DL-Tetrazol-5-ylglycine, a Highly Potent NMDA Agonist: Its Synthesis and NMDA Receptor Efficacy

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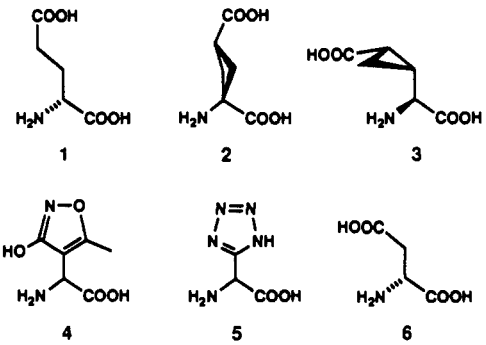
At physiological pH, the spatial arrangement of the three charges of DL-tetrazol-5-ylglycine (**5**) could be viewed as similar to those found in certain conformations of the two excitatory amino acids (EAAs)—aspartic and glutamic acids. Given significant binding to one or more EAA receptors, **5** would offer unique modeling and perhaps biological opportunities. We have previously shown it to be the most potent NMDA agonist known, with a unique and marked in vitro neutrotoxicity at depolarizing concentrations. Now we report the details required for its synthesis, together with its potency and efficacy in two assays of functional activation of the NMDA receptor, namely agonist-influenced [^{3}H]MK801 binding and agonist-induced release of the neurotransmitter [^{3}H]-norepinephrine from brain slices. In both these assays DL-tetrazol-5-ylglycine proved to be more potent and efficacious than NMDA and *cis*-methanoglutamate. It was more potent than, and equally efficacious to, L-glutamate in [^{3}H]MK801 binding. The structural features of **5** may well reflect optimal agonist interaction at the NMDA receptor site. (We considered the possibility that some decarboxylation of DL-tetrazol-5-ylglycine may have occurred during testing. This would give 5-(aminomethyl)tetrazole (**13**), the tetrazole acid analog of glycine; and glycine is involved in NMDA receptor activation. Compound **13** does not affect [^{3}H]glycine binding at the strychnine-insensitive glycine binding site, and [^{3}H]MK801 binding studies showed that the (aminomethyl)-tetrazole, even if is formed, would probably have no effect on the activity of tetrazol-5-ylglycine at the NMDA receptor.)

Knowledge of the glutamatergic excitatory amino acid systems has advanced considerably over the last few years. Several recent publications have described the synthesis and biological properties of compounds with molecular architectures based on the structure of glutamic acid (**1**) itself. Much of this work has tended to center on the NMDA receptor and has led to some potent antagonists.[1] However, there are only a few compounds known to be potently selective agonists for that receptor. Three such agents have recently been reported, namely *cis*-2,4-methanoglutamate (**2**),[2] the (2*S*,3*R*,4*S*) isomer of 1α-(carboxycyclopropyl)glycine (**3**),[3] and, (*RS*)-2-amino-2-(3-hydroxy-5-methylisoxazol-4-yl)acetic acid (AMAA, **4**).[4]

Perusal of the literature revealed that, despite its simplicity, tetrazol-5-ylglycine (**5**) had not yet been synthesized. At physiological pH it could be viewed as having a geometric distribution of charges similar to either glutamic acid or to aspartic acid (**6**), another natural NMDA receptor agonist, albeit less potent than glutamic acid. If it were to exhibit significant activity at one or more of the excitatory amino acid receptors, it would offer unique information for molecular modeling.



Although we have recently published a brief sketch of the synthesis of **5**,[5] some unusual operations are absolutely required for success. This, together with inquiries from

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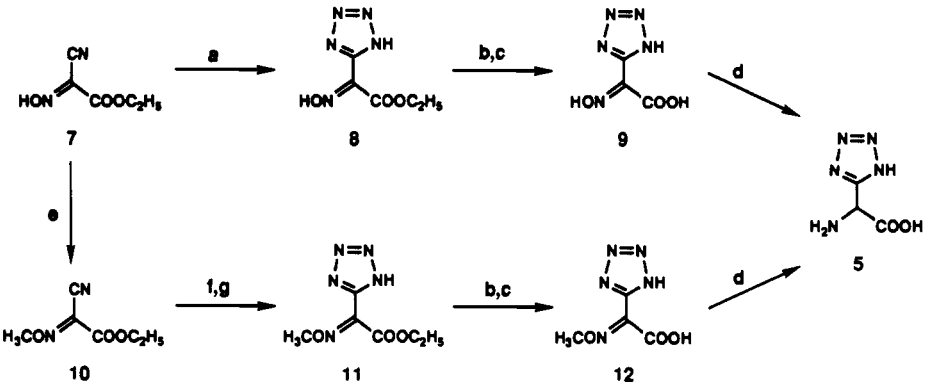
Scheme I [a]



