Design, Synthesis, and Biological Evaluation of Spider Toxin (Argiotoxin-636) Analogs as NMDA Receptor Antagonists

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Purpose. Twelve synthetic spider toxin analogs were prepared in an effort to better understand the structure-activity relationships of the polyamine portion of argiotoxin-636 (Arg-636), a noncompetitive NMDA receptor (NMDAR) antagonist.

Methods. The 1,13-diamino-4,8-diazatridecane portion of the side chain of Arg-636 was systematically modified in an effort to further our knowledge of the structural requirements for the alkyl linker spacing between the amine nitrogens. Systematic isosteric replacement of each of the amine nitrogens in the polyamine moiety with either oxygen or carbon provided a series of compounds which were evaluated *in vitro* for NMDAR antagonist activity.

Results. One-half of the heteroatoms found in Arg-636 were removed to provide analogs which maintained *in vitro* potency below 1 μM. However, these simplified analogs produced similar or more pronounced effects on the cardiovascular system than Arg-636 *in vivo*.

Conclusions. In this set of analogs, a minimum of three basic nitrogens in the side chain was required for maximum potency as NMDAR antagonists. Isosteric nitrogen substitutions in the polyamine chain reduced the *in vitro* potency of these analogs. An analog binding-conformation model was proposed to rationalize the inactivity of these isosterically substituted analogs.

KEY WORDS: glutamate; NMDA; *N*-methyl-D-aspartate; Argiotoxin-636; Arg-636; polyamine spider toxins; Araxin™ compounds; ischemic stroke.

INTRODUCTION

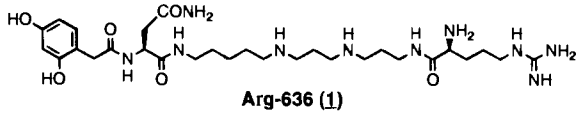
Spider venoms contain a wide variety of bioactive molecules such as proteins, peptides, and small organic compounds. Our interest in venom components has focused on the Araxin™ compounds (1–6), a class of small, highly polar, polycationic arylalkylpolyamine spider toxins, and their ability to selectively block glutamatergic synaptic transmission in the central nervous system (CNS) of vertebrates (7).

Natural products such as the spider toxin argiotoxin-636 (Arg-636, **1**, Figure 1) are of interest in pharmaceutical research because they are excellent probes for the investigation of neurological function and receptor operation. Hesse has recently provided a review concerning the synthesis and structure-activity relationships of synthetic spider and wasp toxin analogs (8). Compound **1** was first isolated from the orb-web spider, *Argiope lobata* (9) and subsequently identified in other spider venoms (10), and was termed Arg-636 based on its molecular weight (11).

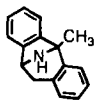
Glutamate, the major excitatory neurotransmitter in the mammalian CNS, acts on at least three subtypes of excitatory amino acid receptors classified pharmacologically as NMDA, AMPA, and kainate receptors, according to their preferred agonists (12–13). Compounds such as Arg-636 exhibit high selectivity for the NMDA subclass of glutamate receptors (1–3,7). It has been proposed that Arg-636 binds to a particular site on the NMDAR (3,14). This unique binding site is pharmacologically distinct from the well-studied polyamine binding site, the site at which polyamines such as spermine potentiate NMDAR function, and also distinct from the dizocilpine (MK-801)/phencyclidine (PCP) binding site (15).

Glutamate receptors have been implicated in the physiology and pathophysiology of various neurological functions and disorders such as ischemic stroke, epilepsy, pain, and neurodegenerative disorders such as Parkinson's disease. Activation of glutamate receptors in an episode of ischemia appears to contribute importantly to the pathological outcome (16). It has been postulated that the NMDAR may play a key role in mediating neuronal damage probably due to its high permeability to calcium, a known mediator of cell damage (17–18).

In animal models of focal ischemia, NMDAR antagonists provide more dramatic and consistent cerebroprotection than any other class of compounds (19). Our interest in exploring the arylalkylpolyamine spider toxins lies in their potent NMDAR antagonist properties coupled with their low affinity for the MK-801/PCP binding site (Figure 1). High affinity at the MK-801/PCP site may be associated with the undesirable psychotomimetic activity of PCP and related compounds seen in humans (19).



Arg-636 (1)



MK-801

	NMDA receptor antagonist potency IC_{50} RCGC (μM)	Inhibition of [^{3}H]-MK-801 binding (μM)
Arg-636 (1)	0.100 ± 0.024 (13)	126 ± 9 (4)
MK-801	0.005 ± 0.008 (4)	0.005 ± 0.001 (6)

Fig. 1. *In vitro* NMDA Receptor Activities of Arg-636 (*1*) and MK-801.

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DESIGN RATIONALE

Several problems exist in developing arylalkylpolyamines such as Arg-636 (**1**) for clinical use, such as polyamine-associated effects on the cardiovascular system (*i.e.,* hypotension and bradycardia) (2–3). Several theories can be presented to explain this problem [*e.g.,* nitric oxide synthase-mediated mechanism (20)]; however, no exact mechanism(s) for these cardiovascular effects has been demonstrated unequivocally.

Another problem with arylalkylpolyamines is their chemical complexity. Extraction of these natural products from arachnid sources in quantities necessary for use as drug products would be impractical. Synthesis of compounds such as Arg-636 involves multistep reaction sequences, and therefore they may be quite difficult to produce on a commercial scale.

The arylalkylpolyamines contain numerous charged residues which may decrease their ability to effectively penetrate the blood-brain-barrier (BBB) and lead to only limited absorption after oral administration. Somewhat surprisingly, it has been demonstrated that certain arylalkylpolyamines cross the BBB and produce CNS effects when administered systemically (2–3). It is possible that these compounds enter the CNS directly via "breakdown" of the BBB or that these compounds are substrates for active polyamine transport mechanisms. The ability of different polyamines to be substrates for such active transport mechanisms greatly increases the complexity of any drug design strategy.

Our design strategy focused on decreasing the polarity of the spider toxins, to simplify their chemical and structural complexity and synthesis, and to minimize their associated cardiovascular side effects.

Previous results from our laboratories indicate that the 2,4-dihydroxyphenylacetamide moiety of Arg-636 is bioisosterically equivalent to the more synthetically accessible and less polar 2-methoxyphenylacetamide group (1–2). We have demonstrated that the internal amino acid residue (D-asparagine) could be replaced with D-phenylalanine to increase lipophilicity without significantly decreasing *in vitro* NMDAR antagonist potency. Structural modifications of the terminal aromatic ring and the internal amino acid residue provide only modest change in the overall polarity. The basic nitrogens were then systematically removed in order to produce a more dramatic effect on the overall polarity of the structure.

