1H, H-2), 11.9 (bs, 1H, H-1). MS (EI) m/z (rel. int.) 230 ($M^{\bullet+}$, 39.2), 186 (59.2), 185 (100).

*4.1.7. 7-Chloro-4-methylamino-quinoline-2-carboxylic acid hydrate (**13**)*

p-Tosyl-isocyanate (1.92 g, 12.6 mmol) was added to a solution of 7-chloro-4-quinolone-2-carboxylic acid methyl ester (**14**) (3 g, 12.6 mmol) in acetonitrile (50 ml) and the mixture was refluxed for 13 h. After evaporation the residue was ground with ethyl acetate to afford crude 7-chloro-4-(toluene-4-sulfonylimino)-1,4-dihydro-quinoline-2-carboxylic acid methyl ester (0.7 g, 14% yield) as a light yellow solid that was utilized without further purification. Methyl iodide (0.16 ml, 2.56 mmol) and potassium carbonate (0.27 g, 1.95 mmol) were added to a solution of 7-chloro-4-(toluene-4-sulfonyl-imino)-1,4-dihydro-quinoline-2-carboxylic acid methyl ester (0.25 g, 0.64 mmol) in acetonitrile (5 ml). The mixture was refluxed for 24 h, then evaporated to dryness. The residue was taken up with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and evaporated to dryness after filtration. The crude 7-chloro-4-methyl-(toluene-4-sulfonyl)-amino-quinoline-2-carboxylic acid methyl ester (0.2 g, 77% yield) as a light yellow solid was utilized without further purification.

A solution of 7-chloro-4-methyl-(toluene-4-sulfonyl)-amino-quinoline-2-carboxylic acid methyl ester (0.2 g, 0.49 mmol) in hydrochloric acid (2 ml) and acetic acid (2 ml) was refluxed for 7 h. The solution was evaporated to dryness and the residue was taken up with water. The insoluble white solid was collected by filtration (0.11 g, 88% yield), m.p. 280.5–285°C. *Anal.* ($C_{11}H_9ClN_2O_2 \cdot H_2O$) C, H, Cl, N. ^{1}H NMR (200 MHz, DMSO): δ 3.17 (d, $J = 4.8$ Hz, 3H, CH_3), 7.20 (s, 1H, H-3), 7.83 (dd, $J = 9.1$, 2.2 Hz, 1H, H-6), 8.29 (d, $J = 2.2$ Hz, 1H, H-8), 8.45 (d, $J = 9.1$ Hz, 1H, H-5), 9.80 (q, $J = 4.8$ Hz, 1H, NH). MS (EI) m/z (rel. int.) 236 ($M^{\bullet+}$, 17), 192 (100).

4.2. Biological testing

4.2.1. Kynurenine-3-hydroxylase assay in the rat liver

The efficacy of the compounds of the invention in the inhibition of the enzyme kynurenine-3-hydroxylase was evaluated in rat liver mitochondrial extract according to the method of Erickson et al. [19] with minor modifications.

This assay is based on the enzymatic synthesis of tritiated water during the hydroxylation reaction. Radio-labelled water was quantified following selective adsorption of the isotopic substrate and its metabolite with activated charcoal.

The assay for kynurenine 3-hydroxylase activity was carried out at 37°C for 30 min. The reaction mixture

(total volume 30 μl) constituted 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-kynurenine, 0.3 μCi L-(3,5-^{3}H)kynurenine (10 Ci/mmol) and 3 μl of different concentrations of inhibitor solutions. After the incubation, the reaction was terminated by the addition of 300 μl of 7.5% (wt./vol.) activated charcoal, and centrifuged for 7 min.

A 75 μl aliquot of supernatant was transferred to an optiplate and 200 μl of liquid scintillation added. The optiplates were vortexed and the radioactivity counted in a scintillation counter.

