Structure−Activity Studies for α-Amino-3-hydroxy-5-methyl-4-isoxazolepropanoic Acid Receptors: Acidic Hydroxyphenylalanines

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Antagonists of α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA) receptors may have therapeutic potential as psychotropic agents. A series of mononitro- and dinitro-2- and 3-hydroxyphenylalanines was prepared, and their activity compared with willardiine, 5-nitrowillardiine, AMPA, and 2,4,5-trihydroxyphenylalanine (6-hydroxydopa) as inhibitors of specific [^{3}H]AMPA and [^{3}H]kainate binding in rat brain homogenates. The most active compounds were highly acidic (pK_a 3−4), namely, 2-hydroxy-3,5-dinitro-DL-phenylalanine (**13**; [^{3}H]AMPA $IC_{50} \approx 25$ μM) and 3-hydroxy-2,4-dinitro-DL-phenylalanine (**19**; [^{3}H]AMPA $IC_{50} \approx 5$ μM). Two other dinitro-3-hydroxyphenylalanines, and 3,5-dinitro-DL-tyrosine, were considerably less active. Various mononitrohydroxyphenylalanines, which are less acidic, were also less active or inactive, and 2- and 3-hydroxyphenylalanine (*o*- and *m*-tyrosine) were inactive. Compounds **13** and **19**, DL-willardiine (pK_a 9.3, [^{3}H]AMPA $IC_{50} = 2$ μM), and 5-nitro-DL-willardiine (pK_a 6.4, [^{3}H]AMPA $IC_{50} = 0.2$ μM) displayed AMPA ≫ kainate selectivity in binding studies. Compound **19** was an AMPA-like agonist, but **13** was an antagonist in an AMPA-evoked norepinephrine release assay in rat hippocampal nerve endings. Also, compound **13** injected into the rat ventral pallidum antagonized the locomotor activity elicited by systemic amphetamine.

Introduction

Excitatory amino acid neurotransmission is ubiquitous in the mammalian central nervous system (CNS).[1] Glutamate and other excitatory amino acid neurotransmitters evoke fast excitatory postsynaptic potentials through activation of ionotropic receptors, one family of which includes those at which depolarizations are elicited by exogenously applied α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA, **1**; Figure 1) or kainate (**2**).[2] Receptors of the AMPA/kainate family are clearly quite diverse and are composed of several subunit proteins;[3] diversity of function is also evident by marked differences in responses to various agonists in various physiological test systems. The quest to understand the workings of the brain, as well as possible therapeutic applications, makes the discovery of compounds that can selectively interact with receptor subtypes an important goal.

Our initial objective was to add to the understanding of structural requirements for AMPA receptor agonists in order to discover new lead compounds that might be converted to antagonists through structural modification.[4] The most potent such agonists identified to date have five-membered heterocylic rings in place of the γ-carboxylate group of glutamate, as in AMPA (**1**) and quisqualic acid (**3**) (Figure 1). The 2,4-imidazolidinedione (hydantoin) **4** was also recently shown to be a potent, AMPA-like agonist in the mammalian hippocampus.[5] Substitution with various alkyl and aryl substituents at the 5-position of **1** has given agonists,[6] while elaboration to the amino acids **5a**−**d** and **6** (Figure 2) provided modestly potent AMPA receptor antagonists.[7]

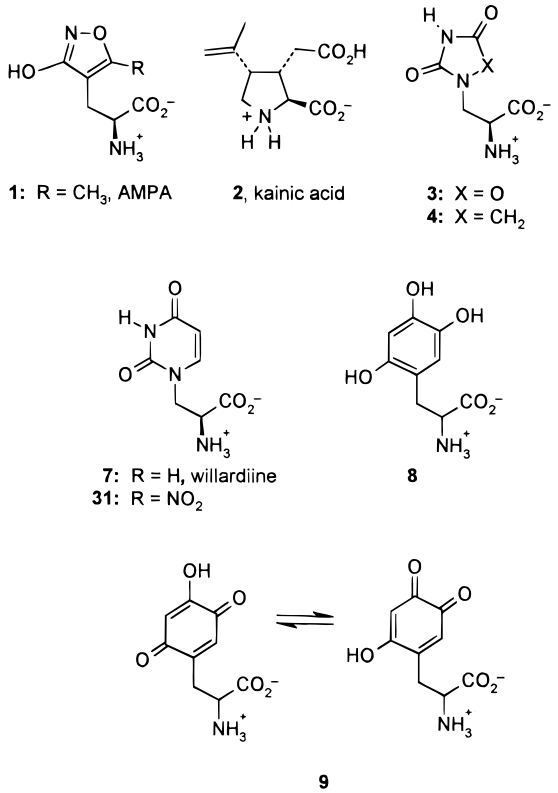

Figure 1. Selected glutamate analogs that are AMPA and kainate receptor agonists or partial agonists.

In this work we addressed six-membered ring systems. Willardiine (**7**; Figure 1) is a β-(1-uracil)-substituted alanine that is approximately equipotent

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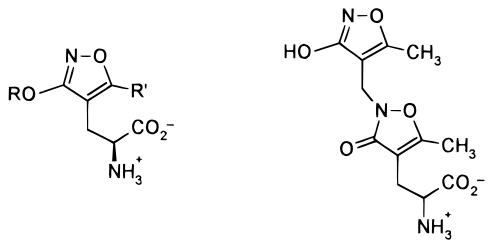


5a: R = CH_2CO_2H, R' = CH_3
5b: R = $CH_2PO_3H_2$, R' = CH_3
5c: R = CH_2CO_2H, R' = $C(CH_3)_3$
5d: R = $CH_2PO_3H_2$, R' = $C(CH_3)_3$

6

Figure 2. Amino acid glutamate antagonists.

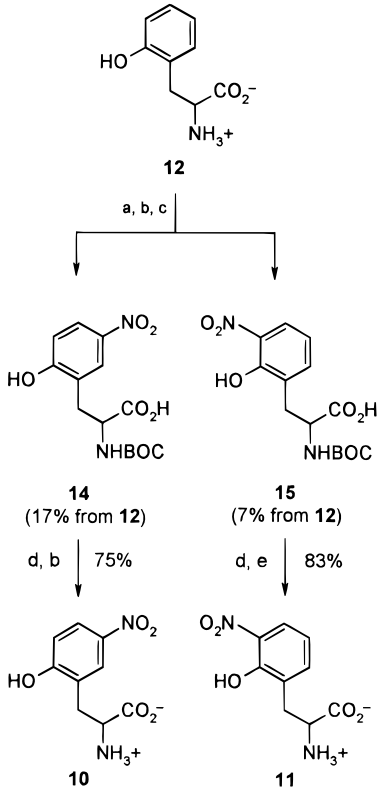
with glutamate in competing for [^{3}H]AMPA binding and in physiological studies.[8] Recently, a number of 5-substituted L-willardiine analogs were shown to be potent AMPA agonists.[9−11] It was also reported that 2,4,5-trihydroxyphenylalanine (6-hydroxydopa, **8**) potently depolarized frog spinal neurons[12] and more recently that **8** inhibited [^{3}H]AMPA binding;[13] it is now clear, however, that the apparent activity of **8** is actually due to the rapid, spontaneous oxidation to the quinone **9** in aqueous solution.[14] We postulated that a phenolate anion with enhanced acidity corresponding to the *o*- or *m*-hydroxyl groups in **8** might serve as a bioisostere for the γ-carboxylate group of glutamate; such an isostere would potentially provide a more flexible template on which to build AMPA antagonists. As a first approach to testing this hypothesis, nitrated 2- and 3-hydroxyphenylalanines were synthesized.

Results

Synthesis of 2-Hydroxyphenylalanine Analogs. Syntheses of **10** from the corresponding benzyl halides have previously been reported,[15,16] but we wished to obtain both the 3- and 5-nitro cogeners (**11** and **10**, respectively) for biological evaluation. The Dall'Asta and Farrario[17] synthesis of 3-nitrotyrosine was adapted for the nitration of commercially available 2-hydroxy-DL-phenylalanine (**12**, Scheme 1). The nitrated product mixture, which contained a small amount of the dinitro product **13**, was treated with BOC-ON; the *tert*-butyloxycarbonyl-protected derivatives **14** and **15** were isolated by preparative chromatography on silica gel, crystallized from ethyl acetate/hexane, and deprotected with 30% trifluoroacetic acid in methylene chloride to give **10** and **11**, respectively. The small amount of BOC-protected dinitro derivative could not be purified. Under one set of conditions intended to increase the amount of **13** (Scheme 2), no significant quantities of any of the three amino acids were detected by HPLC, and compound **16** was isolated in 32% yield. Initially, it was unclear whether **16** was the α-hydroxy or β-hydroxy acid, because the ^{1}H- and ^{13}C-NMR spectra were consistent with either isomer. A peak corresponding to the loss of HO_2CCHO was observed in the high-resolution EI mass spectrum, however, and the carbon−carbon connectivity was ultimately verified using a ^{13}C INADEQUATE experiment.