Several questions were addressed by pharmacologically evaluating the target compounds. How many basic nitrogens are required for potent NMDAR antagonism? How does the spatial positioning of these nitrogens (methylene spacer length) alter potency? Can any of the basic nitrogens be replaced with an isosteric functionality and still maintain high receptor affinity? These manipulations, if successful, would theoretically reduce analog polarity, enhance CNS penetration, and retain an acceptable level of *in vitro* activity.

MATERIALS AND METHODS

Synthetic Chemistry

Apparatus

^{1}H-Nuclear magnetic resonance (NMR) spectra were acquired on a Varian Gemini 300 MHz spectrometer. Capillary GC and low-resolution MS data were obtained using a Hewlett-Packard 5890 Series II Gas Chromatograph coupled to an HP 5971 Series Mass Selective Detector [Ultra-2 Ultra Performance Capillary Column (crosslinked 5% PhMe silicone)]. High-resolution MS data were acquired on a VG Micromass 7050E Double-Focusing High-Resolution Mass Spectrometer, Shrader System GC/MS Data Processing Software (Shrader Analytical and Consulting Laboratories). Analtech Uniplate™ 250-μM silica gel HF TLC plates were used. UV light, ninhydrin, and Dragendorff's spray reagents were used for detecting TLC spots. All compounds tested pharmacologically were purified on a Rainin HPLC system [C_{18}-column, Vydac™ RP-C18 218TP54, 4.6 × 250 mm; particle size, 300 Å; flow rate, 1 mL/min; detector wavelength, 270 nm; gradient elution: 0.1% aq. TFA to 0.1% TFA in acetonitrile, 0–50% over 50 min]. Melting points were determined in open capillary tubes on a Mel-Temp II apparatus. Reagents used were purchased from the Aldrich Chemical Co., Milwaukee, WI; catalysts from the Fluka Chemical Corp., Ronkonkoma, NY; and amino acid analogs from Bachem Bioscience, Inc., Philadelphia, Pennsylvania.

Chemicals (Representative Analog Synthesis)

*N^1-Benzyl-N^5-(tert-butoxycarbonyl)-1,5-diaminopentane (**3**).* To a solution of *N-tert*-butoxycarbonyl-1,5-diaminopentane (21) (9.00 g) in MeOH (100 mL) was added anh. $MgSO_4$ (10.8 g) followed by benzaldehyde (5.20 g). The reaction was stirred for 3 h. $NaBH_4$ (1.87 g) was then added to the solution over a 10-min period. The reaction was subsequently refluxed for 19 h. HOAc (15 mL) was then added and the mixture was stirred for 15 min. The reaction mixture was filtered, the filtrate was rotary evaporated, and the residue was dissolved in $CHCl_3$ (300 mL) and 1.1*N* KOH (220 mL). The organic layer was separated and the aqueous layer was extracted with $CHCl_3$ (200 mL). The combined organic layers were dried (Na_2SO_4) and evaporated yielding 10.3 g which was chromatographed (silica gel, 1:20 MeOH/CH_2Cl_2, 1:10 MeOH/CH_2Cl_2, and MeOH) providing 7.32 g (56.3%) of **3**.

N^1, N^4-Dibenzyl-N^9-(tert-butoxycarbonyl)-4-aza-1,9-diaminononane (4). To a solution of *3* (7.32 g) in acetonitrile (ACN, 100 mL) was added 50% KF/Celite® (5.90 g) followed by *N*-(3-bromopropyl)phthalimide (6.71 g). The reaction was refluxed for 15 h, then cooled to room temperature, filtered through sintered glass, and the filtrate was rotary evaporated. The residue was dissolved in $CHCl_3$ (100 mL) and washed with 0.1*N* NaOH/satd. NaCl. The aqueous layer was back-extracted with $CHCl_3$ (50 mL). The combined organic layers were dried (Na_2SO_4) and evaporated giving 13.5 g. The crude product was chromatographed (silica gel, 3:1 hexane/EtOAc, 1:1 hexane/EtOAc, and EtOAc) yielding 9.98 g (8.31%) of intermediate, TLC (3:1 hexane/EtOAc), R_f 0.17.

To a solution of N^1-benzyl-N^5-(*tert*-butoxycarbonyl)-N^1-(3-phthaloylpropyl)-1,5-diaminopentane (9.95 g) in MeOH (100 mL) was added anh. hydrazine (3.3 mL). The reaction was heated at reflux for 15 h. The mixture was then rotary evaporated and the residue was dissolved in Et_2O (300 mL)/0.1*N* NaOH (100 mL). The ether layer was washed with 0.1*N* NaOH (2 × 100 mL), dried (Na_2SO_4), and evaporated to provide 6.20 g (85.5%).

N^4-Benzyl-N^9-(tert-butoxycarbonyl)-4-aza-1,9-diaminononane (4.95 g) was reductively alkylated (*vide supra*) using benzaldehyde/$NaBH_4$ to provide, after chromatography through

silica gel (1:20 MeOH/CH_2Cl_2, 1:10 MeOH/CH_2Cl_2, and finally MeOH), 3.98 g (63.9%) of **4**.

*N^6, N^{10}-Dibenzyl-N^1-(tert-butoxycarbonyl)-6, 10-diaza-1, 16-diaminohexadecane (**5**).* Following the procedure used for the preparation of N^1-benzyl-N^5-(*tert*-butoxycarbonyl)-N^1-(3-phthaloylpropyl)-1,5-diaminopentane, a solution of *4* (0.57 g) was alkylated with *N*-(6-bromohexyl)-phthalimide to provide 0.98 g which was chromatographed (silica gel, 1:20 MeOH/CH_2Cl_2, 1:10 MeOH/CH_2Cl_2) yielding 0.57 g (65%), TLC (1:10 MeOH/CH_2Cl_2), R_f 0.57.

The above phthalimide was reacted with hydrazine (*vide supra*) providing 0.39 g (90%).

*N^6-$N^1$0-Dibenzyl-N^{16}-benzyloxycarbonyl-6, 10-diaza-1, 16-diaminohexadecane (**6**).* A solution of benzyl chloroformate (0.11 g) in $CHCl_3$ (15 mL) was added dropwise over a 10-min period to a solution of *5* (0.36 g) and triethylamine (0.2 mL) in $CHCl_3$ (15 mL). The reaction was stirred for 69 h. The reaction mixture was then washed with H_2O (15 mL), dried (Na_2SO_4), and evaporated affording 0.46 g. The crude product was chromatographed [silica gel, 1:10 MeOH/CH_2Cl_2/0.5% isopropylamine (IPA)] providing 0.39 g (87%), TLC (1:10 MeOH/CH_2Cl_2), R_f 0.47.