4.2.2. Kynurenine-3-hydroxylase assay in the rat brain

The inhibition of the enzyme kynurenine-3-hydroxylase has been evaluated in rat brain homogenate by the conversion of L-kynurenine to L-3-hydroxykynurenine according to Carpenedo et al. [7].

Brain was homogenized in ice-cold 0.32 M sucrose and centrifuged at 12 000 × *g* for 30 min at 4°C. The pellet was washed three times with 0.32 M sucrose by centrifugation and suspended in 0.14 M KCl in 20 mM K-phosphate buffer at pH 7 (1 g brain in 2 ml buffer).

The reaction mixture contained 75 μl of suspended homogenate, 100 μl of 2 mM $MgCl_2$, 0.4 mM NADPH, 50 μM L-kynurenine in 50 mM K-phosphate buffer pH 7.5 and 25 μl of different concentrations of the tested compound. The reaction was terminated by addition of 200 μl of 1 M $HClO_4$ after 1 h incubation at 37°C. L-3-Hydroxykynurenine formed was quantified by HPLC with coulometric detection at working voltage of + 0.2 V. The column was a 10 cm C 18 reversed phase (3 μm particulate). The mobile phase consisted of 950 ml distilled water, 20 ml acetonitrile, 9 ml triethylamine, 5.9 ml phosphoric acid, 100 mg sodium EDTA and 1.5 g heptanesulfonic acid. The flow rate was 1 ml/min.

4.2.3. Kynureninase assay in the rat liver

The inhibition of the enzyme kynureninase has been evaluated in rat liver according to the method of Takikawa et al. [20] with minor modifications, by the conversion of L-kynurenine to anthranilic acid. Liver tissue (2 g) was homogenized in 10 ml of ice-cold 0.14 M KCl/0.02 M potassium phosphate buffer, pH 7.0 with a Polytron homogenizer (setting 7, twice for 15 min).

The reaction mixture contained 100 μl of homogenate, 40 μl of inhibitor and 60 μl of a substrate-cofactor solution containing 50 μl pyridoxal-5-phosphate, 10 μM L-kynurenine in 100 μM Tris–HCl pH 8.0 (final concentration). The reaction at 37°C was started by adding the substrate and was terminated after 30 min by adding 0.2 ml of 10% trichloroacetic acid. After centrifugation at 10 000 rpm for 15 min the supernatants were directly injected into the HPLC apparatus.

4.2.4. Kynurenine aminotransferase (KAT) assay

KAT activity was quantified in the rat following the forebrain homogenates by the conversion of L-kynurenine to kynurenic acic according to the method by Okuno et al. [21] with minor modifications. Brain tissue was diluted 1:10 in ice-cold 50 μM pyridoxal-5-phosphate, 10 mM 2-mercaptoethanol in 5 mM Tris–HCl pH 8.0, with a Polytron homogenizer (setting 7, twice for 15 s). The reaction mixture consisted of 80 μl of tissue homogenates, 20 μl of inhibitor and 1000 μl substrate-cofactor solution containing 80 μM pyridoxal-5-phosphate, 1 mM α-ketoglutaric acid, 50 μM L-kynurenine in 150 mM Tris–HCl pH 8.0 (final concentration). The reaction was terminated after 60 min of incubation at 37°C by addition of 20 μl of 50% trichloroacetic acid. After centrifugation at 10 000 rpm for 15 min, the supernatants were directly injected into the HPLC system and kynurenic acid was quantified.

4.2.5. p-Hydroxybenzoate hydroxylase (PHBH) assay

The activity of the enzyme PHBH from *Pseudomonas fluorescens* was measured by the conversion of *p*-hydroxybenzoic acid to 3,4-dihydroxybenzoic acid. The enzyme was incubated in the presence of 3 μM FAD, 150 μM NADPH, 25 μM substrate and increasing concentrations of inhibitors (final volume 200 μM) at 30°C. The reaction was terminated after 15 min by adding 200 μl of 1 M $HClO_4$. After centrifugation, supernatants were directly used for the measurement of 3,4-dihydroxybenzoic acid. The concentration of 3,4-dihydroxybenzoic acid was measured on a HPLC system coupled to a coulometric detection (det 1 + 0.05 det 2 + 0.2 V) with a reversed phase (C 18, 3 μm) column. The mobile phase contained water/acetonitrile/trifluoroacetic acid (95:5:0.05). The flow rate was 1 ml/min.