[a] (a) NaN_3, DMF, 70 °C; (b) aqueous NaOH; (c) aqueous HCl; (d) H_2, PtO_2, H_2O; (e) CH_3I, K_2CO_3, acetone; (f) Bu_3SnN_3, 80 °C; (g) HCl, H_2O, Et_2O.

other chemists, leads us to provide detailed experimental procedures to ensure the compound can be available for others to use as a research tool. Additionally, because of the marked receptor-mediated toxicity we observed with DL-tetrazol-5-ylglycine in our previous work,[5] we have further characterized the potency and efficacy of DL-tetrazol-5-ylglycine as an agonist acting at the glutamate recognition site of the NMDA receptor complex.

Chemistry

The synthesis of DL-tetrazol-5-ylglycine was accomplished by two alternative procedures (Scheme I). In the first route the tetrazole ring was constructed using NaN_3 in hot DMF. Heating NaN_3 solutions is reputed to carry some hazard, and in the second procedure tri-*n*-butyltin azide, which is relatively heat stable,[6] was used. The NaN_3 method generally provided purer **5**.

Commercially available ethyl 2-cyano-2-oximinoacetic acid (**7**) was converted to 2-tetrazol-5-yl-2-oximinoacetic acid (**8**) by heating with NaN_3 in DMF for 30 h at 70 °C. Hydrolysis of the ester was accomplished with aqueous NaOH at room temperature for 3 h. However, the resulting Na salt was extremely insoluble and heating with excess aqueous HCl was required to obtain the oxime free acid (**9**). This acid was then hydrogenated to the desired amino acid **5** in H_2O using PtO_2 as the catalyst. The product crystallized out from the hydrogenation mixture, and care had to be exercised to insure it was not lost during removal of catalyst. In the second route compound **7** was first methylated by CH_3I in acetone with K_2CO_3 as the base. The cyanomethoxime **10** was converted to the tetrazole by heating with tri-*n*-butyltin azide at 80 °C for 5 h. Incidentally, **10** could not be converted to **11** with NaN_3 in hot DMF. Hydrolysis of the resulting methoxime ester **11** and acidification to **12** was more straightforward than with the free oxime ester. Again, hydrogenation with PtO_2 catalyst afforded DL-tetrazol-5-ylglycine (**5**).

Pharmacology

DL-Tetrazol-5-ylglycine has proven to be the most potent selective α-amino acid NMDA receptor agonist to date; and, in contrast to NMDA and *cis*-methanoglutamate, it was highly effective in inducing NMDA-receptor mediated in vitro neuronal toxicity at depolarizing concentrations.[5] This made it difficult to assess its agonist potency and efficacy relative to NMDA and *cis*-methanoglutamate. Its unique propensity for NMDA-receptor-mediated toxicity might reflect greater efficacy at the NMDA receptor. Here we report the results of a more complete examination of the potency and efficacy of tetrazol-5-ylglycine, in comparison to NMDA and *cis*-methanoglutamate, using two in vitro assays of functional activation of the NMDA receptor.

In one of these assays the effects of **5** on [^{3}H]MK801 binding was studied. The utility of [^{3}H]MK801 binding to monitor NMDA channel activity is well documented. Both glutamate and glycine stimulate [^{3}H]MK801 binding to membrane preparations.[7] Glycine markedly potentiates NMDA receptor activation at low concentrations.[8] In fact, when glycine is excluded from the receptor environment using the glycine receptor antagonist 7-chlorokynurenic acid, no NMDA receptor response can be elicited.[9] Therefore, an EC_{50} concentration of glycine (0.2 mM) was included in all assays to allow glutamate recognition site enhancement of [^{3}H]MK801 binding. It was found that the rank order of potency to stimulate [^{3}H]MK801 binding was DL-tetrazol-5-ylglycine > L-glutamate > *cis*-methanoglutamate > NMDA (Figure 1 and Table I). DL-Tetrazol-5-ylglycine was slightly more potent ($p < 0.05$) than L-glutamate, and as efficacious. *cis*-Methanoglutamate and NMDA were not fully efficacious, and produced about 80 and 40% of the maximal effect of L-glutamate, respectively. The EC_{50} values we obtained for L-glutamate and NMDA were in good agreement with those previously reported.[7]