To a solution of N^6, N^{10}-dibenzyl-N^{16}-benzyloxycarbonyl-N^1-(*tert*-butoxycarbonyl)-6,10-diaza-1,16-diaminohexadecane (0.37 g) in CH_2Cl_2 (10 mL) was added a solution of trifluoroacetic acid (TFA, 3 mL) in CH_2Cl_2 (3 mL). The reaction was stirred at room temperature for 2 h and the volatiles were subsequently rotary evaporated. The residue was dissolved in $CHCl_3$ (30 mL), washed with 1*N* NaOH (10 mL), dried (Na_2SO_4) and rotary evaporated yielding 0.31 g (98%) of compound **6**.

*N^6, N^{10}-Dibenzyl-N^{16}-benzyloxycarbonyl-N^1-(L-phenylalanine)-6,10-diaza-1,16-diaminohexadecane (**7**).* A solution of *6* (0.31 g) and *N*-Boc-L-phenylalanine-*p*-nitrophenyl ester (0.21 g) in CH_2Cl_2 (20 mL) was stirred for 16 h. The solvent was then rotary evaporated. The residue was dissolved in Et_2O (50 mL) and washed with 0.1*N* NaOH (10 × 10 mL). The combined organic layers were dried (Na_2SO_4) and evaporated affording an oil which was chromatographed (silica gel, 1:10 MeOH/CH_2Cl_2/0.5% IPA) yielding 0.42 g (95%), TLC (1:10 MeOH/CH_2Cl_2); R_f 0.47.

The Boc protecting group of N^6, N^{10}-dibenzyl-N^{16}-benzyloxycarbonyl-N^1-[*N*-(*tert*-butoxycarbonyl)-L-phenylalanine]-6,10-diaza-1,16-diaminohexadecane (0.41 g) was removed as above with TFA to yield 0.35 g (97%) of compound **7**.

*N^1-[N-(2-Methoxyphenylacetamido)-L-phenylalanine]-6,10-diaza-1,16-diaminohexadecane tris(trifluoroacetate) (**8**)* To a solution of **7** (0.35 g) in CH_2Cl_2 (15 mL) was added a solution of *p*-nitrophenyl-2-methoxyphenylacetate (0.14 g). The reaction was stirred overnight and the solvent was subsequently rotary evaporated. The residue was dissolved in Et_2O (50 mL), washed with 0.1*N* NaOH (10 × 10 mL), dried (Na_2SO_4), evaporated, and chromatographed (silica gel, 1:10 MeOH/CH_2Cl_2/0.5% IPA) yielding 0.28 g (66%) of purified product, TLC (1:10 MeOH/CH_2Cl_2), R_f 0.43.

Pearlman's catalyst (0.10 g) was added to a solution of N^6, N^{10}-dibenzyl-N^{16} -benzyloxycarbonyl-N^1-[*N*-(2-methoxyphenylacetamido)-L-phenylalanine]-6,10-diaza-1, 16-diaminohexadecane (0.28 g) in HOAc (50 mL), and was shaken for 20 h under 60 psig H_2 at 25°C on a Parr® Apparatus. The reaction mixture was then filtered through fritted glass/Celite® and the filtrate was rotary evaporated. TFA was added (3 × 20 mL) and evaporated to convert the product to its trifluoroacetate salt (0.55 g). The product was purified by C_{18}-HPLC (0.1% TFA) in H_2O to 0.1% TFA in ACN) yielding 70 mg (24%) of compound **8** as a colorless, crystalline solid.

Molecular Modeling Studies

Computer-aided molecular modeling studies assisted in comparing the structural similarities of Arg-636 (*1*) with compound **8**.

Compound **8** was subjected to simulated annealing to find a possible low-energy conformation. Compound *8* was minimized using steepest-descents minimization and the resulting extended conformation was subjected to molecular-dynamics simulated annealing: temp (time), 1000 K (10 ps), 800 K (15 ps), 600 K (15 ps), 400 K (20 ps), 300 K (30 ps), and then minimized (steepest descents, < 0.001 kcal/mol-Å). The structure shown in Figure 5 represents one possible, low-energy conformation of compound **8**. Interestingly, it was found that two intramolecular hydrogen-bonds, between the carbonyl oxygens and the intrachain nitrogens, were maintained throughout the molecular dynamics simulation at 300 K (Figure 5).

The conformation of Arg-636 (**1**) shown in Figure 5 was achieved by forcing its backbone atoms onto the low-energy conformation of compound **8**. This manipulation allows one to view the overall structural similarities of the various functional groups of Arg-636 with one possible potentially biologically active conformation of analog **8**.

Calculations were performed on a Silicon Graphics Indigo^2 workstation running IRIX 5.3. Molecular mechanics and molecular dynamics calculations used Discover v. 2.95 (Biosym) and InsightII v. 235 (Biosym Technologies, Inc.).

Biology

Preparation of Rat Cerebellar Granule Cell (RCGC) Cultures and Measurement of Cytosolic Ca^{2+}

Following a method of Parks *et al.* (22), primary cultures of RCGCs were prepared and the cultures were incubated with fura-2/acetoxymethylester. Fluorescent signals were calibrated by adding ionomycin to obtain F_{max} and EGTA to obtain F_{min}. A combination of NMDA (50 μM) and glycine (1 μM) was used as the stimulus. Multiple cumulative concentration-response curves were performed for each antagonist tested and IC_{50} values were determined by logit analysis. Four to five different concentrations of each antagonist were used in the determination of each IC_{50}.

[^{3}H]-MK-801 Binding

Following a characterized procedure (23), cerebral cortex and hippocampus were harvested from male Sprague-Dawley rats, homogenized, and purified. MK-801 binding assays were carried out by incubating prepared membranes for 2–3 h with [^{3}H]-MK-801 (5 nM, DuPont NEN, Boston, MA), glycine (100 μM), L-glutamic acid (100 μM), and varying concentrations of antagonist. Non-specific binding was determined by the inclusion of 10 μM MK-801. The assay was terminated by the addition of ice-cold buffer, followed by filtration over glass-fiber filters. The filters were washed with ice-cold buffer and

radioactivity on the filters was measured by liquid scintillation (Beckman 6000IC LS counter). Protein determination was accomplished as described by Lowry *et al.* (24). The data were then analyzed and IC_{50} values were determined.

Measurement of Effects on Blood Pressure and Heart Rate

Adult male Wistar rats (Harlan Sprague Dawley, Indianapolis, IN), weighing 250–350 g, were anesthetized with intraperitoneal pentobarbital (64.8 mg/kg). Polyethylene (PE-50) catheters were inserted into the left femoral artery (arterial pressure) and the left femoral vein. The trachea was cannulated with PE-240 tubing. The arterial catheter was connected to a Gould-Statham transducer coupled to a physiological recorder (Grass 720). Mean arterial pressures (MAP) were monitored continuously and heart rates (HR) were determined from the arterial pulsations. The core body temperature of each rat was maintained at 37°C.