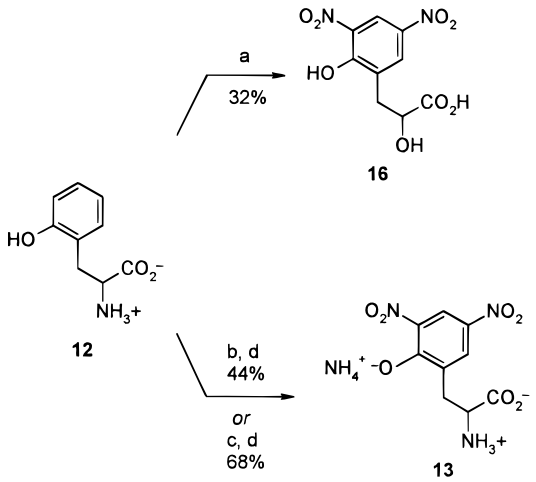
The dinitro compound **13** was produced in 44% yield and free of mononitrated products by adsorbing **12** on dry magnesium sulfate and incubating in a chamber containing fuming nitric acid (Scheme 2). This procedure was costly and difficult to scale, and a more convenient, higher-yielding preparation of **13** was

Scheme 1[a]



[a] (a) 23% HNO_3, 2 h/0 °C; (b) cation exchange chromatography; (c) BOC-ON, Et_3N, H_2O/dioxane; (d) 30% CF_3CO_2H, CH_2Cl_2; (e) Et_3N.

Scheme 2[a]

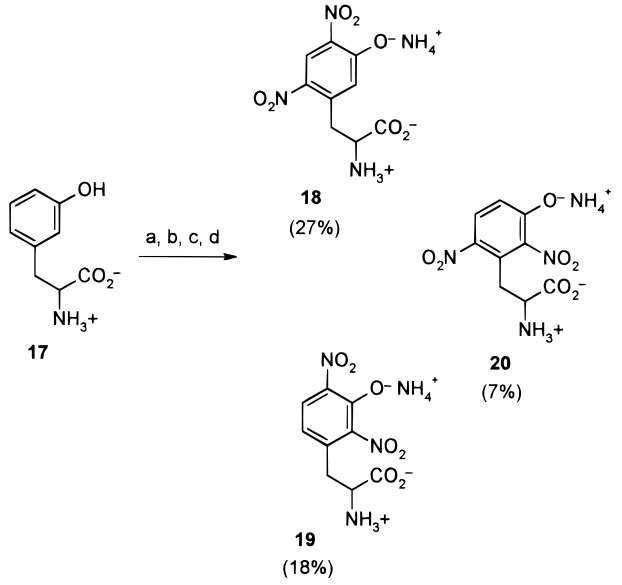


[a] (a) 60% aq HNO_3, 2 h/0 °C, then 5 h/25 °C; (b) adsorb on $MgSO_4$ and dry, incubate over fuming HNO_3; (c) NO_2BF_4/CH_3CN, 2 h/0 °C; (d) cation exchange chromatography.

achieved with nitronium tetrafluoroborate in acetonitrile; the product was isolated as the hydrated ammonium phenolate salt, and this form was found to be stable for at least 1 year. Two separately prepared lots of material assumed the same hydration state ($\cdot^5/_2H_2O$).

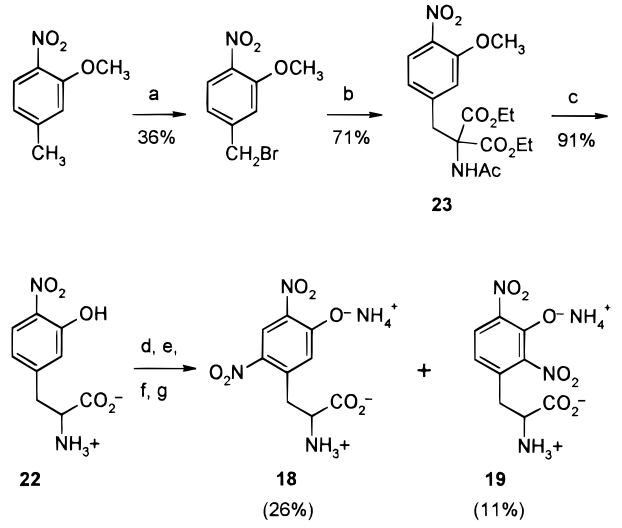
Synthesis of 3-Hydroxyphenylalanine Analogs and 3,5-Dinitro-DL-tyrosine. Nitration of DL-3-hydroxyphenylalanine (**17**; Scheme 3) with nitronium tetrafluoroborate gave a mixture of three products (**18**−**20**); this observation is consistent with the work of Jackson,[18] who nitrated with nitric acid/sulfuric acid and separated the three isomers through extensive fractional crystallization of barium salts. Jackson later confirmed the structures of two of the isomers (4,6- and

Scheme 3[a]



[a] (a) NO_2BF_4/CH_3CN, 27 h/0 °C; (b) cation exchange chromatography; (c) separation of **18** by fractional crystallization; (d) preparative C18 chromatographic separation of **19** and **20**.

Scheme 4[a]

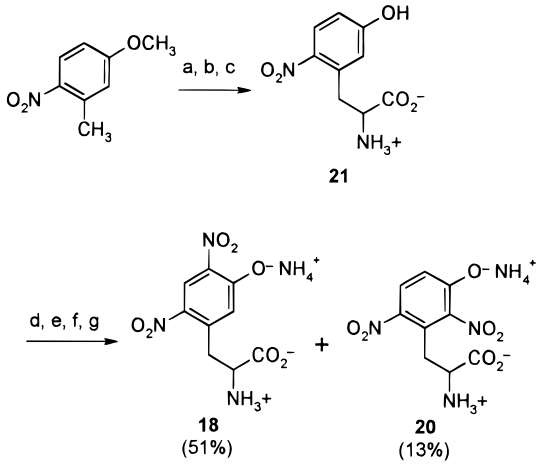


[a] (a) NBS, cat. $PhCO_2OCOPh/CCl_4$, 18 h reflux; (b) $AcNHCH(CO_2Et)_2$, NaOEt/EtOH, 10 min/rt; (c) 48% HBr, 5 h reflux; (d) NO_2BF_4/CH_3CN, 1 h/0 °C; (e) cation exchange chromatography; (f) separation of **18** by fractional crystallization; (g) recrystallization of **19**.

2,6-dinitro) via unambiguous synthesis of the corresponding 3-methoxybenzyl amino acids from the requisite dinitromethoxybenzaldehydes.[19] In the present work, the ammonium salt **18** was found to have a much lower aqueous solubility than **19** or **20**, and **18** could be crystallized from mixed solutions almost quantitatively. Fractional crystallization failed to separate the remaining two compounds, but the mixture could be separated on a medium-pressure liquid chromatography column packed with C18-derivatized silica gel. Either **18** or **19** was the predominant product, apparently depending on the rate at which the nitronium tetrafluoroborate was added (this dependence was not fully characterized). The product ratios were, however, generally similar to the ratio reported by Jackson.[18]

It was impossible to differentiate **19** and **20** using ^{1}H-NMR, and large-scale preparative separation was not practical. These two compounds were, however, synthesized (unambiguously) by further nitrating the two mononitro compounds **21** and **22** to give mixtures of **18** with each of the other two dinitrated products (as in Schemes 4 and 5). The synthesis of **21** by the Scheme 5 route was previously reported in the literature.[20] Compound **22** was synthesized by the same route (Scheme 4), through the known intermediate **23**,[21] and was nitrated to the expected mixture of **18** and **19**. These compounds were directly separated, without resorting to chromatography, by fractional crystallization alone. In this nitration, the hydrobromide salt of **22** could not serve as starting material because additional (unidentified) products were produced in substantial amounts. Also, excess nitronium tetrafluoroborate was needed to ensure complete reaction; otherwise the starting material (**22**) was difficult to remove from the products. The known compound **21** was similarly nitrated to give **18** and **20**, Scheme 5.

Scheme 5[a]



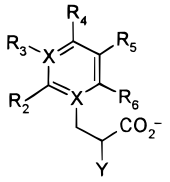
[a] (a−f) as in Scheme 4; (g) recrystallization of **20**.

Finally, 3,5-dinitro-DL-tyrosine (**24**) was smoothly synthesized from DL-tyrosine (**25**), again with nitronium tetrafluoroborate in acetonitrile. This compound was also readily isolated as the hydrated ammonium salt, apparently the first reported instance of this.