Since glycine is involved in activation of the NMDA receptor, we considered the fact that if **5** were to decarboxylate, it would give 5-aminotetrazole (**13**), which is a direct analog of glycine. We were concerned that such

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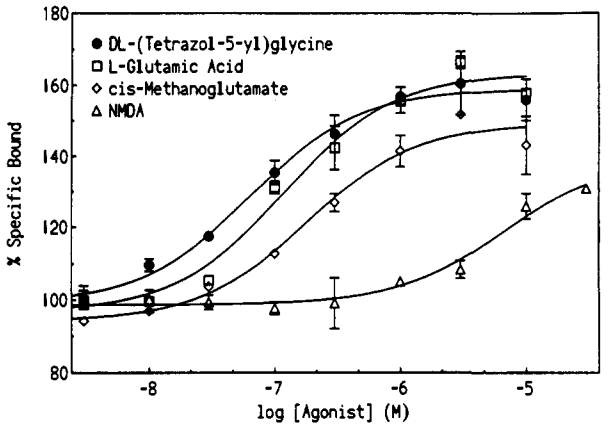

Figure 1. Concentration–effect enhancement of [^{3}H]MK801 binding to well-washed rat cortical membranes by excitatory amino acid agonists. Dose–response curves for DL-tetrazol-5-ylglycine, L-glutamate, *cis*-methanoglutamate, and NMDA. All assays were performed in the presence of 0.2 μM glycine. Percent specific bound was defined as the amount bound in the presence of 0.2 μM glycine minus nonspecific [^{3}H]MK801 bound. The means (±SEM) are from four experiments.

Table I. Comparison of the NMDA Receptor Potencies of DL-Tetrazol-5-ylglycine, *cis*-Methanoglutamate, NMDA, and L-Glutamate

	IC_{50} μM:	EC_{50} (μM)	
	[^{3}H]CGS19755[a] binding	[^{3}H]MK801 binding	[^{3}H]NE release
DL-tetrazol-5-ylglycine	0.098 ± 0.007	0.070 ± 0.004	2.03 ± 0.80
cis-methanoglutamate	0.163 ± 0.048	0.18 ± 0.01	16.5 ± 8.8
NMDA	4.15 ± 0.97	6.86 ± 0.008	75.0 ± 28.0
L-glutamate	0.172 ± 0.055	0.12 ± 0.5	ND[b]
L-aspartate	1.638 ± 0.36		

[a] Except for L-aspartate, the data were adapted from ref 5. [b] Uptake of L-glutamate precludes obtaining meaningful figures with this compound.

decarboxylation could be occurring, to some extent, during testing, with **13** then playing a role. In order to determine the possible impact of **13** on the results, we studied its effects on strychnine-insensitive [^{3}H]glycine binding and on [^{3}H]MK801 binding. At 100 μM it had no effect on strychnine-insensitive [^{3}H]glycine binding, and it did not appreciably stimulate [^{3}H]MK801 binding at up to 1 μM concentration (data not shown).

In the rat hippocampus, NMDA receptor activation is associated with the enhanced release of neurotransmitters such as norepinephrine, and this can be used to characterize potency and efficacy of NMDA receptor agonists.[10] In this assay system NMDA and *cis*-methanoglutamate proved equally efficacious, producing a maximal effect of about 50% total [^{3}H]norepinephrine release (Figure 2). Under the same conditions, DL-tetrazol-5-ylglycine was more efficacious, evoking up to 90% release at a maximally effective concentration of 3 μM (Figure 2). Calculated EC_{50} values for [^{3}H]norepinephrine release showed a relative potency order of DL-tetrazol-5-ylglycine > *cis*-methanoglutamate > NMDA (Table I). [^{3}H]Norepinephrine release evoked by DL-tetrazol-5-ylglycine was NMDA receptor mediated since it could be completely antagonized by either the competitive NMDA receptor antagonist (LY274614)[11] or by 5,7-dichlorokynurenic acid,[12] a competitive antagonist at the glycine modulatory site (Figure 3).