Experimental compounds, dissolved in saline solution, were administered through the femoral venous catheter at a starting bolus dose of 1.5 mg/kg in 1 mL/kg of solution. MAP and HR were monitored before and after the administration of each test substance. If no changes in MAP or HR occurred within 15 min after administration of the test compound, a second dose doubling the initial dose was administered. Changes in MAP and HR were observed up to 120 min post-injection.

All experimental protocols involving the use of laboratory animals were in compliance with U.S. Federal government regulations and NIH guidelines.

RESULTS

Synthesis

The synthesis of Arg-636 (**1**) has been reported using standard chemistry methods (25–29); this area has recently been reviewed by Hesse (8). The following reaction scheme is representative of the syntheses used in preparing the analogs reported in this study.

1,5-Diaminopentane (**2**) was treated with Boc_2O to give the mono-*tert*-butoxycarbonyl-protected diamine (21). The primary amine of *2* was benzylated by reductive amination (benzaldehyde/$NaBH_4$) to provide N^1-benzyl-N^5-Boc-1,5-diaminopentane (**3**). The secondary amine of *3* was alkylated with bromopropylphthalimide in the presence of KF/Celite®. The phthalimide group was removed by treatment with hydrazine giving N^4-benzyl-N^9-Boc-1,9-diamino-4-azanonane, which was then benzylated (*vide supra*) to afford N^1,N^4-dibenzyl-N^9-Boc-1,9-diamino-4-azanonane (**4**). This material was alkylated with bromohexylphthalimide, and the phthalimide group was subsequently removed with hydrazine providing N^1-Boc-N^6,N^{10}-dibenzyl-1,16-diamino-6,10-diazahexadecane (**5**). The primary amine of this compound was protected with a benzyloxycarbonyl (Cbz) group, and the Boc group was cleaved with TFA yielding N^6,N^{10}-dibenzyl-N^{16}-Cbz-1,16-diamino-6,10-diazahexadecane (**6**). The primary amine of *6* was reacted with the ρ-nitrophenyl ester of *N*-Boc-L-phenylalanine to form the amide bond. The Boc group was removed with TFA to provide compound **7**. The primary amine of **7** was reacted with the ρ-nitrophenyl-2-methoxyphenylacetate to afford N^1-(*N*-[2-methoxyphenylacetamido]- N^6,N^{10}-dibenzyl-N^{16}-Cbz-L-phenylalanine)-1,16-diamino-6,10-diazahexadecane. This compound was then fully deprotected by catalytic hydrogenation over Pearlman's catalyst providing target compound **8**.

Compounds **9–17** were prepared in a similar manner. Oxygenated target molecule **19** was synthesized in a sequence starting from the reaction of Boc-protected-5-amino-1-pentanol with acrylonitrile. The nitrile functionality in the resulting Michael addition product was reduced to provide the mono-Boc-protected diamino ether, which was subsequently transformed above to provide compound **19.** Target molecule **18** was also produced in a similar manner using a Michael addition of Boc-protected aminopropanol to acrylonitrile.

The purities of all of the compounds pharmacologically assayed were determined by analytical C_{18}-HPLC (Table 2). Characterization of their chemical structures was based on their ^{1}H-NMR spectral data (Table 3) and the chemical properties of each intermediate in their synthesis. Final products were analyzed by high-resolution MS (exact mass) and were consistent with their proposed structures (Table 2).

Biological Activities

NMDAR Antagonist Activity

Analogs were assayed for functional NMDAR antagonist activity and potency in a [^{3}H]-MK-801 binding assay using published methods (22–23). The results from these assays are shown (Figures 2–4). Arg-636 (**1**) inhibited NMDA/glycine induced increases in intracellular calcium with an IC_{50} of 0.10 μM, and also inhibited MK-801, binding but at three orders of magnitude higher concentrations (IC_{50} = 126 μM). Conversely, MK-801 was found to be equally efficacious with an IC_{50} of 0.005 μM in both assays, suggesting a different site of interaction on the NMDA receptor.

Structures containing three basic nitrogens in the "tail" portion (*i.e.,* compounds **8**, **9**, and **13–15**) all had IC_{50} values around 1 μM as functional NMDAR antagonists (Figures 3–4). Compounds **10–12** and **16–19** were significantly less potent than Arg-636 (Figures 2 and 4). Isosteric replacement analogs (*i.e.,* compounds **16–19**) were significantly less potent than their corresponding triamine analogs (Figures 3 and 4).

Cardiovascular Effects

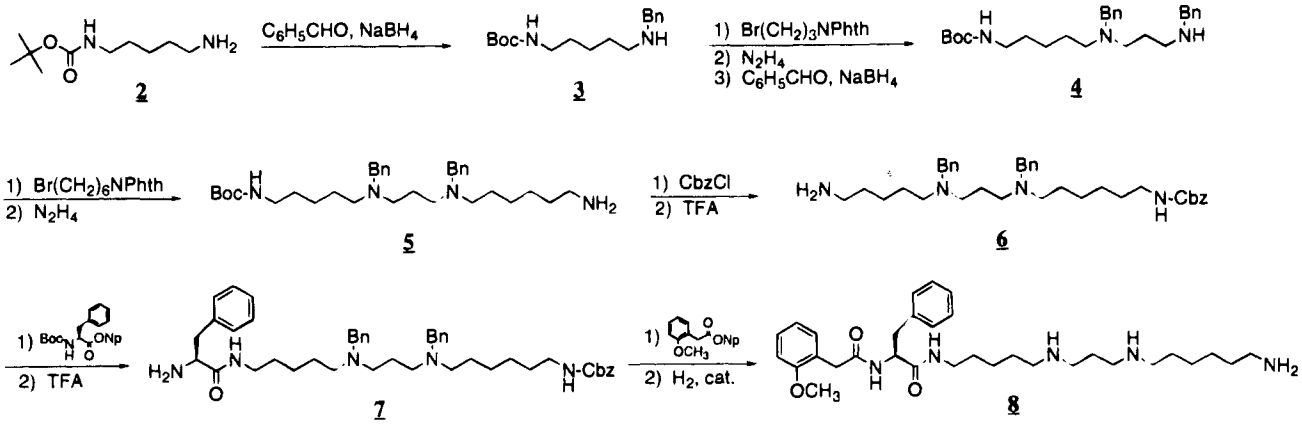
The analog with the highest *in vitro* potency (compound **8**) exhibited significant cardiovascular effects in rats (Table 1) with lethality at doses of 1.5 mg/kg. These cardiovascular problems were significantly more pronounced than those observed with Arg-636 (**1**) and compound **13**.

DISCUSSION

Importance of Nitrogen Spacing

The natural product, Arg-636 (*1*), contains a 5-3-3 methylene-nitrogen arrangement in its polyamine "tail" bonded to a terminal arginine residue. The terminal amino group of compound 8 was positioned at the end of a six-methylene linker to mimic the spatial positioning of the basic α-amino group of the terminal arginine moiety in Arg-636 (see Figure 5). In

Reaction Scheme. Synthesis of compound **8** (representative analog synthesis).



