(silica gel column eluted with CH_2Cl_2) to yield 1.93 g of 4.

Method V. *meso*-3,4-Bis(3-hydroxy-4-iodophenyl)hexane (5). A solution of iodine (5.08 g, 0.02 mol) in 25 mL of THF was added dropwise to a solution of metahexestrol (2.70 g, 0.01 mol) in a mixture of 75 mL of MeOH and 25 mL of concentrated NH_4OH with stirring. After 0.5 h, glacial acetic acid was added to neutralize NH_3. Water was then added, and reaction products were extracted with EtOAc. After removal of the solvent, **5** (2.55 g) was separated by silica gel chromatography with $CHCl_3$ as eluent.

Biological Methods. Estradiol Receptor Binding Assay. The relative binding affinity (RBA) of the test compounds was determined by the displacement of [^{3}H]estradiol. A previously described procedure[15] was used with modifications. Test compounds were incubated with cytosol from calf uteri and [^{3}H]estradiol at 4 °C for 16 h. Incubation was stopped by adding Dextran-coated charcoal. After centrifugation, the radioactivity of a 100-μL supernatant aliquot was counted. The percentage bound radioligand was plotted vs. the concentration of unlabeled test compounds. Six concentrations of the competitors were tested. They were chosen to provide a linear portion on a semilog plot crossing the point of 50% competition. From this plot, the molar concentrations of unlabeled estradiol and of test compounds reducing radioligand binding by 50% were determined.

Estrogen and Antiestrogen Assays. Estrogenic and antiestrogenic activities were determined by stimulation of the uterine growth and by inhibition of the uterine growth stimulated by estrone, respectively, by using immature NMRI mice as described previously.[15] Twenty-day-old female mice (weight 14.5 ± 1.2 g, mean plus or minus SD) were randomly distributed into groups of 10 animals. They were subcutaneously injected daily for 3 days with 0.1 mL of olive oil solutions containing the test compound. The uteri were removed 24 h after the last injection, fixed with Bouin's solution, washed, dried, and weighed.

Acknowledgment. Thanks are due to the Deutsche Forschungsgemeinschaft and to the Verband der Chemischen Industrie, Fonds der Chemischen Industrie, who supported this work by grants. The technical assistance of Gabi Seidl and Fritz Birk is gratefully acknowledged.

Registry No. 1, 71953-72-5; 1 dimethyl ester, 71953-69-0; **1a**, 71953-69-0; **1b**, 76473-05-7; **1c**, 37951-49-8; **2**, 89106-12-7; **2a**, 89106-13-8; **2b**, 89106-14-9; **2c**, 82846-20-6; **3**, 89106-15-0; **3a**, 89106-16-1; **3b**, 89106-17-2; **3c**, 89106-18-3; **4**, 89106-19-4; **5**, 89106-20-7; **6**, 89106-21-8; **7**, 89106-22-9; **8**, 89106-23-0; **8a**, 89106-24-1; **9**, 89106-25-2; **10**, 89106-26-3; **11**, 89106-27-4; **12**, 89106-28-5; **13**, 89106-29-6; **14**, 89106-30-9; **14a**, 89106-31-0; **15**, 89106-32-1; **15a**, 89106-33-2; **16**, 89106-34-3; **16a**, 89106-35-4; **16b**, 89106-36-5; **16c**, 41497-31-8; **17**, 89106-37-6; **17a**, 89106-38-7; **17b**, 89121-16-4; **17c**, 89106-39-8; **18**, 89106-40-1; **18a**, 89106-41-2; **18b**, 89106-42-3; **19**, 89106-43-4; **19a**, 89106-44-5; **19b**, 89106-45-6; **19c**, 89106-46-7; **20**, 89106-47-8; **20a**, 89106-48-9; **20b**, 89106-49-0; **21**, 89106-50-3; **21a**, 89106-51-4; **21b**, 89106-52-5; **21c**, 29578-81-2; 4-fluoro-3′-methoxybenzoic acid, 82846-18-2; 4-fluoro-3-methoxybenzoyl chloride, 82846-19-3; 5-chloro-3-methoxybenzoyl chloride, 89106-53-6; 6-fluoro-3-methoxytoluene, 2338-54-7; 2-methyl-*p*-anisidine, 102-50-1; 6-fluoro-3-methoxybenzoic acid, 367-83-9; 5-chloro-3-methoxybenzoic acid, 82477-67-6; 3,5-dichloroanisole, 33719-74-3; 5-hydroxy-2-methylbenzoic acid, 578-39-2; *o*-toluic acid, 118-90-1; 6-methyl-3-sulfobenzoic acid, 89106-54-7; sodium 6-methyl-3-sulfobenzoate, 52238-48-9.