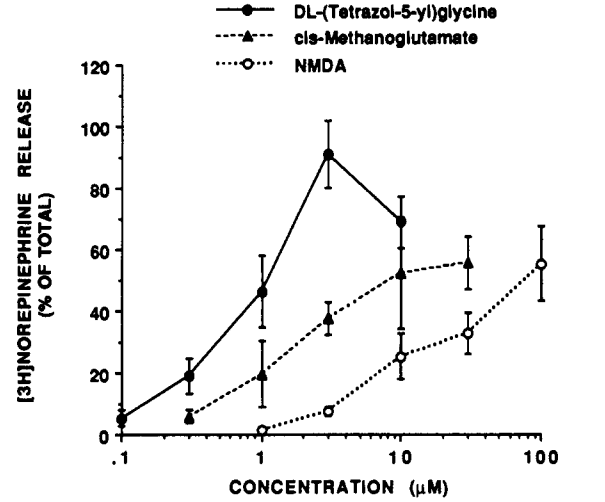

Figure 2. Concentration–effect stimulation of [^{3}H]norepinephrine release by NMDA receptor agonist compounds. Prelabeled rat hippocampal slices were superfused with magnesium-free buffer containing compounds for four 5-min fractions, followed by five additional fractions with buffer only. The total radioactivity released in fractions 4–12 plus that in the tissue was used to calculate percent total [^{3}H]norepinephrine released. Data represent the mean ± SE of four experiments performed in triplicate.

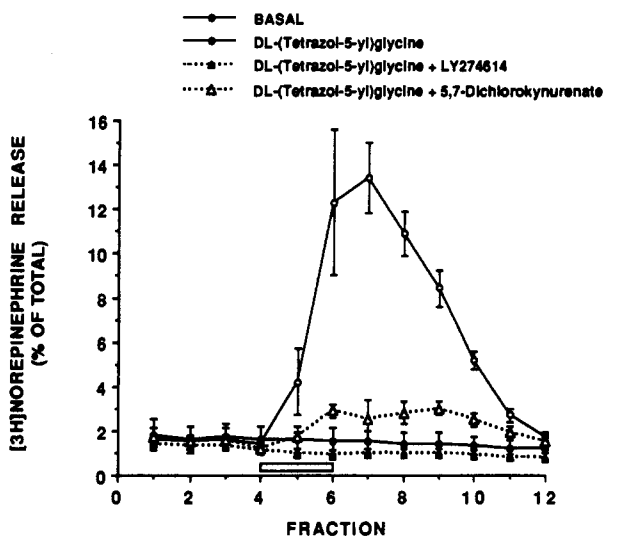

Figure 3. Effect of NMDA receptor antagonists on DL-tetrazol-5-ylglycine-induced release of [^{3}H]norepinephrine. Prelabeled rat hippocampal slices were superfused with magnesium-free buffer and twelve 5-min (1.5 mL) fractions were collected. Antagonist compounds LY274614 (10 μM), 5,7-dichlorokynurenic acid (10 μM), or buffer alone (controls) were present in all fractions. DL-Tetrazol-5-ylglycine was added in fractions 4–6. Data were expressed as the percent of total radioactivity (all fractions plus that in the tissue) released in each fraction and are the mean ± SE of four experiments performed in triplicate.

Discussion

The functional studies indicate that DL-tetrazol-5-ylglycine is a more potent and efficacious NMDA agonist

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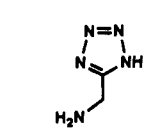
than either *cis*-methanoglutamate or NMDA. Indeed, this might account for its high propensity for inducing NMDA-receptor-mediated in vitro neuronal toxicity at depolarizing concentrations.

