Enzyme-Activated Antagonists of the Strychnine-Insensitive Glycine/NMDA Receptor

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As the physiological importance of the NMDA receptor complex is being unraveled,[1-3] the search for selective modulators has attracted considerable attention in recent years.[4] Potent and selective antagonists recently have been reported for the PCP, glutamic acid, and glycine binding sites[4] which are an integral part of the NMDA receptor complex. Whereas a considerable amount of animal pharmacology has been reported on PCP-like compounds (e.g., MK 801[5]) and competitive glutamate antagonists (e.g., CPP[6], MDL 100,453[7]), relatively few reports have dealt with the glycine-site antagonists due, in part, to problems of brain penetrability. In our attempts to develop therapeutically useful agents acting at the glutamic acid[7,8] and glycine[9,10] binding sites, and to address the bioavailability problem, we have directed some of our efforts to the design of site-selective, glycine antagonists.

Kynurenic acid (**3**) is an endogenous broad spectrum antagonist at the ionotropic excitatory amino acid receptors in the brain, with micromolar affinity for the glycine/NMDA site.[11-14] Compound **3** may, therefore, modulate NMDA receptor function preferentially and play a role in NMDA receptor-mediated pathological processes. Indeed, exogenously applied **3**[15,16] and analogues thereof[17,18] have anticonvulsant and neuroprotective properties, though their central potency is limited by their poor access to the brain.[19] The present set of experiments was designed to examine if the more potent and more receptor-specific kynurenic acid derivatives 7-chloro- (**4**)[20] and 5,7-dichlorokynurenic acid (**5**)[21] can be produced by the action of kynurenine aminotransferase, the enzyme which is singularly responsible for the biosynthesis of **3** from L-kynurenine (**1**) in the brain[22] (Scheme 1). Like L-kynurenine,[23] but in contrast to **3**,[19] the putative bioprecursors of **4** and **5**, L-4-chlorokynurenine (**6**) and L-4,6-dichlorokynurenine (**7**), may possibly cross the blood-brain barrier[19] prior to their transamination to the glycine antagonists **4** and **5**. The utilization of endogenous enzymes to convert bioprecursor molecules to their active form has previously been described by McDonald et al. for the enzymatic production of a series of MAO inhibitors.[24,25]

The syntheses of analogues **6** and **7** were synthetically challenging due in large part to the difficulty in the synthesis of the requisite[26] protected arylstannanes (**12** and **13**) (Scheme 2). Commercially available 2-nitro-4-chloroaniline (**8**) was converted to the iodide (**10**) in four steps in 82% overall yield. Direct iodination of 3,5-dichloroaniline (**9**), followed by amine protection, afforded iodide **11** (36%). Conversion of **10** and **11** to the stannanes **12** and **13**, respectively, required special conditions in order to eliminate intramolecular protonolysis. Thus, addition of a THF solution of **10** to a suspension of NaH in THF at 0 °C prior to metal–halogen exchange (*tert*-butyllithium, −78 °C), followed by quenching with $(CH_3)_3SnCl$, gave **12** in 66% yield after chromatography and recrystallization. Under similar conditions the dichloro analogue (**13**) was obtained in only 40% yield. However, this yield could be increased to 60% by a Pd^0-catalyzed coupling reaction of the preformed Na salt of **11** with hexamethyldistannane. Coupling of **12** and **13** with the acid chloride **14**[26] afforded **15** and **16** in 62% and 53% yield, respectively. Deprotection of **15** and **16** furnished **6** and **7** as pale yellow powders in 56% and 47% yields, respectively.[27]

L-4-Chlorokynurenine (**6**) and L-4,6-dichlorokynurenine (**7**) were found to be good substrates for crude rat cerebral cortex kynurenine aminotransferase and spontaneously cyclized to **4** and **5**, respectively (Figure 1). Compounds **6** and **7** were independently incubated at 37 °C for 2 h