Supplementary Material Available: ^{1}H NMR data (Tables VIII and IX) of the disubstituted metahexestrol derivatives (**1a–21a** and **1–21**) and the disubstituted *cis*-3,4-bis(3-methoxyphenyl)hex-3-enes (**1b–3b**, **16b**, and **19b**) (7 pages). Ordering information is given on any current masthead page.

Ibotenic Acid Analogues. Synthesis and Biological and in Vitro Activity of Conformationally Restricted Agonists at Central Excitatory Amino Acid Receptors

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A number of analogues of ibotenic acid [(*RS*)-3-hydroxy-5-isoxazoleglycine] were synthesized; they were tested as excitants on neurons in the cat spinal cord, by using microelectrophoretic techniques, and as inhibitors of the binding of kainic acid (KA) in vitro, by using synaptic membranes prepared from rat brains. The excitatory effects of the 3-isoxazolol amino acids (*RS*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-7-carboxylic acid (**4**, 7-HPCA), (*RS*)-α-amino-3-hydroxy-5,6-dihydro-4*H*-cyclohept[1,2-*d*]isoxazole-8-propionic acid (**8**, 8-AHCP), (*RS*)-α-amino-3-hydroxy-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazole-4-propionic acid (**12**, 4-AHCP), and (*RS*)-α-(methylamino)-3-hydroxy-5-methyl-4-isoxazolepropionic acid (**15**, *N*-Me-AMPA) were shown to be sensitive to (*S*)-glutamic acid diethyl ester (GDEE), an antagonist at quisqualic acid (QUIS) receptors, and insensitive to (*RS*)-2-amino-5-phosphonovaleric acid (2APV), an antagonist at *N*-methyl-(*R*)-aspartic acid (NMDA) receptors. The compounds **4** and **12** proved to be particularly potent agonists at the former class of receptor, assumed to represent physiological glutamic acid receptors. The amino acids (*RS*)-β-(2-carboxyphenyl)alanine (**19**), an analogue of **12**, and (*RS*)-2-(3-carboxyphenyl)glycine were weak GDEE-sensitive excitants with potencies comparable with that of **8**. All of the compounds were tested as inhibitors of KA binding. With the exception of **12** and **19**, which showed very low affinity for the KA binding sites, the compounds studied were inactive in this in vitro test system.

A number of naturally occurring amino acids, including quisqualic acid (QUIS), kainic acid (KA), and ibotenic acid, which are heterocyclic analogues of (*S*)-glutamic acid (Glu) (Chart I),[1-4] excite neurons in the mammalian central nervous system (CNS).[5,6] These amino acids also destroy neurons when injected into the brain,[7,8] and both excitation and neurotoxicity are assumed to be mediated by neuronal receptors normally operated by Glu and (*S*)-aspartic acid (Asp), putative central excitatory neurotransmitters.[2,5,6,11] The possible involvement of these amino acid transmitters