At physiological pH DL-tetrazol-5-ylglycine will bear three charges, two negative and one positive. The spatial arrangement of the three charges could be viewed as being similar to those found in particular conformations of either aspartic or of glutamic acid. Given similar spatial placement of the α-amino acid portions of all three molecules, the negative charge on the other carboxyl residue of aspartic acid could occupy a location similar to an anionic charge on N(2) of **5**, as shown in **14** and **15**. Glutamic acid could give a charge array approaching that of **14** as shown in **16** (Dreiding models). Since it has been demonstrated that DL-tetrazol-5-yl glycine binds very selectively to the NMDA receptor, relative to the AMPA and KA sites,[5] it is clearly behaving like aspartate, rather than glutamate (which is not selective). This is intriguing because the binding potency of asparate for the NMDA receptor (IC_{50} = 1.638) is about one-tenth that of glutamate (IC_{50} = 0.172) (Table I).



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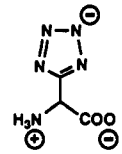

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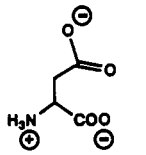

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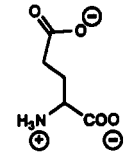

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Experimental Section

Chemistry. Melting points were determined with a Laboratory Devices Mel-temp apparatus and are uncorrected. NMR spectra were obtained on a GE QE-300 spectrometer at 300.15 MHz. Fast atom bombardment (FAB) mass spectral data was recorded with a VG ZAB-2SE mass spectrometer using the indicated matrix.

Ethyl 2-Tetrazol-5-yl-2-oximinoacetate (8). A mixture of 28.4 g (0.2 mol) of ethyl 2-cyano-2-oximinoacetate (**7**) and 14.3 g (0.22 mol) NaN_3 was stirred in 200 mL of dry DMF in an oil bath maintained at 70 °C for 30 h. The reaction mixture was then concentrated under reduced pressure to a thick syrup, which was subjected to rotary evaporation for 8 h to remove most of the DMF. Then 650 mL of ethyl acetate and 175 mL of H_2O were added, and the mixture was stirred until solution was complete. The pH was adjusted from the found 7.46 to 1.29 by the addition of 46 mL of 5 N (0.23 mol) HCl. The phases were separated, and the ethyl acetate was washed with 125 mL of H_2O, dried, and evaporated to give a solid which was subjected to high vacuum for 24 h. The solid was dissolved in 140 mL of boiling acetone, and cyclohexane was added slowly until the solution was cloudy (225 mL of cyclohexane). Standing at room temperature overnight, filtration, and washing with three 100-mL volumes of acetone/cyclohexane 1:2 gave 15.3 g of **8**, mp 170.2–171.5 °C (dg). A second crop of 6.5 g of **8** was obtained by evaporation of the mother liquors and crystallization from acetone/chloroform 1:6. A third crop (1.61 g) could be obtained from the original aqueous phase by thorough evaporation and recrystallization from 90 mL of H_2O. A total of 23.4 g (63%) of **8** was obtained. ^1H NMR (DMSO-d_6) δ: 1.21 (t, J = 7.2 Hz, 3 H), 4.26 (q, J = 7.2 Hz, 2 H). Anal. ($C_5H_7N_5O_3$) C, H, N.

2-Tetrazol-5-yl-2-oximinoacetic Acid (9). A thick suspension of 4.63 g (25 mmol) of ester **8** was stirred in 20 mL of H_2O in an ice bath. To this was added 17.5 mL of 5 N (87.5 mmol) NaOH over a 30-min period to give a pale yellow solution. The ice bath was removed and stirring continued for 3 h. Then, again with cooling in an ice bath, the mixture was acidified by the addition of 18 mL of 5 N (90 mmol) HCl over a 12-min period. At above pH 5.1 the yellow color disappeared, and at pH 2.7 a heavy deposition of crystalline material occurred. This heavy slurry was stirred on a hot plate until complete solution occurred (temperature 65 °C). The solution (pH 0.6) was stood at room temperature for 16 h, refrigerated for 6 h, then filtered, and washed with 25 mL of ice-cold water to give 4.05 g (84%) of **9** as colorless plates: mp 154.5–156.5 (dg). Anal. ($C_3H_3N_5O_3 \cdot 2H_2O$) C, H, N.