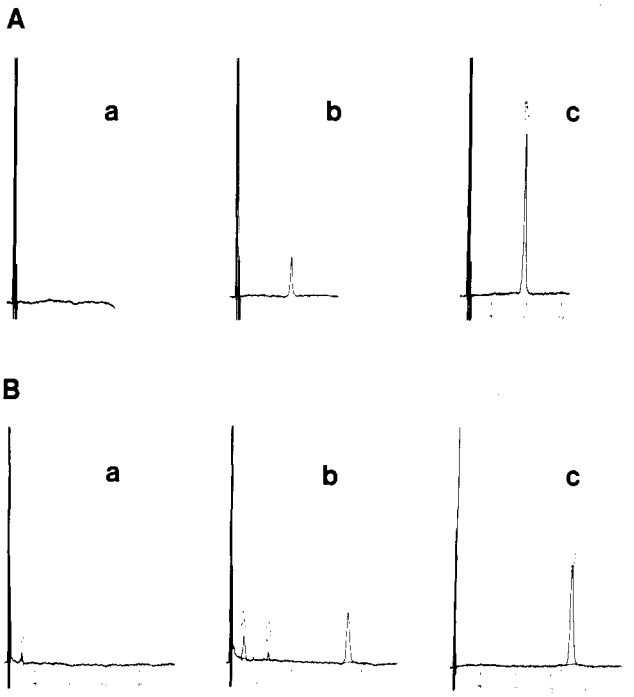

Figure 1. Enzymatic conversion of the kynurenine derivatives **6** (300 pmol; panel A) and **7** (500 pmol; panel B) to the kynurenic acids **4** and **5**, respectively. After incubation of crude rat cerebral cortex tissue homogenate for 120 min at 37 °C with **6** or **7**, the supernatant was applied to a Dowex 50W column (0.5 × 1.0 cm; H^+ form) and the kynurenic acid (KYNA) derivatives were eluted with distilled water.[22] After lyophilization, the samples were resuspended in about 100 μL of distilled water and applied to a 5 μM C_{18} HPLC column (4.6 × 150 mm, Beckman) whereupon the amount of **4** and **5** could be quantitatively measured. Elution conditions: **4** was eluted isocratically with a mobile phase consisting of NH_4OAc (100 mM), pH 4.0, and 10% CH_3CN at a flow rate of 1.2 mL/min; spectrophotometric detection at 340 nm; retention time was approximately 7.7 min; **5** was eluted under the same conditions, but at a flow rate of 1.8 mL/min; retention time was about 14.0 min. Panel A: Production of **4** from **6**: (a) heat-deactivated blank sample; (b) enzymatic product; (c) standard **4** (100 pmol). Panel B: Production of **5** from **7**: (a) heat-deactivated blank sample; (b) enzymatic product; (c) standard **5** (40 pmol).

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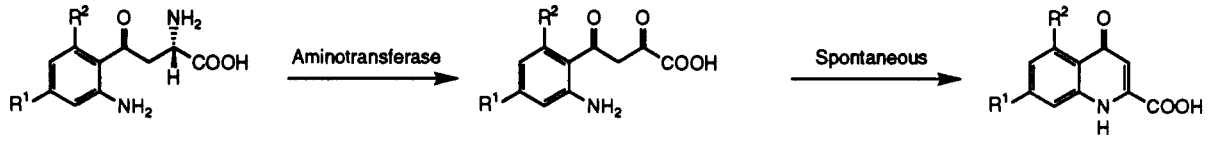
Scheme 1