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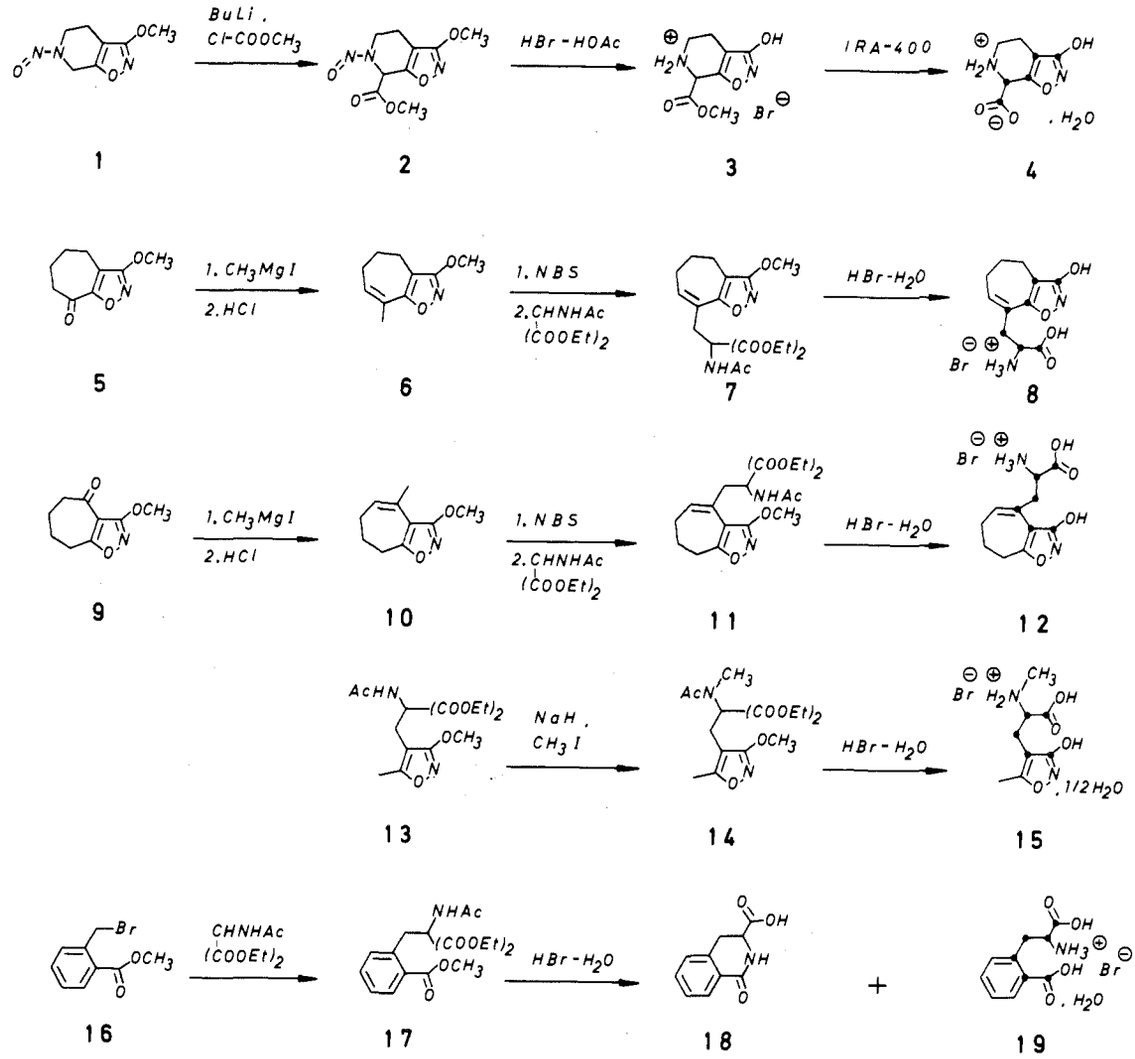
Scheme I



in certain neurological diseases[9] adds further interest to central excitant amino acid receptors as sites for pharmacological and therapeutic attack.

In vivo pharmacological and receptor-binding studies in vitro have disclosed heterogeneity of central excitant amino acid receptors, which at present are most conveniently subdivided into the following three classes:[10-13] (1) QUIS receptors, at which Glu diethyl ester (GDEE) is a relatively selective antagonist; (2) *N*-methyl-(*R*)-aspartic acid (NMDA) receptors, at which (*RS*)-2-amino-5-phosphonovaleric acid (2APV) and various other compounds[11] are selective antagonists; and (3) KA receptors, which are relatively insensitive to GDEE and 2 APV but which can be detected in vitro by using radioactive KA as a ligand. Although the physiological relevance of this receptor classification is unclear, QUIS receptors are assumed to represent the physiological postsynaptic receptors for Glu, and NMDA receptors are assumed to represent those for Asp. Consequently, there is a particular interest in selective agonists and antagonists for these classes of receptor. This paper describes the development of a number of potent and highly selective Glu agonists.

Ibotenic acid (Chart I), which is a constituent of the mushroom *Amanita muscaria*, is a selective NMDA agonist in spite of its structural similarity with Glu.[11,14,15] There is relatively free rotation of the glycine moiety of ibotenic acid (Chart I),[16] and in an attempt to shed light on its receptor-active conformation(s), the semirigid analogue (*RS*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridine-7-carboxylic acid (4, 7-HPCA) (Chart I) was synthesized and tested biologically.

Earlier structure–activity studies on the very potent and GDEE-sensitive excitant (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Chart I) and related ibotenic acid analogues have indicated that the substituents in the 5-position of these compounds force the amino acid side chains into more or less folded conformations.[17,18] This hypothesis prompted the syntheses