4.3. Molecular modelling

The three-dimensional structures of the compounds reported in Figs. 2–4 were built from available structural fragments of the INSIGHTII version 97.2 (MSI, San Diego, CA). The built structures were energy minimized by DISCOVER (version 2.9) with molecular mechanics using the gradient method and cvff.frc force fields. The minimization was carried out until the rms of the gradients was < 0.001 kcal/(mol Å). The structures were then submitted to a complete torsion angle search with increments of 30°, for the bonds free to rotate, discarding the conformations above 5 kcal/mol. The obtained conformations were minimized by molecular mechanics as described above. The conformations of KYN showing an intramolecular hydrogen bond between the carbonylic moiety and the aromatic amino group, leading to a formal 6-atom ring, were at a relative lower energy. This was also the only allowed conformation in the AM1 semi-empirical method (MOPAC program, version 6.0). Indeed, geometry minimiza-

tion of different kynurenine conformations always led to this intramolecular hydrogen bond conformation. A high-energy conformation of kynurenine was submitted to a dynamic simulation at 300 K, either in vacuum or in water (in this case a 15 Å layer of water molecules was used). In both cases after nearly 5 ps, a conversion to the most stable conformation was observed. The compounds remained in this conformation for the rest of the dynamic simulation (100 ps). The superimposition of PNU-156561 and KYN on compound **7a** showed the rms deviations of 0.42 and 0.39 Å, respectively, using the carboxylic group and the phenyl ring for the matching process.

References

[1] N.P. Botting, Chemistry and neurochemistry of the kynurenine pathway of tryptophan metabolism, Chem. Soc. Rev. 24 (1995) 401–412.

[2] K. Saito, M.P. Heyes, Kynurenine pathway enzymes in brain: properties of enzymes and regulation of quinolinic acid synthesis, Adv. Exp. Med. Biol. (Recent Adv. Triptophan Res.) 398 (1996) 485–492.

[3] A.C. Foster, A. Vezzani, E.D. French, R. Schwarcz, Kynurenic acid blocks neurotoxycity and seizures induced in rat by the related brain metabolite quinolinic acid, Neurosci. Lett. 48 (1984) 273–278.

[4] R. Schwarcz, W.O. Whetsell, R.M. Mangano, Quinolinic acid: an endogenous metabolite that causes axon-sparing lesions in rat brain, Science 219 (1983) 316–318.

[5] K. Saito, T.S. Nowak, S.P. Markey, M.P. Heyes, Mechanism of delayed increases in kynurenine pathway metabolism in damaged brain regions following transient cerebral ischemia, J. Neurochem. 60 (1993) 180–192.

[6] R. Pellicciari, B. Natalini, G. Costantino, M.R. Mahmoud, L. Mattoli, B.M. Sadeghpour, Modulation of the kynurenine pathway in search for new neuroprotective agents. Synthesis and preliminary evaluation of (*m*-nitrobenzoyl)alanine, a potent inhibitor of kynurenine-3-hydroxylase, J. Med. Chem. 37 (1994) 647–655.

[7] R. Carpenedo, A. Chiarugi, P. Russi, G. Lombardi, V. Carla, R. Pellicciari, L. Mattoli, F. Moroni, Inhibitors of kynurenine hydroxylase and kynureninase increase cerebral formation of kynurenate and have sedative and anticonvulsant activities, Neuroscience 61 (1994) 237–244.