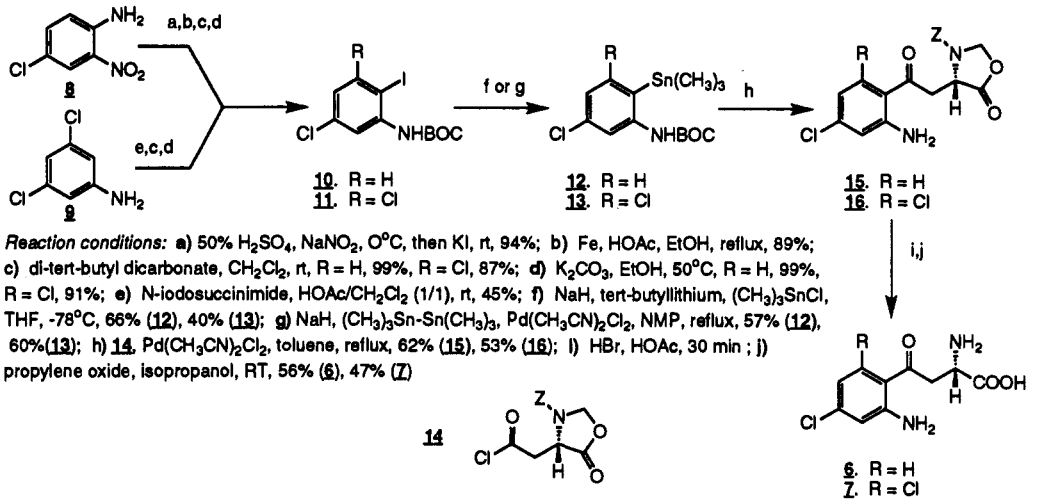


1. $R^1, R^2 = H$
6. $R^1 = Cl; R^2 = H$
7. $R^1, R^2 = Cl$

2.

3. $R^1, R^2 = H$
4. $R^1 = Cl; R^2 = H$
5. $R^1, R^2 = Cl$

Scheme 2



8 9

10. R = H
11. R = Cl

12. R = H
13. R = Cl

15. R = H
16. R = Cl

Reaction conditions: a) 50% H_2SO_4, $NaNO_2$, 0°C, then KI, rt, 94%; b) Fe, HOAc, EtOH, reflux, 89%; c) di-tert-butyl dicarbonate, CH_2Cl_2, rt, R = H, 99%, R = Cl, 87%; d) K_2CO_3, EtOH, 50°C, R = H, 99%, R = Cl, 91%; e) N-iodosuccinimide, HOAc/CH_2Cl_2 (1/1), rt, 45%; f) NaH, tert-butyllithium, $(CH_3)_3SnCl$, THF, -78°C, 66% (12), 40% (13); g) NaH, $(CH_3)_3Sn\text{-}Sn(CH_3)_3$, $Pd(CH_3CN)_2Cl_2$, NMP, reflux, 57% (12), 60%(13); h) 14, $Pd(CH_3CN)_2Cl_2$, toluene, reflux, 62% (15), 53% (16); I) HBr, HOAc, 30 min ; j) propylene oxide, isopropanol, RT, 56% (6), 47% (7)

14

6. R = H
7. R = Cl

with a crude enzyme preparation from rat cerebral cortex. HPLC analysis of the assays run against heat-deactivated controls and the standard products (compounds 4 and 5) confirmed the conversions. Further studies were performed with partially purified enzyme and analog 6 (Figure 2), the better of the two substrates. Thus, using standard Michaelis–Menten kinetics, conversion of 1 to 3 was shown to be competitively inhibited by 6, indicating competition of the compound with L-kynurenine for the active site of the enzyme. In addition, HPLC analyses of competing substrate reactions as described in the literature showed that the V/K values for the two substrates were essentially equal (0.23 and 0.27 for 1 and 6, respectively).[28]

To ascertain whether 6 could be similarly metabolized in the brain *in vivo*, 6 (10 mM) was administered to the brain for 7 h via microdialysis probes positioned vertically

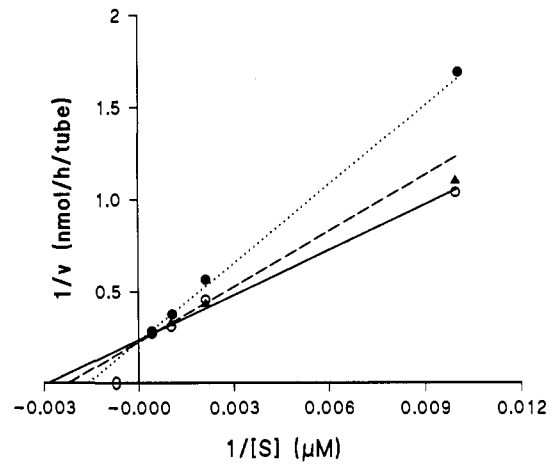


Figure 2. Kinetic analysis of the effect of 6 on partially purified rat brain kynurenine aminotransferase. The protein was purified[31] and its activity was determined in 5 μL of the enzyme preparation (54 pmol/h) as described previously.[22] Kinetic analysis was performed using different concentrations of L-kynurenine (2 μM to 2 mM) in the absence (○) or presence of 50 μM (▲) or 200 μM (●) 6. The data were analyzed by fitting two rectangular hyperbola according to the Michaelis equation using a computerized least-square fit method and represented as double-reciprocal plots.