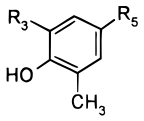
pK_a Studies. The protolysis of various compounds was studied by UV spectrophotometric methods[22] at a constant ionic strength of 0.1 M. Unexpectedly, the measured ionization constants for **10**, **11**, and **13** (Table 1) were nearly 1 log unit lower than the literature values[23] for 2,4-dinitro-*o*-cresol (**26**; see structure accompanying Table 1, pK_a 4.35), *o*- and *p*-nitrophenols (pK_a 7.23 and 7.15, respectively), and 4-nitro-*o*-cresol (**27**; pK_a 7.85 by titration in 50% methanol).[24] We determined the protolysis constants for the appropriate *o*-cresols (**26**−**28**) under the same conditions as for the amino acids (Table 1) and found them to be in excellent agreement with the literature values.[25] Clearly, the amino acid side chain exerts a substantial electron-withdrawing effect on the aromatic ring in these compounds, and most of this effect must result from the positively charged α-ammonium group because the pK_a of the α-hydroxy acid **16** is similar to that of the *o*-cresol **26** and considerably higher than that of the α-amino acid **13**.

Radioligand Binding Studies. None of the unsubstituted hydroxyphenylalanines, namely, 2-hydroxy-DL-phenylalanine (**12**), 3-hydroxy-DL-phenylalanine (**17**), or DL-tyrosine (**25**), competed significantly for specific [^{3}H]AMPA binding in rat cortex homogenates (Table 1);

Table 1. pK_a Values and Inhibition of [^{3}H]AMPA Specific Binding at 10^{-4} M Concentrations of Various Hydroxyphenylalanines and Related Compounds



7-8, 10-13, 16-22, 24-25, 29-31

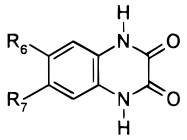


26: $R_3 = R_5 = NO_2$
27: $R_3 = H$, $R_5 = NO_2$
28: $R_3 = NO_2$, $R_5 = H$

compd	configuration	R_2	R_3	R_4	R_5	R_6	X	Y	analytical wavelength (nm)	pK_a[a]	[^{3}H]AMPA inhibtn[b]
8	DL	OH	H	OH	OH	H	C	NH_3^+		ND	~50
7	DL	OH	H	OH	H	H	N	NH_3^+	258	9.3	100
31	DL	OH	H	OH	NO_2	H	N	NH_3^+	326	6.4	100
12	DL	OH	H	H	H	H	C	NH_3^+		8.67[c]	0, 0, 4
10	DL	OH	H	H	NO_2	H	C	NH_3^+	413	6.4 (**27**, 7.2)[d]	20, 57
11	DL	OH	NO_2	H	H	H	C	NH_3^+	430	6.6 (**28**, 7.7)[d]	37, 35
13	DL	OH	NO_2	H	NO_2	H	C	NH_3^+	380	6.4 (**26**, 4.4)[d]	>90
16	*R,S*	OH	NO_2	H	NO_2	H	C	OH	371	4.2	0
17	DL	H	OH	H	H	H	C	NH_3^+		ND	0, 0, 0
21	DL	H	OH	H	H	NO_2	C	NH_3^+	398	6.8	ND
22	DL	H	OH	NO_2	H	H	C	NH_3^+	417	6.6	0
18	DL	H	OH	NO_2	H	NO_2	C	NH_3^+	360	3.8	~50
19	DL	NO_2	OH	NO_2	H	H	C	NH_3^+	410	3.2	100
20	DL	NO_2	OH	H	H	NO_2	C	NH_3^+	381	3.6	~50
25	DL	H	H	OH	H	H	C	NH_3^+		ND	0, 0, 0
24	DL	H	NO_2	OH	NO_2	H	C	NH_3^+		ND	33
29	L	H	NO_2	OH	H	H	C	NH_3^+	429	6.8	0, 30
30	L	H	NO_2	OH	NO_2	H	C	NH_3^+	444	3.2	0,0

[a] Protolysis constant at an ionic strength of 0.1 M. [b] Percent inhibition of specific [^{3}H]AMPA binding by 100 μM test compound; replicate results represent determinations in different tissue preparations. [c] Protolysis constant at ionic strength = 0.2 (KCl).[31] [d] pK_a of the corresponding *o*-cresol; see accompanying structure and text for discussion. ND, not determined.

Table 2. Inhibition of Specific [^{3}H]AMPA and [^{3}H]Kainate Binding, and Functional Activity in Hippocampal Nerve Ending Norepinephrine Release Assay



32: $R_6 = R_7 = NO_2$
39: $R_6 = CN$, $R_7 = NO_2$

	IC$_{50}$ (μM)[a]		NE release	
compound	[^{3}H]AMPA	[^{3}H]kainate	EC$_{50}$ (μM)[b]	IC$_{50}$ (μM)[c]
L-glutamate	1.2 ± 0.7	0.7 ± 0.3	ND	
AMPA (**1**)	0.013 ± 0.003	92 ± 8	6.7 ± 2.4	
kainate (**2**)	ND	0.0054 ± 0.0013	3.7 ± 1.2	
DL-willardiine (**7**)	1.8 ± 0.1	97 ± 1.7	ND	
31	0.12 ± 0.025	3.7 ± 0.9	15 ± 1.6	
19	4.9 ± 0.9	34 ± 6	74 ± 5.1	
13	25 ± 2.5	>100		730 ± 88
DNQX (**32**)	0.93 ± 0.42	2.2 ± 0.4		41 ± 6
CNQX (**39**)	0.54 ± 0.14	2.4 ± 0.4		ND

[a] 50% inhibition of specific radioligand binding, mean ± standard error of the mean of at least three independent determinations. [b] In vitro stimulation of norepinephrine release from hippocampal nerve endings; see text for experimental details. [c] Inhibition of the agonist activity elicited by the simultaneous presence of 100 μM AMPA.

however, the more acidic mononitro compounds **10** and **11**, and the dinitro compound **13**, did. For the series **12**, **11**, **10**, and **13**, there is a suggestive correlation between extent of ionization at physiological pH and affinity for binding sites labeled by [^{3}H]AMPA. The α-hydroxy acid **16** was inactive, reiterating the requirement for the α-amino group. Of the 3-hydroxyphenylalanine analogs, **19** exhibited substantial activity, whereas the other two dinitro analogs (**18** and **20**) were considerably less active. 3-Nitro-L-tyrosine (**29**) and 3,5-dinitro-L-tyrosine (**30**) were inactive; however, 3,5-dinitro-DL-tyrosine (**24**) gave some displacement of [^{3}H]AMPA in the 100 μM screening assay, comparable to that observed with **18** and **20**.

Compounds effecting greater than 50% inhibition of specific [^{3}H]AMPA binding at 100 μM concentration were further evaluated for potency as inhibitors of both [^{3}H]AMPA and [^{3}H]kainate specific binding (Table 2). The 2- and 3-hydroxyphenylalanines **13** and **19** inhibited [^{3}H]AMPA binding with low-micromolar potency. These two compounds were somewhat less active as inhibitors of [^{3}H]kainate specific binding, and the ratio

of IC_{50} values provides one index of relative selectivity. The 2-hydroxyphenylalanine **13** was approximately 5-fold selective for AMPA over kainate binding inhibition and the 3-hydroxyphenylalanine **19** about 10-fold selective.

DL-Willardiine (**7**) is surprisingly potent considering that the 1-substituted uracil ring has a pK_a of about 9.3. We had predicted that the 5-nitro group of DL-5-nitrowillardiine (**31**) would, *via* increased acidity, confer as much as 2 orders of magnitude increase in potency; at pH 7.4, willardiine (pK_a 9.3) is about 1% ionized, compared with 90% for 5-nitrowillardiine (pK_a 6.4). (This work was carried out in 1989−1990, prior to the reports of Patneau et al.,[9] Wong et al.,[11] and Hawkins et al.,[10] and we first reported the pK_a and preliminary binding data for **31** in early 1991.[4]) Compound **31** was, in fact, about 10-fold more potent than willardiine as an inhibitor of specific [^{3}H]AMPA binding (Table 2). Compounds **7** and **31** were also quite selective for AMPA vs kainate receptors in these binding studies.

Functional Pharmacological Studies. The most potent compounds were further studied in functional assays. One assay is based on the fact that AMPA/kainate receptor activation induces norepinephrine release from hippocampal nerve endings.[26,27] Compound **19** was an agonist, albeit less potent than AMPA and 5-nitrowillardiine (**31**) (Table 2). Compound **13**, however, was an antagonist that was somewhat less potent than 1,4-dihydro-6,7-dinitro-2,3-quinoxalinedione (**32**, DNQX; see structure in Table 2), in agreement with the results of the equilibrium binding studies.