[8] C. Speciale, M. Varasi, M. Cini, A. Molinari, M. Marconi, R. Bormetti, C. Caccia, L. Curatolo, N. Carfagna, H.Q. Wu, R. Schwarcz, Multiple ways to modulate the content of quinolate and kynurenate in the central nervous system, Adv. Exp. Med. Biol. (Recent Adv. Triptophan Res.) 398 (1996) 512–517.

[9] A.F. Miranda, R.J. Boegman, R.J. Beninger, K. Jhamandas, Protection against quinolinic acid-mediated excitotoxicity in nigrostriatal dopaminergic neurons by endogenous kynurenic acid, Neuroscience 78 (1997) 967–975.

[10] A. Giordani, L. Corti, M. Cini, R. Bormetti, M. Marconi, O. Veneroni, C. Speciale, M. Varasi, Enantiospecific synthesis and in vitro activity of selective inhibitors of rat brain kynureninase and kynurenine-3-hydroxylase, Adv. Exp. Med. Biol. (Recent Adv. Triptophan Res.) 398 (1996) 531–534.

[11] F.D. Popp, Developments in the chemistry of Reissert compounds, Adv. Heterocycl. Chem. 24 (1979) 187.

[12] K.N. Campbell, C.H. Helbing, J.F. Kervin, Studies in the quinoline series. V. The preparation of some α-dialkylaminoethyl-2-quinolinemethanols, J. Am. Chem. Soc. 68 (1946) 1840–1843.

[13] N.D. Heindel, I.S. Bechara, P.D. Kennewell, J. Molnar, C.J. Ohnmacht, S.M. Lenke, T.F. Lenke, Cyclization of aniline–acetylenedicarboxylate adducts. A modified Conrad–Limpach method for the synthesis of potential antimalarials, J. Med. Chem. 11 (1968) 1218–1221.

[14] R. Gordon, R. McWright, A simple one-pot conversion of alkyl 4-oxo-1,4-dihydroquinoline-2-carboxylates to 4-aminoquinoline-2-carboxylates using reactive isocyanates, Synthesis (1984) 1058–1061.

[15] A. Giordani, P. Pevarello, M. Cini, R. Bormetti, F. Greco, S. Toma, C. Speciale, M. Varasi, 4-Phenyl-4-oxo-butanoic acid derivatives inhibitors of kynurenine 3-hydroxylase, BioMed. Chem. Lett. 8 (1998) 2907–2912.

[16] S. Röver, A.M. Cesura, P. Huguenin, R. Kettler, A. Szente, Synthesis and biochemical evaluation of *N*-(4-phenylthiazol-2-yl)benzenesulfonamides as high-affinity inhibitors of kynurenine 3-hydroxylase, J. Med. Chem. 40 (1997) 4378–4385.

[17] M.J.S. Dewar, E.G. Zoebisch, E.F. Healy, J.J.P. Stewart, AM1: a new general purpose quantum mechanical molecular model, J. Am. Chem. Soc. 107 (1985) 3902–3909.

[18] M.A. Khan, J. Ferreira da Rocha, Tricyclic heteroaromatic system II (1). A convenient synthesis of 1*H*-pyrrolo[3,2-*c*]-quinolines (**2a, b**), J. Heterocycl. Chem. 15 (1978) 913–921.

[19] J.B. Erickson, E.M. Flanagan, S. Russo, J.F. Reinhard, A radiometric assay for kynurenine 3-hydroxylase based on the release of ^3H_2O during hydroxylation of L-[3,5-^{3}H]kynurenine, Analyt. Biochem. 205 (1992) 257–262.

[20] O. Takikawa, R. Yoshida, R. Kydo, O. Hayashi, Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase, J. Biol. Chem. 261 (1986) 3648–3653.

[21] E. Okuno, W. Schmidt, D.A. Parks, M. Nakamura, R. Schwarcz, Measurement of rat brain kynurenine aminotransferase at physiological conditions, J. Neurochem. 57 (1991) 533–540.