Bn = benzyl; Boc = *tert*-butoxycarbonyl; Cbz = benzyloxycarbonyl; NPhth = phthalimide; ONp = *p*-nitrophenyl ester; TFA = trifluoroacetic acid; and cat. = $Pd(OH)_2/C$.

Table 1. Physiological Effects of **1**, **13**, and **8** on Rat Mean Arterial Blood Pressure (MAP) and Heart Rate (HR).

Compound	Dose (mg/kg i.v.)	MAP (% decrease)	HR (% decrease)	Other observations
Arg-636 (**1**)	1.6	14–30	18–19	30–40 min duration
13	1.5–3	29–48	28–32	dyspnea at 3 mg/kg
8	1.5	56–62	18–42	convulsions, death

compound **9** (a 5-3-3 nitrogen side-chain analog) the terminal arginine group Arg-636 has been removed and yet retains *in vitro* NMDAR antagonist activity (IC_{50} = 1.5 μM; Figure 3). Therefore, for *in vitro* receptor interaction the guanidino functionality is not specifically required. Compound **8** (containing a 5-3-6 side-chain) was shown to be approximately three times as potent as its corresponding 5-3-3 analog (compound **9**). These results support our hypothesis that the terminal amino moiety in compound *8* may be spatially mimicking the α-amino group of the arginine residue in Arg-636 (**1**; Figure 5).

Effects of Varying the Number of Nitrogens in the "Tail" Moiety

Three compounds (**10–12**) were synthesized to explore the effect of varying the number of "tail" nitrogens required to maintain *in vitro* potency. Systematic truncation of the "tail" from the terminal amine nitrogen of compound **9** dramatically reduced *in vitro* NMDA antagonism (Figure 2). These results suggest that the three basic nitrogens in the "tail" moiety of compound **9** are required for micromolar activity. A structure-

Table 2. Physical Data of Compounds Prepared for Pharmacological Testing

Compound	Molecular Formula	Exact Mass (Calculated)	Exact Mass (Observed)	Mp (°C)	RP-HPLC[a] t_R, min (% purity)
1	Arg-636	—	—	—	14.0 (94.0)
8	$C_{32}H_{51}N_5O_3 \cdot 3CF_3CO_2H$	554.40702	554.40353	164–166	31.1 (96.5)
9	$C_{29}H_{45}N_5O_3 \cdot 3CF_3CO_2H$	512.36006	512.35993	198–200	31.6 (100.0)
10	$C_{23}H_{31}N_3O_3 \cdot CF_3CO_2H$	398.24437	398.24293	58–65	33.3 (99.6)
11	$C_{26}H_{38}N_4O_3 \cdot 2CF_3CO_2H$	455.30222	455.30064	111–113	31.8 (99.9)
12	$C_{27}H_{39}N_3O_3 \cdot CF_3CO_2H$	454.30697	454.30560	semisolid	40.2 (96.0)
13	$C_{29}H_{45}N_5O_3 \cdot 3CF_3CO_2H$	512.36005	512.35817	179–180	30.4 (99.7)
14	$C_{27}H_{41}N_5O_3 \cdot 3CF_3CO_2H$	484.32876	484.32876	158–161	30.0 (98.5)
15	$C_{31}H_{48}N_6O_4 \cdot 3CF_3CO_2H$	569.38153	569.38405	169–170	30.8 (100.0)
16	$C_{32}H_{49}N_5O_4 \cdot 2CF_3CO_2H$	568.38628	568.38460	46–50	34.4 (100.0)
17	$C_{32}H_{49}N_5O_4 \cdot 2CF_3CO_2H$	568.38628	568.38245	semisolid	37.5 (97.4)
18	$C_{29}H_{44}N_4O_4 \cdot 2CF_3CO_2H$	513.34408	513.34034	semisolid	32.1 (99.3)
19	$C_{29}H_{44}N_4O_4 \cdot 2CF_3CO_2H$	513.34408	513.34136	semisolid	34.1 (97.6)

[a] Trifluoroacetic acid (TFA) salts of the above compounds were purified by reversed-phase C_{18}-HPLC and then analyzed for chromatographic purity using the following conditions: column, Vydac™ RP-C18 218TP54, 4.6 × 250 mm; particle size, 300 Å; flow rate, 1 mL/min; detector wavelength, 270 nm; 20 μg of each test substance (dissolved in H_2O) were applied to the column; gradient elution: 0.1% aq. TFA to 0.1% TFA in acetonitrile, 0–50% over 50 min.

Table 3. 300 MHz ^{1}H-NMR Data of Compounds in Reaction Scheme

N^1-Benzyl-N^5-(*tert*-butoxycarbonyl)-1,5-diaminopentane (**3**); δ 1.31–1.56 [15H, m, Boc + -CH_2-(CH_2)$_3$-CH_2-], 2.12 (1H, bs, -NH-benzyl), 2.63 (2H, t, -CH_2-NH-benzyl), 3.11 (2H, dt, Boc-NH-CH_2-), 3.79 (2H, s, -CH_2-phenyl), 4.60 (1H, bs, Boc-NH-), and 7.25–7.40 (5H, m, aromatic).

N^1-benzyl-N^5-(*tert*-butoxycarbonyl)-N^1-(3-phthaloylpropyl)-1,5-diaminopentane; δ 1.27–1.35 [2H, m, -(CH_2)$_2$-CH_2-(CH_2)$_2$-], 1.41–1.53 (13H, m, Boc + -CH_2-CH_2-CH_2-CH_2-CH_2-), 1.83 (2H, tt,-N-CH_2-CH_2-CH_2-NPhth), 2.41 (2H, t, -CH_2-N-benzyl), 2.47 (2H, t, -CH_2-N-benzyl), 3.09 (2H, dt, Boc-NH-CH_2), 3.52 (2H, s, -CH_2-phenyl), 3.69 (2H, t, -CH_2-NPhth), 4.65 (1H, bs, Boc-NH-), 7.21–7.32 (5H, m, benzyl aromatic), and 7.69–7.72 + 7.82–7.85 (4H, 2m, phthalimide).

N^4-Benzyl-N^9-(*tert*-butoxycarbonyl)-4-aza-1,9-diaminononane; δ 1.26–1.32 [2H, m, -(CH_2)$_2$-CH_2-(CH_2)$_2$-], 1.38–1.50 (13H, m, Boc + -CH_2-CH_2-CH_2-CH_2-CH_2-), 1.59 (2H, tt, -N-CH_2-CH_2-CH_2-NH_2), 1.89 (2H, bs, -NH_2), 2.39 (2H, t, -CH_2-N-benzyl), 2.43 (2H, t, -CH_2-N-benzyl), 2.68 (2H, t, -CH_2-NH_2), 3.07 (2H, dt, Boc-NH-CH_2-), 3.51 (2H, s, -CH_2-phenyl), 4.65 (1H, bs, Boc-NH-), and 7.21–7.32 (5H, m, benzyl aromatic).