Another test derives from the observation that some glutamatergic neurons become activated in parallel with certain behavioral responses to amphetamine. One example is that the AMPA/kainate antagonist DNQX, when injected into the rat ventral pallidum, attenuates the locomotor response elicited by systemic amphetamine (Figure 3; cf. Willins et al.[28]). The 2-hydroxyphenylalanine **13** had a similar effect, while 6-amino-7-fluoro-1,4-dihydro-2,3-quinoxalinedione (**33**), which has very low potency in competing for [^{3}H]AMPA binding sites, was inactive. The correlation between potency in the binding assay and functional ability to antagonize effects of amphetamine, coupled with the antagonist action of **13** in the neurotransmitter release assay, strongly suggests that the locomotor antagonism of **13** is brought about by AMPA or kainate receptor blockade. In another functional study, compound **13** was unable to stimulate acetylcholine release in rat striatal slices, a model in which NMDA produces a marked effect (data not shown), or to block the effect of NMDA in this test system. Compound **13** also did not antagonize the specific binding of [^{3}H]NMDA, [^{3}H]-MK801, or [^{3}H]PCP to rat brain preparations.[29]

Discussion

The notion that a phenolate anion might be bioisosteric for the γ-carboxyl group in glutamate is certainly not new; in fact, Curtis and Watkins tested the activity of 2-hydroxyphenylalanine (**12**) in their early structure−activity studies (ca. 1960).[30] They found, however, that **12** could not depolarize cat dorsal horn interneurons, Renshaw cells, or motoneurons. The lack of activity is not surprising, since the pK_a of the phenolic hydroxyl group in **12** is about 8.7,[31] in contrast to the γ-carboxyl group of glutamate (pK_a 4.3)[23] or the heterocyclic rings

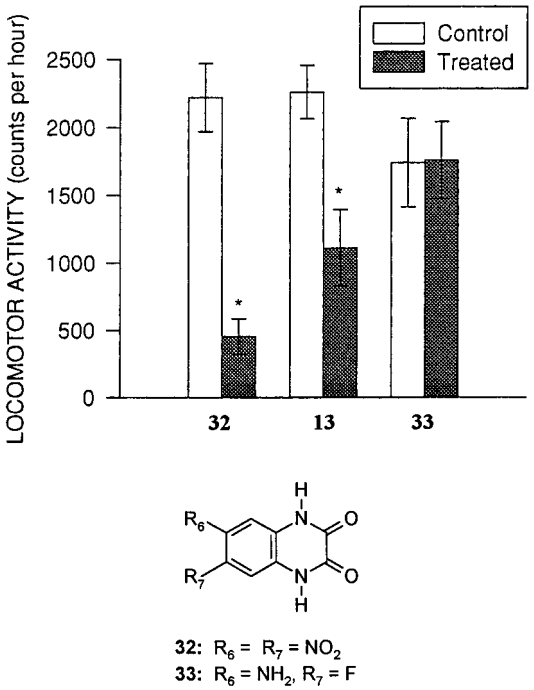


Figure 3. Effect of AMPA receptor antagonists injected into the ventral pallidum on hypermotility elicited by systemic injection of stimulants. Microinjections of 1 μg of DNQX (**32**), 1 μg of compound **33**, 1 μg of compound **13**, or vehicle were made into the ventral pallidum of anesthetized rats. Immediately following this injection, the rats were given 0.5 mg/kg amphetamine. Ambulatory locomotor activity was monitored during a 1 h period immediately following recovery from anesthesia. Control hypermotility (systemic stimulant; vehicle in ventral pallidum) was 2000−3000 locomotor counts with the doses of stimulants used, and baseline motility in the absence of any drugs was approximately 500 locomotor counts. Data are expressed as mean ± SEM for groups of 6−8 animals. See main text for further details.

of AMPA (pK_a 4.8)[32] or quisqualate (pK_a 4.5).[33] Nitrotyrosines **10** and **21** were previously studied for the pH dependence of their uptake in Ehrlich ascites tumor cells *via* the glutamic acid uptake system.[34] The rate of uptake was well correlated with the degree of protonation of these two compounds in pH-dependence studies, as it was for glutamate. Exploiting this same concept, a 4-hydroxy-3-cyclobutene-1,2-dione (squaric acid) analog was recently reported to be a potent AMPA-like agonist.[35] Even more recently, Matzen et al.[6] reported that the less acidic isothiazole analog of AMPA (pK_a 7.00 for the 3-isothiazolol group) was about 50 times less potent than AMPA as an inhibitor of [^{3}H]-AMPA specific binding.

Despite the compelling correlation between acidity of the γ-carboxyl group surrogate and activity, willardiine (pK_a >9) retains good biological activity. The considerable potency of willardiine might be accounted for if the acidity of willardiine is enhanced by appropriate receptor interactions, such as a hydrogen bond to the 4-oxo group. Nonetheless, 5-nitrowillardiine (**31**) was about 10-fold more potent than willardiine in our binding studies, and electron-withdrawing groups have been found by others[9−11] to enhance the potency of willardiines at AMPA-preferring receptors.

Regarding the antagonist action of **13**, the *o*-nitrophenolate moiety would be expected to engage the receptor in a different manner than the γ-carboxylate group of glutamate or the carboxylate isosteres of agonists such as AMPA or willardiine; all of the three

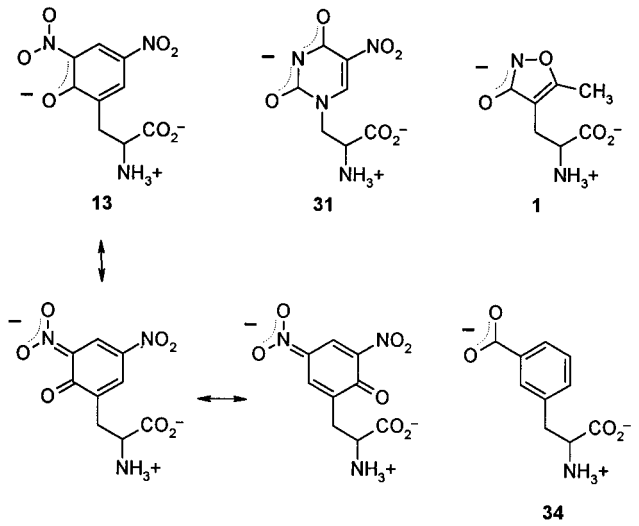


Figure 4. Structural comparison of the AMPA antagonist **13** with AMPA (**1**), 5-nitrowillardiine (**31**), and 3-carboxyphenylalanine (**34**).

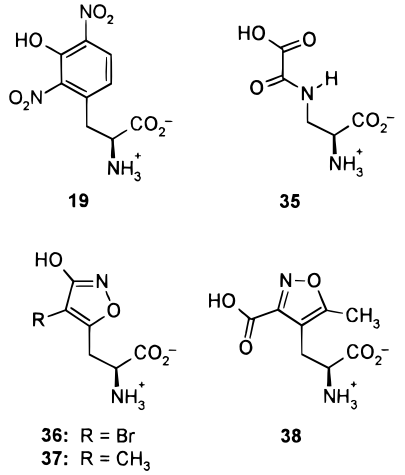


Figure 5. Structural comparison of the agonist **19** to selected glutamate homologs.

latter compounds have a pair of heteroatoms positioned to interact with the receptor in a similar fashion, and different than for the antagonist **13** (see Figure 4). Furthermore, it is noteworthy that certain invertebrate neurons are depolarized by 3-carboxyphenylalanine (**34**; Figure 4).[36] In **13**, either of the two nitro groups could occupy the same region of receptor space as the 3-carboxy group in **34**. The 3-hydroxyphenylalanine **19**, on the other hand, elicited the anticipated agonist activity; the glutamate homologs β-oxalylaminoalanine (**35**),[37] 4-bromo- and 4-methylhomoibotenic acids (**36** and **37**),[38] and compound **38**[39] (Figure 5) are also all potent AMPA receptor agonists.

It is now known[40] that the enantioselectivity for compound **13** is the same as for AMPA (L >> D).[41] The screening data for 3,5-dinitro-L-tyrosine (**30**) and 3,5-dinitro-DL-tyrosine (**24**) suggest the opposite enantioselectivity (Table 1). The enantiospecific synthesis of L-**13** was sufficiently challenging that it will be the subject of a subsequent publication.