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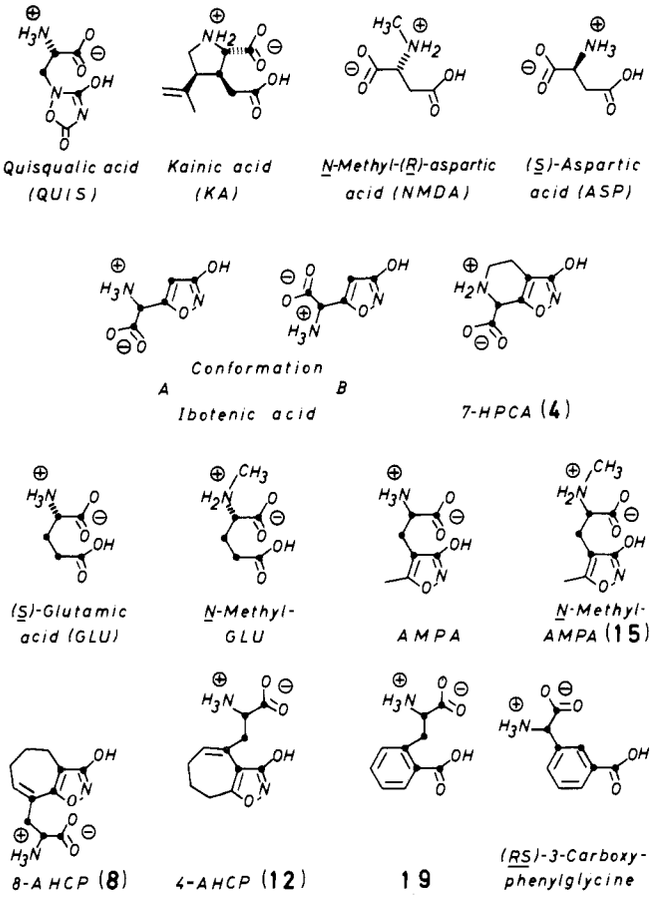
Chart I. Structures of (*S*)-Glutamic Acid, Ibotenic Acid, and a Number of Ibotenic Acid Analogues and Related Amino Acids[a]



[a] Two minimum-energy conformations of ibotenic acid are shown.

and biological testing of (*RS*)-α-amino-3-hydroxy-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazole-4-propionic acid (**12**, 4-AHCP) and some related amino acids (Chart I), assumed to reflect folded conformations of AMPA.

While Glu-induced excitation can be selectively antagonized by GDEE,[6,10,11] *N*-Me-Glu appears to be an NMDA agonist.[4] In order to study the effect of N-methylation of AMPA, (*RS*)-α-(methylamino)-3-hydroxy-5-methyl-4-isoxazolepropionic acid (**15**, *N*-Me-AMPA) was synthesized and studied biologically.

Chemistry. Since ibotenic acid is relatively easily decarboxylated, a reaction sequence for the synthesis of the cyclic ibotenic acid analogue **4** (Chart I) was developed, in which the carboxylate group was unmasked in the last step under mild conditions (Scheme I). A methoxycarbonyl group was introduced regiospecifically into the 7-position of **1** under strongly basic conditions. However, even in the presence of a large excess of methyl chloroformate, a mixture of **2** and the starting material (**1**), containing at least 30% of **1**, was isolated. Attempts to increase the yield of **2** by using other bases, including sodium hydride and potassium *tert*-butylate, failed to give detectable amounts of **2**. The stepwise deprotection of **2** was initiated by passing a vigorous stream of hydrogen bromide gas through a solution of **2** in glacial acetic acid, until the formation of a brown volatile product, assumed to be nitrosyl bromide, ceased after 5–10 min. If this initial treatment with hydrogen bromide gas was omitted, a complex reaction mixture was formed, in which **3** was only a minor constituent. The ^{1}H NMR spectra of **3**, as well as **4**, recorded with deuterium oxide as a solvent, revealed that the respective C-7 protons were quantitatively replaced by deuterium. Treatment of **3** with a strongly basic ion-exchange resin gave **4** and only vanishing amounts of the decarboxylation product 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol (THIP). The structure of **4**·H_2O has recently been confirmed by X-ray crystallographic methods.[19]

The isomeric compounds **8** and **12** were synthesized as outlined in Scheme I. Complete conversions of **6** and **10** into the corresponding bromomethyl derivatives could not be achieved under the conditions used. Acid-catalyzed deprotection under various conditions of **7** and **11** to give **8** and **12**, respectively, were accompanied by substantial decomposition, and only low yields of the final products could be isolated.

The syntheses of **15** and (*RS*)-β-(2-carboxyphenyl)alanine hydrobromide (**19**) are shown in Scheme I. The preparation of **19** by acid deprotection of **17** was accompanied by the formation of substantial amounts of **18**. (*RS*)-2-(3-carboxyphenyl)glycine (Chart I) was synthesized by a published procedure.[22]

For all new compounds, the ^{1}H NMR, IR, and UV data were consistent with the proposed structures. In the ^{1}H NMR spectrum of **12**, very different chemical-shift values (δ 2.76 and 3.18) were observed for the two side-chain methylene protons. With the exception of **8**, which was obtained in very small quantities, the structure determinations were supported by elemental analyses. For the final products **4**, **8**, **12**, **15**, and **19** and for the compounds for which satisfactory elemental analyses could not be obtained, spectroscopic data are reported.