Ethyl 2-Cyano-2-methoximinoacetate (10). A solution of 71.0 g (0.5 mol) of ethyl 2-cyano-2-oximinoacetate (**7**) was stirred in 500 mL of acetone while 82.9 g (0.6 mol) of K_2CO_3 was added in one portion. Copious gas evolution occurred and the temperature rose from 23 to 28 °C, and 10 min later the deep yellow suspension was cooled to 7 °C in an ice–acetone bath. At this point the bath was removed and CH_3I (177.4 g, 1.25 mol) was added, with stirring, over a 13-min period. When this addition was complete, there was a steady gas evolution and the temperature was 22 °C. Minor cooling was used to maintain the temperature of the reaction mixture at 18–20 °C for the next 1–3 h. By this time gas evolution had almost ceased and the raspberry-red mixture was carefully concentrated under a reduced pressure of no less than 8 mm Hg to a thick sludge. Evaporation was terminated when there were signs of condensate in the trap between the flask and the rotary evaporator. Then 600 mL of CH_2Cl_2 and 375 mL of H_2O were added, and the phases were gently mixed and separated. (Backlighting was necessary to be able to see the meniscus.) The aqueous phase was further extracted with 150 mL of CH_2Cl_2. The CH_2Cl_2 extracts were combined, washed with two 200-mL volumes of water, dried, and carefully evaporated to a thin oil. The oil was flash chromatographed on a short (3-in. height, 5-in. diameter) column of Merck silica gel 60, by eluting with CH_2Cl_2 until the rate of elution was about 1 mg/mL (2100 mL of CH_2Cl_2). Careful evaporation gave a straw-colored oil, which was distilled (bp 89–91 °C at 4 mmHg). This gave 33.8 g (43%) of **10**. ^1H NMR (DMSO-d_6) δ:1.23 (t, J = 7.1 Hz, 3 H), 4.21 (s, 3 H), 4.27 (q, J = 7.1 Hz, 2 H). Anal. (capsule) ($C_6H_8N_2O_3$) C, H, N.

Ethyl 2-Tetrazol-5-yl-2-methoximinoacetate (11). A mixture of 9.52 g (61 mmol) of oxime **10** and 25.4 g (76 mmol) of Bu_3SnN_3 was stirred in an 80 °C oil bath for 4.8 h. After the mixture cooled to room temperature, 120 mL of Et_2O was added, and this solution was vigorously stirred with cooling in acetone–ice, while 16.9 mL of 5 N (84.5 mmol) of HCl was added. Removal of the cooling bath, vigorous stirring for a further 10 min, and addition of 160 mL of cyclohexane in a steady stream led to the deposition of crystals. The mixture was carefully evaporated to a volume of about 160 mL and a further 100 mL of cyclohexane added. The slurry was filtered, and the insolubles were thoroughly pressed and washed with 10-mL volumes of cyclohexane, pressing the filtercake each time, until the filtrate was absolutely clear (a total of 100 mL of cyclohexane was necessary). Washing with a further 20 mL of cyclohexane and air-drying gave 9.36 g (77%) of **11**, mp 109.7–111.5 °C. ^1H NMR (DMSO-d_6) δ: 1.22 (t, J = 7.2 Hz, 3 H), 4.08 (s, 3 H), 4.28 (q, J = 7.2 Hz, 2 H). Anal. ($C_6H_9N_5O_3$) C, H, N.

2-Tetrazol-5-yl-2-methoximinoacetic Acid (12). A suspension of 1.99 g (10 mmol) of ester **11** in 10 mL of H_2O was stirred in an ice bath and 4.5 mL of 5 N (22.5 mmol) NaOH was added over 2 min, leading to complete solution. The cold bath was removed and stirring continued for 1.7 h. Ice cooling was reinstigated, 4.7 mL of 5 N (23.5 mmol) HCl was added, and the resulting colorless solution (pH 1.2) was evaporated, under reduced pressure, to a white powder. This was stirred with 20 mL of acetone and filtered, and the insolubles were washed with four 5-mL volumes of acetone. The combined filtrate and washings were evaporated to give 1.85 g (99%) of **12** as an oil, which crystallized, mp 127.5–129.4 °C (dg), with previous softening at 90–95 °C, followed by rehardening until melting. Anal. ($C_4H_5N_5O_3 \cdot 0.9H_2O$) C, H, N.