Conclusions

Of the nitrated hydroxyphenylalanines tested for inhibition of specific [^{3}H]AMPA binding in rat brain homogenates, acidic hydroxy groups at the 2- and 3-positions of the phenyl ring conferred substantial activity. We have thus shown that the phenolate anion can serve as a bioisosteric replacement for the γ-carboxylate of glutamate in AMPA receptor ligands. In one case (**13**), antagonist properties were conferred, and a suitably positioned *o*-nitrohydroxylate moiety is therefore an important structural lead.

Experimental Section

General Procedures. Melting points were determined on a Thomas-Hoover melting point apparatus; emergent stem corrections were not applied. Unless otherwise noted, ^{1}H-NMR spectra were acquired using an IBM AF/250 spectrometer (250 MHz). Some ^{13}C-NMR and other special experiments were conducted with IBM AF/270 (270 MHz) or Brüker AMX-500 (500 MHz) spectrometers. Infrared spectra were obtained using a Laser Precision Analytical RFX-40 FTIR. High-resolution electron impact (EI) mass spectra were obtained at The Ohio State University Chemical Instrumentation Center using a Kratos MS-30 spectrometer, while fast atom bombardment (FAB) mass spectra were acquired using either a VG 70-250S or Finnigan MAT-90 spectrometer on "magic bullet" or 3-nitrobenzyl alcohol, using DMSO as an additional solvent when necessary. High-pressure liquid chromatography (HPLC) was carried out on a Beckman 421 system, equipped with Model 112 pumps and a Model 153 fixed-wavelength detector, or a Beckman System Gold equipped with a Model 167 scanning UV−visible detector, Model 406 analog interface, and Model 110B pumps. Unless otherwise noted, chromatograms were obtained on an IBM 5 μm EC-C8 column, 4.6 mm i.d. × 15 cm length, using a mobile phase flow rate of 2.0 mL/min and detection by ultraviolet absorption at 254 nm. Ultraviolet/visible spectra were acquired with a Kontron UVIKON 860, IBM Model 9420, or Gilford REPONSE spectrometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, Oneida Research Services, Inc., Whitesboro, NY, or Atlantic Microlab, Inc., Norcross, GA.

Miscellaneous Compounds. DL-Willardiine (**7**) was synthesized by the method of Martinez and Lee.[42] DL-5-Nitrowillardiine (**31**) was reported as a synthetic intermediate by Martinez, Lee, and Goodman;[43] we readily repeated their procedure for the nitration of willardiine. 3-Nitro-L-tyrosine (**29**) and 3,5-dinitro-L-tyrosine (**30**) were obtained from Sigma.

pK_a Determinations. An adaptation of published spectrophotometric methods[22] served to determine the proton dissociation constants for various compounds. Compound (1−2 mg) was dissolved in a solution that contained 0.01 N NaOH and 0.09 M KCl (solution A) to make the ionic strength equal to 0.1; the pH of the resulting solutions was ca. 11.8, and the volume was sufficient to give an optical density of ca. 1 AU at a suitable wavelength (Table 1). The solution was titrated with 0.1 N HCl, graphical estimates of the pK_a and the acid- and base-form absorbance values were obtained, and the pK_a was then calculated by fitting the data to the Henderson−Hasselbach equation (recast in terms of UV absorbances) using the program NONLIN84.[44]

***N*-[[(1,1-Dimethylethyl)oxy]carbonyl]-2-hydroxy-5-nitro-DL-phenylalanine (14) and *N*-[[(1,1-dimethylethyl)oxy]carbonyl]-2-hydroxy-3-nitro-DL-phenylalanine (15), Method A.** Compound **12** (2-hydroxy-DL-phenylalanine; 5.0 g, 27.6 mmol) was suspended in 20.7 mL of H_2O, and 5.2 mL of 50% HNO_3 was added during 30 min at room temperature while stirring. The solution was cooled in an ice bath, and an additional 11.0 mL of 50% HNO_3 was added dropwise during 2 h. The solution was allowed to warm to room temperature during 5 h and then placed in a refrigerator overnight. H_2O (50 mL) was added, and the mixture was extracted with benzene (2 × 100 mL). The aqueous fraction was neutralized with Na_2CO_3, again extracted with benzene (2 × 100 mL), and evaporated to dryness in vacuo using *i*-PrOH for azeotropic removal of the last portion of H_2O. The solid residue was taken up in 100 mL of 1.2 N HCl (a black solid remained undissolved) and eluted through a short 1 in. i.d. column containing 5 g of Bio-beads (SM-16, 20−50 mesh; Biorad, Inc.). The solution was diluted 10-fold with H_2O and eluted through a cation exchange column (2.5 cm i.d. × 9 cm length, Biorad AG50W-

X12, sulfonic acid type, hydrogen form, 200−400 mesh). The column was washed with H_2O (ca. 1500 mL) and then eluted with 0.75 N NH_4OH. Evaporation to dryness in vacuo gave 2.57 g of a solid that was estimated by ^{1}H-NMR to contain **10** and **11** in about a 7:3 (*para*:*ortho*) ratio, along with <10 mol % of **13**.

A 1.5 g portion (ca. 6.6 mmol) of the above mixture was dissolved in 100 mL of 1:1 dioxane−H_2O, and 4.0 mL (30 mmol) of Et_3N and 3.59 g (14.6 mmol) of 2-[[(*tert*-butyloxycarbonyl)-oxy]imino]-2-phenylacetonitrile ("BOC-ON") was added. After stirring for 16 h at room temperature, 200 mL of H_2O was added and the solution was extracted with EtOAc (2 × 200 mL). The aqueous phase was acidified with 5% citric acid (75 mL) and 1.2 N HCl (20 mL) and extracted with EtOAc (2 × 150 mL). The extracts were dried ($MgSO_4$) and concentrated in vacuo. This solution was divided into two portions, and each was chromatographed on fresh silica gel (40−63 μm, E. Merck, dry-packed in a 51 mm i.d. × 43 cm length Michel-Miller column (Ace Glass, Inc.) and pre-equilibrated with $CHCl_3$), eluting with 8% *i*-PrOH/2% MeOH/0.7% CH_3CO_2H in $CHCl_3$ at 20 mL/min. Appropriate fractions were combined, concentrated in vacuo, and coevaporated with toluene to dryness. The residues were taken up in EtOAc and triturated with hexane to yield 0.88 g of **14** (pale-yellow crystals, 16.8% from **12**) and 0.35 g of **15** (yellow crystals, 6.6% from **12**).

Analytical data for **14**: dec 205 °C; ^{1}H-NMR (acetone-d_6, TMS) δ 8.13 (d, 1H, J = 2.7 Hz, ArH), 8.03 (dd, 1H, J = 8.9, 2.7 Hz, ArH), 7.03 (d, 1H, J = 8.9 Hz, ArH), 6.26 (bd, J = 7.2 Hz, NH), 4.54−4.62 (bm, 1H, H_α), 3.41 (dd, 1H, J = 4.6, 13.6 Hz, CH_2), 3.00 (dd, 1H, J = 9.8, 13.6 Hz, CH_2), 1.31 (s, 9H, CH_3); HPLC (5 μm IBM RP-phenyl, 4.6 mm i.d. × 15 cm length, 3 min at 10% MeOH/0.1% CF_3CO_2H/0.2% CH_3CO_2H/H_2O, then a linear gradient during 15 min to 21% MeOH; see also General Procedures) indicated <1% contamination of the product with **15**. Anal. ($C_{14}H_{18}N_2O_7$) C, H, N.

Analytical data for **15**: dec 139−140 °C; ^{1}H-NMR (acetone-d_6, TMS) δ 8.04 (d, 1H, J = 8.1 Hz, peak-broadening due to *meta*-coupling to δ 7.67 proton, ArH), 7.67 (d, 1H, J = 6.8, ∼1 Hz, ArH), 7.03 (*asym* t, 1H, ArH), 6.18 (bd, J = 7.9 Hz, NH), 4.59 (bdd, 1H, J = 4.7, 9.8 Hz, H_α), 3.44 (dd, 1H, J = 4.7, 13.6 Hz, CH_2), 3.03 (dd, 1H, J = 9.8, 13.6 Hz, CH_2), 1.31 (s, 9H, CH_3); HPLC (same conditions as for **14**) showed the product to contain ∼2% of **14**, and there were no other significant peaks. Anal. ($C_{14}H_{18}N_2O_7$) C, H, N.