N^1, N^4-Dibenzyl-N^9-(*tert*-butoxycarbonyl)-4-aza-1,9-diaminononane (**4**); δ 1.22–1.31 [2H, m, -(CH_2)$_2$-CH_2-(CH_2)$_2$-], 1.36–1.54 (13H, m, Boc + -CH_2-CH_2-CH_2-CH_2-CH_2-), 1.69 (2H, tt, -N-CH_2-CH_2-CH_2-NH-), 2.12 (1H, bs, -NH-benzyl), 2.38 (2H, t, -CH_2-N-benzyl), 2.45 (2H, t, -CH_2-N-benzyl), 2.65 (2H, t, -CH_2-NH-benzyl), 3.07 (2H, dt, Boc-NH-CH_2-), 3.51 (2H, s, -N-CH_2-phenyl), 3.75 (2H, s, -NH-CH_2-phenyl), 4.58 (1H, bs, Boc-NH-), and 7.20–7.40 (10H, m, aromatic).

N^1, N^4-Dibenzyl-N^9-(*tert*-butoxycarbonyl)-N^1-(6-phthaloylhexyl)-4-aza-1,9-diaminononane; δ 1.22–1.34 [6H, m, -NH-(CH_2)$_2$-CH_2-(CH_2)$_2$-N- + -N-(CH_2)$_2$-(CH_2)$_2$-(CH_2)$_2$-NPhth], 1.38–1.52 (15H, m, Boc + -NH-CH_2-CH_2-CH_2-CH_2-CH_2-N- + -N-CH_2-CH_2-(CH_2)$_4$-NPhth), 1.59–1.72 (4H, m, -N-CH_2-CH_2-CH_2-N- + -CH_2-CH_2-NPhth), 2.35–2.44 (8H, m, -CH_2-N-benzyl), 3.07 (2H, dt, Boc-NH-CH_2-), 3.52 (4H, s, -CH_2-phenyl), 3.65 (2H, t, -CH_2-NPhth), 4.53 (1H, bs, Boc-NH-), 7.18–7.36 (10H, m, benzyl aromatic), and 7.69–7.72 + 7.82–7.85 (4H, 2m, phthalimide).

N^6, N^{10}-Dibenzyl-N^1-(*tert*-butoxycarbonyl)-6,10-diaza-1,16-diaminohexadecane (**5**); δ 1.21–1.33 (6H, m, -NH-(CH_2)$_2$-CH_2-(CH_2)$_2$-N- + -N-(CH_2)$_2$-(CH_2)$_2$-(CH_2)$_2$-NH_2), 1.38–1.53 (17H, m, Boc + -NH-CH_2-CH_2-CH_2-CH_2-CH_2-N- + -N-CH_2-CH_2-(CH_2)$_2$-CH_2-CH_2-NH_2), 1.59–1.70 (2H, m, -N-CH_2-CH_2-CH_2-N-), 2.31–2.48 (8H, m, -CH_2-N-benzyl), 2.65 (2H, t, -CH_2-NH_2), 3.07 (2H, dt, Boc-NH-CH_2-), 3.51 (4H, s, -CH_2-phenyl), 4.58 (1H, bs, Boc-NH-), and 7.19–7.38 (10H, m, aromatic).

N^6, N^{10}-dibenzyl-N^{16}-benzyloxycarbonyl-N^1-(*tert*-butoxycarbonyl)-6,10-diaza-1,16-diaminohexadecane; δ 1.19–1.30 [6H, m, -NH-(CH_2)$_2$-CH_2-(CH_2)$_2$-N- + -N-(CH_2)$_2$-(CH_2)$_2$-(CH_2)$_2$-NH-Cbz], 1.37–1.54 [17H, m, Boc + -NH-CH_2-CH_2-CH_2-CH_2-CH_2-N- + -N-CH_2-CH_2-(CH_2)$_2$-CH_2-NH-Cbz], 1.59–1.67 (2H, m, -N-CH_2-CH_2-CH_2-N-), 2.33–2.47 (8H, m, -CH_2-N-benzyl), 3.07 (2H, dt, Boc-NH-CH_2-), 3.15 (2H, t, CH_2-NH-Cbz), 3.51 (4H, s, -N-CH_2-phenyl), 4.51 (1H, bs, Boc-NH-), 4.79 (1H, bs, -NH-Cbz), 5.09 (2H, s, -CO-O-CH_2-phenyl), and 7.21–7.44 (15H, m, aromatic).

N^6, N^{10}-dibenzyl-N^{16}-benzyloxycarbonyl-N^1-[*N*-(*tert*-butoxycarbonyl)-L-phenylalanine]-6,10-diaza-1,16-diaminohexadecane; δ 1.08–1.16 + 1.18–1.33 + 1.36–1.55 [23H, 3m, Boc + -NH-CH_2-(CH_2)$_3$-CH_2-N- + -N-CH_2-(CH_2)$_4$-CH_2-NH-], 1.59–1.68 (2H, m, -N-CH_2-CH_2-CH_2-N-), 2.30–2.48 (8H, m, -CH_2-N-benzyl), 2.99–3.22 (6H, m, phenyl-CH_2-CH- + -CH-CO-NH-CH_2- + -CH_2-NH-Cbz), 3.51 (4H, s, -N-CH_2-phenyl), 4.26 (1H, bs, -NH-CH-CO-NH-), 4.87 (1H, bs, -NH-Cbz), 5.09 (2H, s, -CO-O-CH_2-phenyl), 5.16 (1H, bs, CH-CO-NH-), 5.69 (1H, bs, Boc-NH-), and 7.17–7.49 (20H, m, aromatic).

N^1-[*N*-(2-Methoxyphenylacetamido)-L-phenylalanine]-6,10-diaza-1,16-diaminohexadecane tris(trifluoroacetate) (**8**); δ 1.04–1.13 + 1.19–1.28 + 1.35–1.58 [14H, 3m, -NH-CH_2-(CH_2)$_3$-CH_2-N-+ -N-CH_2-(CH_2)$_4$-CH_2-NH-], 1.59–1.68 (2H, m, -N-CH_2-CH_2-CH_2-N-), 2.31–2.47 (8H, m, -CH_2-N-benzyl), 2.97 (2H, m, phenyl-CH_2-CH-), 3.05 (2H, m, -CH-CO-NH-CH_2-), 3.15 (2H, m, -CH_2-NH-Cbz), 3.52 (4H, s, -N-CH_2-phenyl), 3.54 (2H, s, 2-methoxyphenyl-CH_2-), 3.71 (3H, s, -O-CH_3), 4.53 (1H, dt, -NH-CH-CO-NH-), 4.85 (1H, bs, -NH-Cbz), 5.09 (2H, s, -CO-O-CH_2-phenyl), 5.77 (1H, bs, CH-CO-NH-), 6.42 (1H, bs, 2-methoxyphenyl-CH_2-CO-NH-), 6.80–7.03 (4H, m, 2-methoxyphenyl aromatic), and 7.13–7.36 (20H, m, phenyl aromatic).