DL-Tetrazol-5-ylglycine (5). A solution of 386 mg (2 mmol)

of oxime **9** in 15 mL of H_2O was hydrogenated over 96.5 mg of PtO_2 at room temperature and 60 psi for 16 h. The resulting mixture was heated to 50 °C for 5 min and filtered through talcum powder, three 8 mL rinses of H_2O, each at 50 °C, being used to wash the insolubles. The filtrate and washes were rotary evaporated (bath temperature 30 °C) to give a crystalline solid. The solid was dissolved in 6 mL of H_2O at 60 °C and filtered hot through a small cotton plug; this was followed by a hot 1 mL H_2O rinse. The filtered solution was swirled at 60 °C while 8.5 mL of 2-propanol was slowly added. The resulting turbid solution was stood at room temperature for 10 min (crystals), sonicated briefly, allowed to stand for 3 h at room temperature, refrigerated for 2 h, then filtered, and washed with H_2O/2-propanol 2:3 (3 mL) and air-dried to give 232 mg (72%) of **5** as white crystals, mp 127 °C (dg). ^{13}C NMR (D_2O) δ: 48.7, 153.6, 167.9. High-resolution FABMS (glycerol/oxalic acid): m/z 144.0529 (MH^+) (calcd for $C_3H_6N_5O_2$ 144.0522). Hydrogenation of **12** likewise gave **5**. Anal. $C_3H_5N_5O_2 \cdot 1.05H_2O$) C, H, N.

Biology. [^{3}H]MK-801 Binding. Rat brain cortices were extensively washed to remove endogenous glutamate and glycine. The cortices were homogenized in 20 volumes of ice-cold 5 mM Tris (pH 7.4) and centrifuged at 45000*g* for 10 min. The tissue pellet was washed two additional times with buffer. The resulting pellet was frozen on dry ice and thawed at room temperature. The thawed pellet was washed an additional three times with buffer. The final tissue pellet was frozen and stored at −80 °C. On the day of the assay, the pellet was thawed at room temperature and resuspended in 70 vol/g wet weight of original tissue in ice-cold 5 mM Tris (pH 7.4). Final protein concentrations for each assay were determined using the Lowry method.[13] [^{3}H]MK801 binding to well-washed rat cortical membranes was conducted in the presence of added glycine (0.2 μM). Competing compounds were dissolved in 5 mL of Tris (pH 7.4) and incubated with [^{3}H]MK801 (2.5 nM) and membrane aliquots (0.5–1.0 mg of protein/reaction) in a final volume of 1 mL at 27 °C for 2 h. Nonspecific [^{3}H]MK801 binding was determined in the presence of 0.5 μM MK801. Assays were terminated by filtration, using a Brandel cell harvester, over Whatman GF/B glass-fiber filters, presoaked in 0.05% polyethylenimine, followed by a 10-mL ice-cold saline wash. The values for half-maximal effective concentrations were determined from four experiments using a four parameter logistic equation (Graphpad Software, San Diego, CA). Data were analyzed for statistical significance by the Student's t-test when comparing two means. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Measurement of [^{3}H]Norepinephrine ([^{3}H]NE) from Superfused Rat Hippocampal Slices. Cross-chopped slices of hippocampus from Sprague-Dawley rats (250–300 g) were prelabeled by incubating (37 °C for 15 min) with [^{3}H]norepinephrine (0.1 μM final) in magnesium-free (oxygenated; 95% O_2, 5% CO_2) Krebs–Hensleit bicarbonate (pH 7.4) buffer containing 0.2 μM ascorbic acid and 1 μM clorgyline. Prelabeled slices were washed twice with fresh buffer and transferred to superfusion chambers (Brandel Superfusion System, Series B-18). Tissue was supported on Whatman 6F-B filter discs and superfused with magnesium-free Krebs–Hensleit buffer containing 1 μM desipramine at 37 °C (0.3 mL/min). After 90 min to obtain a steady base line, 5 min (1.5 mL) fractions were collected and counted using liquid scintillation spectrophotometry. After three base-line fractions were collected, drugs were added in fractions 4–7 and switched back to buffer for fractions 8–12. At the end of the experiment, the filter and the tissue were removed, solubilized, and counted. Percent release of [^{3}H]norepinephrine was calculated from the formula: (DPM ^{3}H in fraction(s)/total DPM in fraction(s) plus tissue) × 100. ED_{50} values from dose–response experiments were calculated using the median-effect plot of Chou and Talalay.[14]

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