Method B. A suspension of **12** (2.94 g, 16.2 mmol) in 50 mL of reagent-grade CH_3CN under dry argon was cooled to −18 °C, and 85% NO_2BF_4 (2.53 g, 16.2 mmol) was added in small portions during 30 min. After another 30 min of cooling, the reaction was quenched by pouring the mixture into 470 mL of H_2O and adding 28 mL of 1.2 N HCl. The solution was allowed to stand overnight, filtered to remove a brown precipitate, and eluted through a cation exchange column as in method A. The ammoniacal eluates were evaporated to dryness in vacuo, the residue was suspended in 10 mL of H_2O, and the grayish solid was collected, washed with *i*-PrOH, and dried to a constant weight of 2.26 g. ^{1}H-NMR (DCl/DMSO) showed the mixture to contain **10**, **11**, and **12**, ca. 33:22:45 (molar basis), corresponding to 22% and 14% yields of **10** and **11**, respectively, and 30% recovery of unreacted **12**.

A portion of this mixture (2.03 g, ca. 9.0 mmol) was treated with BOC-ON as in method A. The EtOAc extracts were concentrated in vacuo to a volume of ca. 5 mL and diluted to 40 mL with $CHCl_3$; this solution was chromatographed as in method A, but in a single portion. The two mononitro products were crystallized as before to give 0.73 g of **14** (15% from **12**) and 0.58 g of **15** (12% from **12**).

2-Hydroxy-5-nitro-DL-phenylalanine (10). Compound **14** (0.50 g, 1.5 mmol) was suspended in CH_2Cl_2 (14 mL) and treated with CF_3CO_2H (6 mL) while stirring. The starting material dissolved, and stirring was continued for 3 h at room temperature. The resulting white precipitate was collected and air-dried to yield 0.42 g (80%) of the trifluoroacetate salt, dec 208−209 °C. Reversed-phase HPLC (see General Procedures for column and detection; eluting 8 min with 8% CH_3CN/0.1% CF_3CO_2H/H_2O, then a linear gradient to 70% CH_3CN during 10 min) showed the material to be free of detectable (<0.01%) quantitities of **11**. A portion was converted to the zwitterionic form: 230 mg of the trifluoroacetate salt was dissolved in 90 mL of 0.1 N HCl, and this solution was eluted through a 2.5 cm i.d. × 3.5 cm length cation exchange column (see above). The column was washed with 600 mL of H_2O, and the product was eluted with 550 mL of 0.75 N NH_4OH. The volume was reduced in vacuo to ca. 15 mL, *i*-PrOH (50 mL) was added, and the volume was again reduced to ca. 20 mL. The product was collected and washed successively with *i*-PrOH and Et_2O. Recovery from the column was ca. 94%: dec 246−256 °C (lit.[15] dec 252−257 °C); ^{1}H-NMR (DCl/D_2O, DSS) δ 8.15 (s, 1H, ArH, overlapped with δ 8.13), 8.13 (dd overlapped with δ 8.15, 1H, $J \approx$ 2.8 Hz, ArH), 7.04 (dd, 1H, J = 7.8, 1.6 Hz, ArH), 4.48 (dd, 1H, J = 5.8, 7.3 Hz, H_α), 3.44 (dd, 1H, J = 5.8, 14.5 Hz, CH_2), 3.27 (dd, 1H, J = 7.3, 14.5 Hz, CH_2); MS (FAB) *m/e* 227 (M + 1). Anal. ($C_9H_{10}N_2O_5$) C, H, N.

2-Hydroxy-3-nitro-DL-phenylalanine (11). Compound **15** (0.20 g, 0.61 mmol) was suspended in 7 mL of CH_2Cl_2 and treated with 3 mL of CF_3CO_2H while stirring. After 7 h at room temperature, the reaction mixture was neutralized with ca. 30 mL of 10% Et_3N in CH_2Cl_2 to give 0.12 g (83%) of **11** in two crops as a yellow powder: dec 236−237 °C; ^{1}H-NMR (DCl/D_2O, DSS) δ 8.07 (dd, 1H, J = 8.5, 1.3 Hz, ArH), 7.67 (dd, 1H, J = 7.4, 1.3 Hz, ArH), 7.10 (*asym* t, 1H, ArH), 4.24 (*asym* t, 1H, H_α), 3.37 (dd, 1H, J = 6.9, 14.0 Hz, CH_2), 3.24 (dd, 1H, J = 7.3, 14.1 Hz, CH_2); MS (FAB) *m/e* 227 (M + 1); reversed-phase HPLC showed the product to contain <0.5% of **10**. Anal. ($C_9H_{10}N_2O_5$) C, H, N.

2-Hydroxy-3,5-dinitro-DL-phenylalanine, Ammonium Salt, $^5/_2$ Hydrate (13). A suspension of **12** (0.50 g, 2.76 mmol) in reagent-grade CH_3CN (8 mL) was cooled to 0−5 °C, and 1.0 g (6.4 mmol) of 85% NO_2BF_4 was added in small portions during 10 min. Cooling and stirring were continued for 8 h, H_2O (120 mL) was added, and the mixture was filtered after standing overnight to remove a dark-brown solid. The filtrate was eluted through a 2.5 cm i.d. × 6 cm length cation exchange column (Biorad AG50W-X12, see above), and the column was washed with H_2O (2500 mL). The product was eluted with 0.4 N NH_4OH, the volume was reduced to ca. 10 mL in vacuo, *i*-PrOH (100 mL) was added, and the suspension was heated briefly. After cooling, Et_2O (500 mL) was slowly added and the flask placed in a refrigerator overnight. The product was collected, washed with small amounts of *i*-PrOH and Et_2O, and dried in air to give 0.625 g (68%) of a fluffy, yellow-orange powder: melting behavior—heating at about 5 °C/min, the material visibly changed form at ca. 160 °C, discolored at >180 °C, and rapidly decomposed at >190 °C; ^{1}H-NMR (D_2O, DSS) δ 8.78 (d, 1H, J = 3.1 Hz, ArH), 8.05 (d, 1H, J = 3.1 Hz, ArH), 4.02 (dd, 1H, J = 3.6, 7.7 Hz, H_α), 3.27 (dd, 1H, J = 3.6, 14.6 Hz, CH_2), 3.00 (dd, 1H, J = 7.7, 14.6 Hz, CH_2); in DMSO-d_6 there was a broad peak centered at ∼7.5 ppm (7H, NH_3^+ and NH_4^+); MS (FAB) *m/e* 272 (M + 1), 226 (−NO_2). Anal. ($C_9H_9N_3O_7 \cdot NH_3 \cdot {}^5/_2H_2O$) C, H, N.

2-Hydroxy-3-(2′-hydroxy-3′,5′-dinitrophenyl)propanoic Acid (16). A solution of concentrated HNO_3 (5 mL) in H_2O (6 mL) was cooled in an ice bath, and **12** (1.00 g, 5.52 mmol) was added in small portions during 10 min while stirring. After 2.5 h of continued stirring at 0−5 °C, fuming HNO_3 (11 mL) was added during 15 min. After 1 h, the ice bath was removed and the solution stirred 7 h longer. Water (400 mL) was added, and the solution was extracted twice with 400-mL portions of 1:9 CH_3CN/EtOAc. The extracts were shaken with 200 mL of brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Hexane was added portionwise during 2 days while crystallization proceeded, yielding 0.48 g (32%) of pale-yellow crystals: mp 140−141 °C after an additional recrystallization from EtOAc/hexane: ^{1}H-NMR (acetone-d_6, TMS) δ 8.85 (d, 1H, J = 2.8 Hz, ArH), 8.53 (d, 1H, J = 2.8 Hz, ArH), 4.57 (dd, 1H, J = 4.2, 8.9 Hz, H_α), 3.46 (dd, 1H, J = 4.2, 14.2 Hz, CH_2), 3.11 (dd, 1H, J = 8.9, 14.2 Hz, CH_2); ^{13}C-NMR (acetone-d_6, TMS) δ 175 (s, C1), 158.1 (s, C2′), 140.1 (s, C5′), 134.2 (s, C3′), 133.0 (d, C6′), 131.9 (s, C1′), 120.7 (d, C4′), 69.7 (d, C2), 35.2 (t, C3); these assignments were confirmed by ^{1}H−^{13}C heteronuclear correlation and by a ^{13}C INADEQUATE experiment; MS (EI) *m/e* 272.025 (calcd 272.028, M^+), 254 (−H_2O), 227 (−CO_2H), 198 (−HO_2CCHO), 76 (base). Anal. ($C_9H_8N_2O_8 \cdot {}^1/_2H_2O$) C, H, N.