Effects on Kainic Acid Binding. The affinities of **4**, **8**, **12**, **15**, **19**, and (*RS*)-2-(3-carboxyphenyl)glycine for the KA binding sites on purified synaptic membranes prepared from rat brains were studied as described earlier[17] on the basis of a published procedure.[20] While Glu (IC_{50} = 0.40 μM) and KA (IC_{50} = 0.013 μM) were potent inhibitors of [^{3}H]KA binding, the compounds under study were weak or inactive. Only **12** (IC_{50} = 85 μM) and **19** (IC_{50} = 80 μM) showed detectable affinities, verging on insignificance (IC_{50} > 100 μM).

Single-Cell Pharmacology. Microelectrophoretic techniques[21] were used to compare the effects on single neurons (Renshaw cells and interneurons) in the cat spinal cord of AMPA, NMDA, **4**, **8**, **12**, **15**, **19**, and (*RS*)-2-(3-carboxyphenyl)glycine. In agreement with earlier findings,[17,18] AMPA was shown to be a very potent excitant, the effects of which were reduced by GDEE but not by the NMDA antagonist 2APV (Figure 1). NMDA-induced excitation was also reduced on a few neurons by GDEE, as shown in Figure 1.

Compound **4** was tested on 10 dorsal horn cells in three cats, and on all cells studied, **4** was more potent than ibotenic acid. In general, the potency and pharmacological profile of **4** was similar to that of AMPA (Figure 1), although the slow onset and offset of the excitation produced

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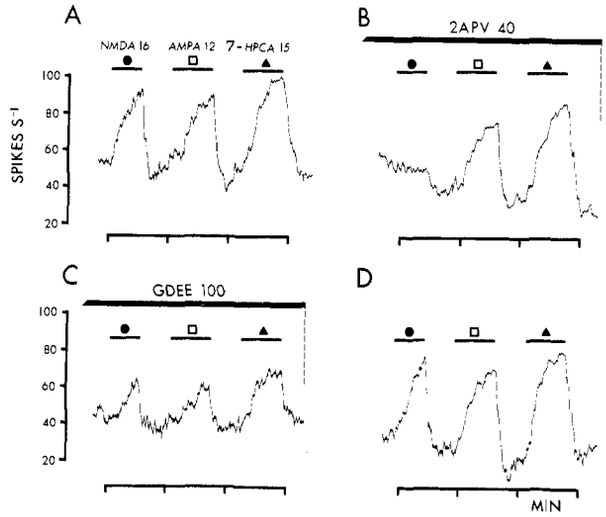

Figure 1. Effects of 2APV and GDEE on the excitation of a spontaneously firing cat spinal interneuron by NMDA, AMPA, and **4** (7-HPCA) ejected with the indicated currents (nanoamperes) for the times shown by the horizontal bars and symbols: (A) before and (B) during 2APV (40 nA), starting and ceasing as indicated by the horizontal and vertical broken lines; (C) after recovery from 2APV and during GDEE (100 nA); (D) 1 min after C. Ordinates: firing rate, spikes per second; abscissas: time, minutes.

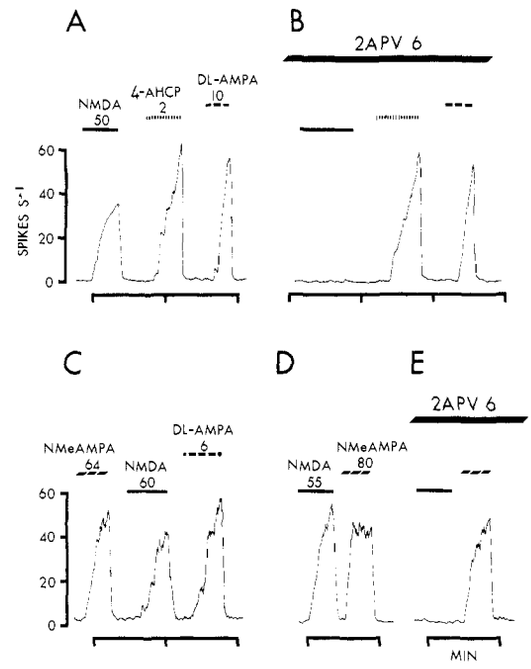

Figure 2. Effects on two cat spinal interneurons (A, B, and C–E) of NMDA, **12** (4-AHCP), AMPA, and **15** (*N*-Me-AMPA) ejected with the indicated currents (nanoamperes) for the times shown by the horizontal bars: (A) before and (B) during the administration of 2APV, which commenced 1 min earlier; recovery (not illustrated) of the excitatory action of NMDA was incomplete 5 min after B; (C) comparison of the effects of **15**, NMDA, and AMPA; (D) before and (E) during the administration of 2APV, which commenced 2 min earlier. Recovery (not illustrated) occurred 4 min after E. Ordinates: firing rate, spikes per second; Abscissas: time, minutes.

by **4** was even more pronounced than in the case of AMPA. With seven cells, excitation by **4** was not affected by simultaneously administered 2APV in amounts sufficient to block NMDA-induced excitation, whereas the effect of **4** was reduced by concentrations of GDEE, which also reduced AMPA- and QUIS-induced excitation.