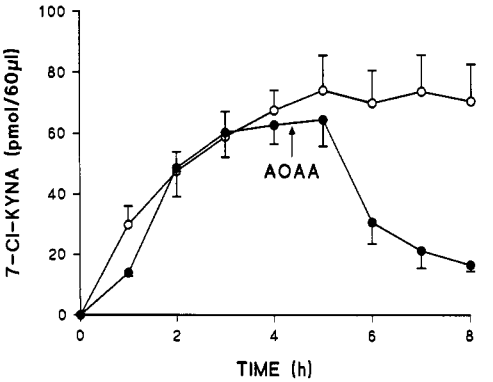


Figure 3. Production of 4 from 6, assessed by microdialysis in freely moving animals. Male Sprague–Dawley rats (200–250 g) were anesthetized with chloral hydrate (360 mg/kg, ip) and mounted in a stereotaxic frame. Guide cannulae for microdialysis probes were positioned bilaterally 1 mm below dura over the dorsal hippocampus. One day after surgery, the microdialysis probes were inserted through the guide cannulae, extending vertically throughout the hippocampi. Both hippocampi were perfused through the probe for 8 h with 10 mM 6, dissolved in Ringer solution, at a flow rate of 1 μL/min. In one hippocampus, 500 μM (aminooxy)acetic acid (AOAA) was added to the perfusion solution for 4 h (●), while the other hippocampus served as a control (○). Samples were collected every 60 min. Data represent the mean ± SEM of four animals. The amount of 4 (7-Cl-KYNA) formed was determined by HPLC using a 5-μm C_{18} column (4.6 × 100 mm, esa) and a mobile phase containing 50 mM NH_4OAc and 5% methanol at a flow rate of 0.5 mL/min, and spectrophotometric detection at 340 nm (retention time of 4: approximately 7.5 min). Two-way ANOVA with Newman-Keul post-hoc analysis revealed a significant effect of the (aminooxy)acetic acid treatment [F (4,24) = 15.96, $P < 0.01$].

through both dorsal hippocampi of male Sprague–Dawley rats. After 4 h, the nonspecific transaminase inhibitor, (aminooxy)acetic acid,[30] was added to the perfusion medium in one hippocampus. The production of 4 was detected within 1 h, and the levels increased until a steady state was reached at about 3 h (Figure 3). Within 1 h after the introduction of (aminooxy)acetic acid (AOAA), the concentration of extracellular 4 decreased to 23% of the control side after an additional 3 h.

Taken together, these experiments demonstrate that minor variations to the L-kynurenine structure are tolerated by brain kynurenine aminotransferase and that spontaneous cyclization of the putative keto intermediates leads to chlorine-substituted kynurenic acid derivatives which are among the most potent glycine antagonists known to date.[32] We are currently evaluating the bioavailability, pharmacological profile, and efficacy of compounds 6 and 7 in a number of biochemical and pharmacological models.

Supplementary Material Available: Experimental data for compounds mentioned in this paper (6 pages). Ordering information is given on any current masthead page.

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(27) Compound 7 gave a satisfactory combustion analysis (C, H, N); after deprotection, compound 6 was further purified by HPLC (Vydac C_{18} radical pack 50- × 300-mm column, 15–20 μM. Elution: isocratic 10% CH_3CN in 0.1% TFA/H_2O).

(28) *V*/*K* values were determined from competitive substrate experiments as described in Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, p 112. Experimental details are described in the supplementary material.

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(32) It will be important to determine to what extent compounds 6 and 7 are partitioned through other enzymatic steps of the kynurenine pathway and how these potential metabolites reflect upon the efficacy of the compounds.