5-Hydroxy-2,4-dinitro-DL-phenylalanine, Ammonium Salt (18) and 3-Hydroxy-2,6-dinitro-DL-phenylalanine, Ammonium Salt, 7/2 Hydrate (20). Commercially obtained 3-hydroxy-DL-phenylalanine (**17**; 1.00 g, 5.52 mmol) was suspended in 15 mL of reagent-grade CH_3CN under dry argon. This suspension was cooled in an ice bath and treated with solid 85% NO_2BF_4 (2.07 g, 13.2 mmol) in small portions during 10 min with constant stirring and cooling. The resulting deep-red solution was stirred for 2 h more in the ice bath and then allowed to come to room temperature during another 5.5 h. The reaction vessel was placed in a freezer overnight, warmed to room temperature during 2 h, and diluted with H_2O to 500 mL. After cation exchange chromatography and concentration of the ammoniacal eluates, **18** crystallized almost quantitatively. After several days of refrigeration, the nearly pure crystals of **18** were collected (the mother liquor was set aside), washed twice with H_2O (1−2 mL), and dried in air overnight and in a dessicator for 3 days, yielding 0.43 g (27%) of **18** as the anhydrous ammonium salt. The product was recrystallized from H_2O: melting behavior—heating at about 5 °C/min, the yellow-orange salt slowly discolored at temperatures above 200 °C and rapidly turned dark-brown at ∼245 °C; ^{1}H-NMR (DCl/D_2O, DSS) δ 9.02 (s, 1H, ArH), 7.33 (s, 1H, ArH), 4.52 (*asym* t, 1H, H_α), 3.77 (dd, 1H, J = 7.6, 13.8 Hz, CH_2), 3.58 (dd, 1H, J = 7.5, 13.8 Hz, CH_2); MS (FAB) *m/e* 289 ($M\cdot NH_4^+$), 272 (M + 1). The amounts of the other two isomers in this material were quantitated by HPLC (mobile phase 8% CH_3CN/0.1% CF_3CO_2H/H_2O), using authentic standards due to the considerable differences in response factors: **19** (<0.5%) and **20** (<0.5%). Anal. ($C_9H_9N_3O_7\cdot NH_3$) C, H, N.

The mother liquors from above, containing **19** and **20**, were slowly treated with *i*-PrOH to a volume of ca. 400 mL; Et_2O (150 mL) was then added. The suspension was concentrated in vacuo to ca. 50 mL, *i*-PrOH (150 mL) and Et_2O (150 mL) were added, and crystallization was allowed to proceed overnight at room temperature. The solid was collected and air-dried to yield 0.63 g of material that contained **19** and **20** in a 2:1 molar ratio (^{1}H-NMR). These compounds were separated by preparative reversed-phase (C18) chromatography as follows: 10 60-mg portions were each dissolved in 1 mL of 1.2 N HCl and injected onto a 47 mm i.d. × 38 cm length Michel-Miller column packed with 40−63 μm silica gel (E. Merck) that had been derivatized with octadecyltrichlorosilane. The compounds were eluted with 15% CH_3CN/1% CF_3CO_2H/H_2O at 20 mL/min, monitoring by UV at 280 nm. The fractions were analyzed by HPLC (4% CH_3CN/0.1% CF_3CO_2H/H_2O, 2.0 mL/min); those containing **20** were combined, and HCl was added to make the solution 0.1 N. Cation exchange chromatography and evaporation of the ammoniacal eluates gave a damp cake. Water (10 mL) was added, and the solution was treated with *i*-PrOH to the point of initial cloudiness. Crystallization was continued for 1 week in a freezer, and the product was collected, washed with *i*-PrOH, and dried in air to give 0.13 g (6.7%) of **20**: dec 170−180 °C; ^{1}H-NMR (D_2O, DSS) δ 8.07 (d, 1H, J = 9.6 Hz, ArH), 6.58 (d, 1H, J = 9.6 Hz, ArH), 3.92 (dd, 1H, J = 6.9, 8.7 Hz, H_α), 3.50 (dd, 1H, J = 6.9, 14.3 Hz, CH_2), 3.04 (dd, 1H, J = 8.7, 14.3 Hz, CH_2); MS (FAB) *m/e* 272 (M + 1). The amounts of the other two isomers in this product, quantitated by HPLC vs authentic standards, were ∼0.35% of **19** and ∼0.04% of **18**. Anal. ($C_9H_9N_3O_7\cdot NH_3\cdot 7/2H_2O$) C, H, N.

The fractions containing mostly **19** were combined, and HCl was added to make the solution 0.1 N. Cation exchange chromatography was carried out, the eluates were concentrated to ca. 5 mL, *i*-PrOH was added to the point of cloudiness, and crystallization was allowed to proceed slowly in the freezer. The crystals were collected, washed with *i*-PrOH, and dried in air to yield compound **19** (0.31 g, ca. 19%), which was contaminated with **20** (3 mol %) and **18** (1 mol %). This material was not purified further because an alternate synthesis was in progress (see below). The total yield for the three isolated compounds (**18**−**20**) was 53%.

Diethyl 1-Acetamido-2-(3′-methoxy-4′-nitrophenyl)-1,1-ethanedicarboxylate (23). Sodium metal (1.77 g, 77.0 mmol) was dissolved in 375 mL of EtOH, and acetamidomalonic acid diethyl ester (15.2 g, 70.0 mmol) was added. The solution was heated to 60 °C and treated with 17.22 g (70.0 mmol) of 4-(bromomethyl)-2-methoxy-1-nitrobenzene[21] with strong stirring. The reaction was rapid; after 4−5 min, strong precipitation occurred and the suspension quickly became an unstirrable cake, whereupon heating was discontinued. After another 15 min, the reaction flask was placed in a refrigerator. After 3 days, 50 mL of EtOH was added and the cake was broken up, collected by filtration, resuspended in 400 mL of EtOH, and stirred vigorously for 1 h. The solid was again collected, washed (EtOH, 2 × 25 mL), and dried in air to give **23**, 18.98 g (70.9%): mp 165.0−165.5 °C (lit.[21] 163−164 °C); ^{1}H-NMR ($CDCl_3$, TMS) δ 7.76 (d, 1H, J = 8.3 Hz, ArH), 6.73 (d, 1H, J = 1.3 Hz, ArH), 6.66 (dd, 1H, J = 8.3, 1.3 Hz, ArH), 6.59 (s, 1H, NH), 4.20−4.36 (m, 4H, CH_2), 3.90 (s, 3H, $ArOCH_3$), 3.73 (s, 2H, $ArCH_2$), 2.04 (s, 3H, $COCH_3$), 1.31 (t, 6H, J = 7.1 Hz, CH_3); MS (EI) *m/e* 382.136 (calcd 382.138, M^+), 323 ($-CH_3CONH_2$), 276, 43 (base, CH_3CO^+). Anal. ($C_{17}H_{22}N_2O_8$) C, H, N.

3-Hydroxy-4-nitro-DL-phenylalanine (22) and 3-Hydroxy-4-nitro-DL-phenylalanine Hydrobromide. A suspension of 9.56 g (25.0 mmol) of **23** in 95 mL of 48% HBr was brought to gentle reflux. Heating was discontinued after 20 h, and the HBr salt slowly crystallized from solution. After 3 days in a freezer, the salt was collected, and the damp cake was dissolved in H_2O (50 mL) and titrated to pH 5 with concentrated NH_4OH. After overnight refrigeration another 50 mL of H_2O was added; the product was collected, washed with H_2O, and dried in air and then under high vacuum for 18 h to yield 4.31 g (76.3%) of **22** as light-yellow crystals: dec 229 °C (at 2 °C/min from 226 °C); ^{1}H-NMR (DCl/D_2O, DSS) δ 8.13 (d, 1H, J = 8.7 Hz, ArH), 7.15 (d, 1H, J = 1.7 Hz, ArH), 7.04 (dd, 1H, J = 8.7, 1.7 Hz, ArH), 4.48 (dd, 1H, J = 6.1, 7.5 Hz, H_α), 3.47 (dd, 1H, J = 6.1, 14.5 Hz, CH_2), 3.26 (dd, 1H, J = 7.5, 14.5 Hz, CH_2); MS (FAB) *m/e* 227 (M + 1), 181 ($-NO_2$). Anal. Calcd for $C_9H_{10}N_2O_5$: C, 47.79; H, 4.46; N, 12.39. Found: C, 47.12; H, 4.38; N, 12.23.

In a separate trial (2/5 scale), the hydrolysis took only 5 h (monitored by HPLC), and the HBr salt was isolated by washing the filter cake thrice with *i*-PrOH. Drying in air and then under high vacuum for 3.5 h gave **22**·HBr: mp 238−239 °C (at 2 °C/min from 235 °C). Anal. ($C_9H_{11}BrN_2O_5$) C, H, N.