In Figure 2 the excitatory effects of **12** and **15** are compared with those of AMPA and NMDA on two different cat spinal interneurons. As illustrated, **12** was more potent than AMPA and much more potent than NMDA as a neuronal excitant. While the effects of NMDA on both cells were completely blocked by 2APV, excitation by **12** and AMPA were insensitive to this antagonist (Figure 2) but were reduced by GDEE (not illustrated). A comparison of **12** and QUIS on spinal interneurons indicated approximate equipotency as neuronal excitants.

From Figure 2C it is evident that N-methylation of AMPA resulted in a substantial decrease in potency, and allowing for the different dilutions of the test solutions, **15** was weaker than NMDA as an excitant. The sensitivity of these two excitatory amino acids, both containing methylamino groups, to antagonists was, however, distinctly different. In contrast to NMDA, excitation by **15** was insensitive to 2APV (Figure 2), whereas **15**- and AMPA-induced excitations were sensitive to GDEE.

The potency and sensitivity of **8** to antagonists were compared with those of **12**, QUIS, and NMDA on a number of cat spinal neurons. Compound **12** was a more potent excitant on 18 cells than NMDA, in agreement with the relative potencies depicted in Figure 2, whereas **8** was substantially less active than NMDA (data not shown). Like **12**, compound **8** excited neurons in a GDEE-sensitive and 2APV-insensitive manner, although the effects of these antagonists on **8**-induced excitation were not as clear, as in the case of **12**, because of the much lower potency of **8**.

In spite of the structural similarity between **12** and **19**, the latter amino acid was approximately two orders of magnitude weaker than **12** as a neuronal excitant, and (*RS*)-2-(3-carboxyphenyl)glycine (Chart I) was significantly weaker than **19** (not illustrated). The effects of **19**, as well as of (*RS*)-2-(3-carboxyphenyl)glycine, could be reduced by GDEE but not by 2APV.

Discussion

The aim of this work was to use ibotenic acid as a lead structure for the development of specific agonists at the population of central excitant amino acid receptors at which QUIS is a powerful agonist and GDEE a relatively selective antagonist.[10–13] The potent excitatory effects of ibotenic acid appears to be the result of activation of NMDA receptors.[14] To some extent the excitatory effect of this amino acid is obscurred by its conversion, within spinal tissue in vivo, to a depressant amino acid, presumably muscimol.[15] Such conversion, which results in a prolonged depression of neuronal excitability after excitation by ibotenic acid, was not apparent with the synthetic analogues described in this paper. The pharmacology of these compounds was studied microelectrophoretically on single cat spinal neurons with GDEE and 2APV as selective antagonists at the QUIS and NMDA receptors, respectively, supplemented by receptor-binding studies, with [^{3}H]KA as a ligand.[20]

Molecular orbital calculations show that a conformation of the fully charged molecule of ibotenic acid similar to conformation B (Chart I) is energetically favorable in the conservative state.[16] Allowance for an aqueous medium predicts a change in the conservative-molecule minimum-energy conformation from B into A.[16] These conformations may reflect receptor-active conformations of ibotenic acid at different types of amino acid receptors.[23] In order to shed light on these aspects, we have synthesized and tested

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4, a conformationally restricted analogue of ibotenic acid representing conformation A. Compound 4 proved to be a very active GDEE-sensitive, 2APV-insensitive neuronal excitant (Figure 1) without significant affinity for the KA receptor sites in vitro, suggesting that ibotenic acid could interact with QUIS receptors in a conformation similar to A.

Like 4, AMPA is a powerful and highly selective agonist at QUIS receptors.[17,18] Although the conformation of AMPA[24] and 4[19] in the crystalline states, established by X-ray analyses, are dissimilar, computer-aided force-field calculations[25] have indicated that energy barriers less than 29 kJ/mol separate the conformation of AMPA in the crystalline state from conformations similar to that found for 4 in crystals.[19,23] These studies account for 4 and AMPA having very similar pharmacological profiles.

We have earlier proposed that the methyl group of AMPA forces the molecule to adopt preferentially folded conformations and, consequently, that AMPA and Glu may interact with QUIS receptors in somewhat folded conformations.[17,18] This hypothesis prompted us to synthesize 12 and the isomeric compound 8 (Chart I), of which 12 was designed as a model compound reflecting folded conformations of AMPA. While 8 was only a weak GDEE-sensitive excitant, 12 was shown to be a very powerful excitatory amino acid sensitive to GDEE and insensitive to 2APV (Figure 2). On most cells studied, 12 and QUIS were approximately equipotent, but whereas QUIS is a potent inhibitor of KA binding,[20] 12 was virtually devoid of affinity for these receptor sites. In addition to AMPA and 4, which are analogues of Glu, compound 12, the backbone of which consists of six carbon atoms (Chart I), appears to be a useful tool for studies of the receptors involved in the central excitatory neurotransmission.