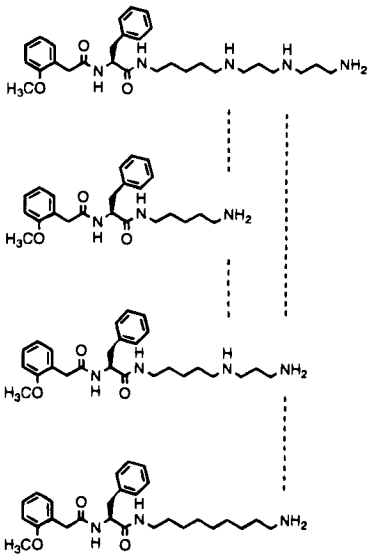
activity relationship (SAR) study of a series of philanthotoxin (PhTX) analogs reported in the literature (30) suggests that a minimum of three basic nitrogens (PhTX analogs) are required for NMDAR antagonist activity.

Carbon-for-Nitrogen Substitution

Two compounds (**16** and **17**) were synthesized in an effort to explore the effect of substituting carbon for nitrogen in the polyamine "tail" portion, using compound **15** for comparison. The results suggest that replacing either of the intrachain nitrogens in the "tail" moiety of **15** with methylenes dramatically reduces NMDAR antagonist potency (Figure 4).

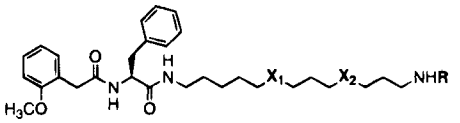
Oxygen-for-Nitrogen Substitution

Compounds **18** and **19** were synthesized to determine if oxygen could be substituted for nitrogen in the polyamine "tail". Oxygen substitution, we believed, would be a more compatible replacement than the more lipophilic carbon substitution discussed above. The oxygenated analogs which were synthesized (*i.e.*, compounds **18** and **19**) were essentially inactive in our functional *in vitro* NMDAR antagonist assay. The dramatic loss of NMDAR antagonism seen in compounds **16–19** indicates that neither of the basic intrachain nitrogens can be replaced with sterically similar functionalities.



Compound	IC_{50} RCGC (μM)
9	1.5 (2)
10	28 (1)
11	21 (2)
12	117 (1)

Fig. 2. Modification of the number of basic side-chain nitrogens.



Compound	X_1	X_2	R	IC_{50} RCGC (μM)
9	-NH-	-NH-	-H	1.5 (2)
15	-NH-	-NH-	-GLY	0.96 (2)
16	-NH-	$-CH_2-$	-GLY	71 (1)
17	$-CH_2-$	-NH-	-GLY	19 (3)
18	-NH-	-O-	-H	>100 (1)
19	-O-	-NH-	-H	53 (2)

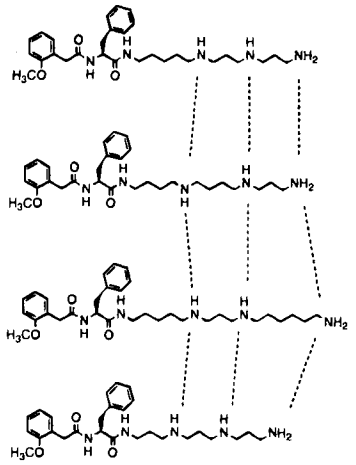
Fig. 4. Substitution of intrachain nitrogen with either carbon or oxygen.

CONCLUSIONS

In vitro results suggest that a minimum of three basic nitrogens are required for high NMDAR antagonist potency in this series of Arg-636 analogs. Attempts to increase lipophilicity by isosteric substitution of either of the two intrachain nitrogens with carbon or oxygen resulted in significantly less active analogs.

Our molecular-modeling studies suggest that intramolecular hydrogen bonds may be important for these analogs to assume a specific bioactive conformation (Figure 5). Analogs in which these hydrogen bonds cannot form (*e.g.*, those analogs lacking intrachain nitrogens, *i.e.*, hydrogen-bond donor atoms) would therefore reside in non-hydrogen-bonded conformations. A second, more plausible explanation for the loss of *in vitro* potency in compounds **16–19** is that a minimum of three amine functionalities are required for intermolecular hydrogen-bonding to the NMDAR.

The results of this study have shown that structural manipulations of argiotoxin-636 can be made in an effort to decrease the overall polarity and net molecular charge of the natural product lead compound, while maintaining *in vitro* NMDAR antagonist potency. The *in vivo* results indicate that while the *in vitro* potency of one analog (compound **8**) was maintained, the cardiovascular toxicity of this analog increased dramatically when compared to Arg-636 (Table 1). One objective of this research was to develop a compound with high *in vitro* NMDAR antagonist potency while minimizing the cardiovascular effects



Compound	IC_{50} RCGC (μM)	MK-801 binding (μM)
9	1.5 (2)	49% @ 100 μM
13	1.2 (1)	33% @ 100 μM
8	0.49 (2)	63% @ 10 μM
14	3.1 (1)	not tested

Fig. 3. Modification of the alkyl length between the three basic side-chain nitrogens.

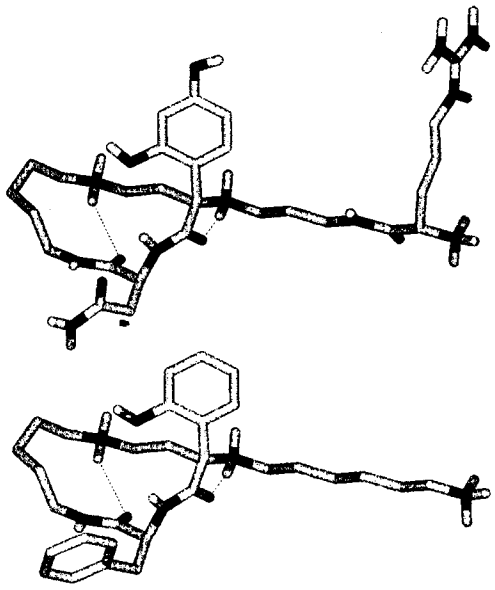


Fig. 5. The spatial relationship of the pharmacophoric groups in one pair of possible, potentially biologically active, conformations for Arg-636 (**1**, top) and active analog **8** (bottom) are shown. Hydrogen bonds are shown with dashed lines.

of these analogs. The cardiovascular toxicity associated with compound **8** thus precludes its use medicinally as an NMDAR antagonist. Therefore, the compounds presented in this paper were not tested further in *in vivo* NMDAR antagonist assays.