5-Hydroxy-2,4-dinitro-DL-phenylalanine, Ammonium Salt (18) and 3-Hydroxy-2,4-dinitro-DL-phenylalanine, Ammonium Salt, Dihydrate (19). Compound **22** (free zwitterion, 2.26 g, 10.0 mmol) was suspended in CH_3CN (10 mL) under dry argon gas and cooled to 0−4 °C. NO_2BF_4 (85%, 3.9 g, 25 mmol) was added during 5 min with continued cooling. After 2.7 h, the contents were poured into 0.1 N HCl (1000 mL), and the solution was eluted through a cation exchange column. The column was washed (H_2O, 600 mL) and eluted (0.3 N NH_4OH, 1000 mL), and the eluates were evaporated in vacuo to a damp residue. H_2O (10 mL) was added, and after 5 days of refrigeration the crystals were collected (setting the mother liquor aside), washed twice with H_2O, and dried in air to a constant weight (ca. 4 days), yielding 0.75 g (26%) of **18**.

The mother liquor was slowly treated with *i*-PrOH just to the point of persistent cloudiness; after 3 weeks in a freezer, the product was collected, washed (*i*-PrOH), and dried in air to give 0.27 g of **19** as deep-orange crystals of the ammonium salt dihydrate: slow dec >170 °C, rapid dec >185 °C. This crop contained 0.9% **18** (HPLC, 8% CH_3CN/0.1% CF_3CO_2H/H_2O at 2.0 mL/min). A second crop was obtained (0.04 g, total 11%): ^{1}H-NMR (D_2O, DSS) δ 7.96 (d, 1H, J = 8.9 Hz, ArH), 6.38 (d, 1H, J = 8.9 Hz, ArH), 3.98 (dd, 1H, J = 5.2, 9.0 Hz, H_α), 3.20 (dd, 1H, J = 5.2, 14.8 Hz, CH_2), 2.92 (dd, 1H, J = 9.0, 14.8 Hz, CH_2); the spectrum in DMSO-d_6 gave a broad peak centered near 7.5 ppm (NH_4^+ and RNH_3^+); MS (FAB) *m/e* 272 (M + 1). Anal. Calcd for $C_9H_9N_3O_7\cdot NH_3\cdot 2H_2O$: C, 33.34; H, 4.97; N, 17.28. Found: C, 33.76, 33.81; H, 4.69, 4.73; N, 17.03, 17.33.

5-Hydroxy-2,4-dinitro-DL-phenylalanine, Ammonium Salt (18) and 3-Hydroxy-2,6-dinitro-DL-phenylalanine, Ammonium Salt (20). Compound **21**[20] (1.70 g, 7.52 mmol) was nitrated under conditions similar to those for compound **22**, except the NO_2BF_4 was added in portions during 12 min. The products (**18** and **20**) were isolated by ion exchange chromatography and fractional crystallization in similar fash-

ion (the volume of the mother liquor from the first crystallization of **18** was 8 mL). The yield of **18** was 1.10 g (50.7%), and the yield of **20** was 0.35 g (13%).

4-Hydroxy-3,5-dinitro-DL-phenylalanine, Ammonium Salt, Monohydrate (24). DL-Tyrosine (**25**; 1.0 g, 5.5 mmol) was suspended in 16 mL of CH_3CN, and NO_2BF_4 (95%, 1.8 g, 13 mmol) was added portionwise during a period of 20 min while the temperature was maintained at 0−5 °C. Stirring was continued until no starting material or 3-nitrotyrosine was in evidence by HPLC (ca. 1 h); the mixture was then poured onto 50 g of ice and refrigerated for ca. 12 h. The solution was filtered and diluted with H_2O (1000 mL). Cation exchange chromatography, concentration of the eluates in vacuo to ca. 10 mL, addition of *i*-PrOH (100 mL) followed by Et_2O (500 mL), and refrigeration for ca. 12 h gave orange crystals. These were collected, washed with Et_2O (10 mL), and air-dried to yield 0.96 g (57%) of **24**: melting behavior—darkened at 190 °C, dec >200 °C; ^{1}H-NMR (90 MHz, D_2O, DSS) δ 7.97 (s, 2H, ArH), 3.92 (dd, 1H, J = 4.9, 8.3 Hz, H_α), 3.18 (m, 1H, CH_aH_b), 2.91 (m, 1H, CH_aH_b); ^{13}C-NMR (90 MHz, D_2O, DSS) δ 176.6 (C=O), 161.7 (C4), 145.0 (C3 and C5), 136.0 (C2 and C6), 118.8 (C1), 58.6 (CH), 37.8 (CH_2). Anal. ($C_9H_9N_3O_7 \cdot NH_3 \cdot H_2O$) C, H, N.

Pharmacology. Binding Studies. The interaction of compounds with AMPA receptors was assessed by inhibition of specific [^{3}H]AMPA binding in washed membrane preparations of rat brain following the procedure originally published by Honore et al.[45] After the freeze/thaw cycle and two additional washings, the preparation was incubated with 0.04% Triton X-100 at 37 °C for 30 min to remove an inhibitor protein. After another wash, binding was performed by one of two procedures.

Procedure A: Membranes were incubated with [^{3}H]AMPA (1.0 nM final concentration) and solutions of test compounds at appropriate concentrations in 30 mM Tris-acetate containing 2.5 mM $CaCl_2$ and 100 mM KSCN, pH 7.4. The mixture was incubated on ice for 30 min, and bound ligand was then separated from free ligand by centrifugation. The pellet was surface-washed and then dissolved in scintillation fluid for counting.

Procedure B: Membranes were incubated with [^{3}H]AMPA (1.4 nM final concentration) and solutions of test compounds at appropriate concentrations in 50 mM Tris-HCl buffer containing 2.5 mM $CaCl_2$ and 100 mM KSCN, pH 7.2. The mixture was incubated on ice for 60 min with periodic shaking, following which bound ligand was separated from free ligand by rapid filtration through Whatman GF/C filter paper using a Brandel M-12R cell harvester (Gaithersburg, MD). The resulting filter disks, containing membrane-bound radioligand, were dissolved in Scintiverse-E cocktail for scintillation counting. For both procedures, nonspecific binding was determined in the presence of 1 mM glutamate. For determination of potency, a range of test compound concentrations (0.01−100 μM) was tested. IC_{50} values were calculated using nonlinear regression analysis (InPlot software, GraphPad, Inc.).

Interaction of compounds with kainate receptors was assessed by inhibition of specific [^{3}H]kainate binding sites.[45] Tissue preparation was exactly the same as for the AMPA studies to the point of the freeze/thaw cycle. After thawing, the membranes were washed twice with 50 mM Tris-HCl buffer containing 2.5 mM $CaCl_2$, pH 7.1. Binding was then performed by incubating membranes with [^{3}H]kainate (1 nM final concentration) and solutions of test compounds at appropriate concentrations, in a 50 mM Tris-citrate buffer, pH 7.4. The mixture was incubated for 60 min on ice before separating bound and free ligand by one of the two methods described above.

Neurotransmitter Release Assay. This assay is based on the fact that AMPA receptor activation induces norepinephrine release from hippocampal nerve endings,[26] and is adapted from Desai et al.[27] Briefly, mouse hippocampi were dissected out and chopped into 0.3 mm × 0.3 mm slices. Slices were incubated with 0.2 μM [^{3}H]norepinephrine in Krebs buffer for 30 min, then transferred to superfusion chambers, and washed for 60 min with warmed, oxygenated Krebs buffer at 0.3 mL/min. Following this, 10 5-min fractions were collected. After the third fraction was collected, buffer containing 50 μM cyclothiazide was introduced. (Cyclothiazide diminishes AMPA receptor desensitization, thus allowing for a more readily quantifiable AMPA response.) After fraction 4, buffer containing test compound, cyclothiazide, and 100 μM AMPA was introduced for 5 min, followed by normal buffer until the end. For initial screening purposes, tissue slices were exposed to 1 mM test compound alone (test for possible agonist activity) run in parallel with tissue slices exposed to test compound plus 100 μM AMPA (test for antagonist). Each condition was run in triplicate. At least two experiments were conducted to generate $n = 6$ for each treatment. For dose−response data, a minimum of four concentrations of each test compound were run against 100 μM AMPA (for antagonists) or alone (for agonists). Determination of EC_{50} or IC_{50} values was accomplished utilizing median effect plot analysis.[46]

Locomotor Studies. Microinjections of 1 μg of DNQX (**32**), 1 μg of **33**, 1 μg of **13**, or vehicle were made into the ventral pallidum of anesthetized rats using a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Immediately following this injection, the rats were given 0.5 mg/kg amphetamine ip. Ambulatory locomotor activity was monitored during a 1-h period immediately following recovery from anesthesia using activity cages equipped with infrared beams and photocells (Columbus Instruments, Columbus, OH). Control hypermotility (systemic stimulant; vehicle in ventral pallidum) was 2000−3000 locomotor counts with the doses of stumulants used, and baseline motility in the absence of any drugs was approximately 500 locomotor counts. Study groups contained 6−8 animals.

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