In continuation of the structure–activity studies of 12, we synthesized the structurally related amino acid 19 and performed comparative studies on 12, 19, and (*RS*)-2-(3-carboxyphenyl)glycine (Chart I). Compound 19, as well as (*RS*)-2-(3-carboxyphenyl)glycine were GDEE-sensitive, 2APV-insensitive excitants. The former compound was the more potent excitant, but both amino acids were much weaker than 12, indicating a high degree of structural specificity of the QUIS receptors. The relative conformational mobility of 12 and 19 have not been studied in detail yet, but the structure and rotational freedom of their ω-acid groups obviously are different. It is possible that the rigidity of the 3-isoxazolol unit and the adjacent parts of the backbone of 12 facilitates the interaction of 12 with QUIS receptors.

While *N*-Me-Glu, in contrast to Glu, appears to be a selective agonist at NMDA receptors, a similar change in pharmacological profile was not observed after N-methylation of AMPA (Figure 2). AMPA- and 15-induced excitations were selectively reduced by GDEE, but AMPA is the more potent neuronal excitant. These differential effects of N-methylation cannot be explained at the present time, but studies of these aspects are in progress using a variety of different model compounds.

Experimental Section

Melting points are corrected and were determined in capillary tubes. Elemental analyses were performed by P. Hansen, Chemical Laboratory II, University of Copenhagen. IR spectra, obtained on a Perkin-Elmer Grating Infrared spectrophotometer, Model 247, were taken in KBr pellets (crystalline compounds) or by the film technique. ^{1}H NMR spectra were recorded on a Varian T60, a JEOL FX 900, (compounds 8, 15, and 19), or a Bruker HX-270S spectrometer in the Fourier-transform mode to obtain the spectrum with a width of 5000 Hz and with 16K data points and quadrature detection (compound 12). Me_4Si was used as an internal standard, except for the compounds dissolved in D_2O, where sodium 3-(trimethylsilyl)propanesulfonate was used. UV spectra were recorded with methanol as a solvent on a Perkin-Elmer ultraviolet–visible spectrophotometer, Model 402. The mass spectrum of 2 was obtained from a Varian CH5 spectrometer. Thin-layer chromatography (TLC) and gravity column chromatography (CC) were performed with silica gel F_{254} plates (Merck) and silica gel (Woelm, 0.063–0.200 mm), respectively. Compounds containing the 3-isoxazolol unit were visualized on TLC plates with UV light and a $FeCl_3$ spraying reagent (yellow color), and compounds containing amino groups were visualized by a ninhydrin spraying reagent. All evaporations were performed at ~15 mmHg on a rotatory evaporator.

(*RS*)-Methyl 3-Methoxy-6-nitroso-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-7-carboxylate (2). To a stirred solution of 1[26] (495 mg, 2.7 mmol) in dry THF (10 mL) kept at −75 °C was added butyllithium (2.0 mL of a 1.5 M solution in hexane, 3.0 mmol), and after 1 min, methyl chloroformate (2.6 mL, 27 mmol) was added during a period of 1 min. After stirring at −75 °C for 5 min, the solution was evaporated to give a semicrystalline product. TLC [eluent: dichloromethane–ethyl acetate (9:1); visualizer: UV light] of this product revealed the presence of two components: 1 (R_f 0.58) and a compound with R_f 0.64. CC [silica gel, 15 g; eluent: dichloromethane–ethyl acetate (9:1)] gave 1 (93 mg) and 2 (231 mg, 36%): mp 85.0–86.0 °C (ethyl acetate–hexane); mass spectrum: *m/e* 211 (100, M − 30), 182 (79, M − 59), 152 (79, M − 30–59), 124 (24), 109 (12), 94 (15), 82 (17), 67 (29), 59 (49). Anal. ($C_9H_{11}N_3O_5$) C, H, N.

(*RS*)-3-Hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-7-carboxylic Acid Hydrate (4). Through a solution of 2 (231 mg, 0.96 mmol) in glacial acetic acid (12 mL) kept at 10 °C was passed a stream of hydrogen bromide gas, until the color of the solution became pale yellow (5 min). The solution was kept at ~25 °C for 18 h and then evaporated to give TLC-pure 3 (203 mg) [eluent: 1-butanol–glacial acetic acid–water (4:1:1); R_f 0.59]: ^{1}H NMR (D_2O) δ 3.74 (3 H, s), 3.55 (2 H, t, J = 4.5 Hz), 2.65 (2 H, t, J = 4.5 Hz). Without further characterization, 3 (203 mg) was dissolved in water and transferred to a column containing an ion-exchange resin [Amberlite IRA-400 (OH); 100 mL]. After 5 h the column was eluted with acetic acid (1 M) to give 4 (111 mg, 53% based on 2): mp 220 °C dec (water); ^{1}H NMR (D_2O) δ 3.55 (2 H, slightly perturbed t), 2.67 (2 H, slightly perturbed t). Anal. ($C_7H_8N_2O_4 \cdot H_2O$) C, H, N.