In conclusion, maximal potency was achieved in analogs with a 5-3-6 arrangement of methylenes and nitrogens in the "tail" moiety. Compound **8** was the most potent analog in this series. This compound is a structurally simplified spider toxin analog in which a total of five nitrogen and three oxygen atoms present in the original natural product lead (Arg-636) have been deleted.

Although the analogs prepared in this study are not currently candidates for clinical development, the research surrounding this class of spider toxin analogs provides important insight into the structure and function of NMDAs and will hopefully facilitate the discovery and development of clinically useful medicinal agents acting through this mechanism. NMDA receptor-operated ion channels are important theraputic targets, *e.g.,* for neuroprotection and epilepsy, and remain a highly active area of clinical and preclinical investigation.

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REFERENCES

1. B. C. Van Wagenen, E. G. DelMar, S. T. Moe, S. M. Shimizu, D. L. Smith, R. M. Barmore, R. Trovato, J. L. Rhodes, A. P. Miaullis, M. F. Balandrin, L. D. Artman, N. Alasti, and A. L. Mueller. *209th American Chemical Society National Meeting,* MEDI 134, April 2–6, 1995.
2. A. L. Mueller, B. C. Van Wagenen, E. G. Delmar, M. F. Balandrin, S. T. Moe, and L. D. Artman. PCT Intl. Appl. WO 95/21612, August 17, 1995. *Chem. Abstr. 123(23): P 306618t (1995).* U.S. Pat. Appl. 14,813, February 8, 1994.
3. A. L. Mueller, L. D. Artman, Y. Chien, J. L. Raszkiewicz, N. Alasti, E. G. DelMar, B. C. Van Wagenen, S. T. Moe, M. F. Balandrin, D. L. Smith, S. M. Shimizu, J. L. Rhodes, R. Trovato, and R. M. Barmore. *Keystone Symposia on Molecular and Cellular Biology: Ion Channels as Theraputic Targets,* Tamarron, CO, February 4–10, 1996.
4. N. A. Saccomano, R. A. Volkmann, H. Jackson, and T. N. Parks. *Annu. Rep. Med. Chem.* **24**:287–293 (1989).
5. D. L. Smith, S. T. Moe, L. D. Artman, and A. L. Mueller. *Joint 50th Northwest/12th Rocky Mountain Regional American Chemical Society Meeting,* No. 221, June 14–16 (1995).
6. S. T. Moe, D. L. Smith, L. D. Artman, and A. L. Mueller. *Joint 50th Northwest/12th Rocky Mountain Regional American Chemical Society Meeting,* No. 222, June 14–16 (1995).
7. A. L. Mueller, R. Roeloffs, and H, Jackson. *In* G. A. Cordell (Ed.), *The Alkaloids,* Academic Press, NY, 1995, Vol. 46, pp. 63–94.
8. A. Schäfer, A. Benz, W. Fiedler, A. Guggisberg, S. Beinz, and M. Hesse. *In* G. A. Cordell and A. Brossi (Eds.), *The Alkaloids: Chemistry and Pharmacology,* Vol. 45, Academic Press, NY, 1993, pp. 1–125.
9. E. V. Grishin, T. M. Volkova, A. S. Arseniev, O. S. Reshetova, and V. V. Onopreinko. *Bioorg. Khim.* **12**:1121–1124 (1986).
10. A. Bateman, P. Boden, A. Dell, I. R. Duce, D. L. J. Quicke, and P. N. R. Usherwood. *Brain Res.* **339**:237–244 (1989).
11. T. Budd, P. Clinton, A. Dell, I. R. Duce, S. J. Johnson, D. L. J. Quicke, G. W. Taylor, P. N. R. Usherwood, and G. Usoh. *Brain Res.* **448**:30–39 (1988).
12. J. E. Watkins and R. H. Evans. *Annu. Rev. Pharmacol. Toxicol.* **21**:165–204 (1981).
13. A. C. Foster and G. E. Fagg. *Brain Res. Rev.* **1**:103–104 (1984).
14. A. L. Mueller, B. C. Albenzi, A. H. Ganong, L. S. Reynolds, and H. Jackson. *Synapse* **9**:244–250 (1991).
15. R. W. Ransom and N. L. Stec. *J. Neurochem.* **51**:830 (1988).
16. D. W. Choi. *Neuron* **1**:623–634 (1988).
17. A. B. MacDermott, M. L. Mayer, G. L. Westbrook, S. J. Smith, and J. L. Barker. *Nature* **321**:519–522 (1986).
18. F. A. Schanne, A. B. Kane, E. E. Young, and J. L. Farber. *Science* **206**:700–702 (1979).
19. B. S. Meldrum and A. G. Chapman. *In* G. L. Collingridge and J. C. Watkins (Eds.), *The NMDA Receptor,* 2nd ed., Oxford University Press, NY, 1994, pp. 457–468.
20. P. L. Feldman, O. W. Griffith, and D. J. Stuehr. *Chem. Eng. News* **71**:26–38 (1993).
21. P. A. Krapcho and C. S. Kuell. *Synth. Commun.* **20**:2559–2564 (1990).
22. T. N. Parks, L. D. Artman, N. Alasti, and E. F. Nemeth. *Brain Res.* **552**:13–22 (1991).
23. K. Williams, C. Romano, and P. B. Molinoff. *Molec. Pharmacol.* **36**:575–581 (1989).
24. O. H. Lowry, N. H. Rosenbrough, A. C. Farr, and R. J. Randall. *J. Biol. Chem.* **193**:265–275 (1951).
25. T. L. Shih, J. R. Sanchez, and H. Mrozik. *Tetrahedron Lett.* **28**:6015–6018 (1987).
26. M. E. Adams, R. L. Carney, F. E. Enderlin, E. T. Fu, M. A. Jarema, J. P. Li, C. A. Miller, D. A. Schooley, M. J. Shapiro, and V. J. Venema. *Biochem. Biophys. Res. Commun.* **148**:678–683 (1987).
27. É. A. Elin, B. F. de Masedo, V. V. Onoprienko, N. E. Osokina, and O. B. Tikhomirova. *Bioorg. Khim.* **14**:704–706 (1988) (translation by Plenum Publishing Co., 1989).
28. V. J. Jasys, P. R. Kelbaugh, D. M. Nason, D. Phillips, N. A. Saccomano, and R. A. Volkmann. *Tetrahedron Lett.* **29**:6223–6226 (1988).
29. S.-K. Choi and K. Nakanishi. *Tetrahedron* **49**:5777–5790 (1993).
30. N. Anis, S. Sherby, R. Goodnow, Jr., M. Niwa, K. Konno, T. Kallimopoloulos, R. Bukownik, K. Nakanishi, P. Usherwood, A. Eldefrawi, and M. Eldefrawi. *J. Pharmacol. Exptl. Therap.* **254**:764–773 (1990).