3-Methoxy-8-methyl-5,6-dihydro-4*H*-cyclohept[1,2-*d*]isoxazole (6). To a solution of methyl iodide (1.17 g, 8.2 mmol) in dry ether (4 mL) was added magnesium chips (200 mg, 8.3 mmol). After the solution was stirred for 30 min at 0 °C, a solution of 5[27] (1.30 g, 7.2 mmol) in dry ether (25 mL) was added at 0 °C during a period of 30 min, and stirring was continued at 0 °C for 30 min. Upon addition of sulfuric acid (15 mL, 4 M), the mixture was extracted with ether (4 × 10 mL). The combined ether phases were dried ($MgSO_4$) and filtered, and the filtrate was evaporated to give crude 6 (1.20 g). CC [silica gel, 50 g; eluent: toluene–ethyl acetate (5:1)] afforded TLC-pure 6 (728 mg, 57%) as an oil [R_f 0.75; eluent: toluene–ethyl acetate (5:1)]: UV 263 nm (log ε 3.89) nm; ^{1}H NMR (CCl_4) δ 6.0–5.6 (1 H, m), 3.93 (3 H, s), 2.7–2.2 (4 H, m), 2.03 (3 H, s), 1.9–1.6 (2 H, m). Anal. ($C_{10}H_{13}NO_2$) H; C: calcd, 67.02; found, 64.89; N: calcd, 7.82; found: 7.40.

Ethyl α-(Ethoxycarbonyl)-α-acetamido-3-methoxy-5,6-dihydro-4*H*-cyclohept[1,2-*d*]isoxazole-8-propionate (7). To a refluxing solution of 6 (450 mg, 2.5 mmol) in tetrachloromethane (10 mL) was added, during a period of 3 h, *N*-bromosuccinimide (NBS; 670 mg, 3.8 mmol) in portions of ~100 mg and benzoyl peroxide (10 mg) in portions of ~2 mg. The reaction mixture was filtered, and the filtrate was evaporated to give crude 3-methoxy-8-(bromomethyl)-5,6-dihydro-4*H*-cyclohept[1,2-*d*]isoxazole, identified by a signal at δ 4.38 (~2 H, s, CH_2Br) in the ^{1}H NMR spectrum (CCl_4). TLC (eluent: toluene; visualizers: UV light and iodine vapor) revealed the presence of a main reaction

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product (R_f 0.23) and a number of minor constituents, including **6** (R_f 0.20). A solution of this crude product in ethanol (2 mL) was added to a solution of diethyl acetamidomalonate (545 mg, 2.5 mmol) in a solution of sodium ethoxide prepared from sodium (58 mg, 2.5 mmol) and ethanol (5 mL). After reflux for 4 h, evaporation, and addition of water (10 mL), the mixture was extracted with dichloromethane (5 × 10 mL). The combined organic phases were washed with sodium hydroxide (50 mL, 1 M, 0 °C) and evaporated. CC [silica gel, 35 g; eluent: dichloromethane–ethyl acetate (5:1)] gave **7** (153 mg, 22%) as an oil: UV 259 nm (log ε 4.10); ^{1}H NMR ($CDCl_3$) δ 6.75 (1 H, br s), 5.87 (1 H, br d), 4.33 and 4.28 (4 H, 2 q, J = 6 Hz), 3.99 (3 H, s), 3.71 and 3.50 (2 H, dd, J = 5 Hz), 2.8–1.5 (m), 2.11 and 2.05 (2 s) (a total of 9 H), 1.30 and 1.25 (6 H, 2 q, J = 6 Hz). Anal. Calcd for $C_{19}H_{26}N_2O_7$: C, 57.86; H, 6.64; N, 7.10. Found: C, 55.98; H, 7.16; N, 5.89.

(*RS*)-α-Amino-3-hydroxy-5,6-dihydro-4*H*-cyclohept[1,2-*d*]isoxazole-8-propionic Acid Hydrobromide (8). A solution of **7** (20 mg, 0.051 mmol) in hydrobromic acid (0.5 mL, 48%) was refluxed for 15 min. The solution was filtered, and the filtrate evaporated to give a dark brown oil, from which crude **8** crystallized. Recrystallization (methanol–ether) afforded **8** (2.3 mg, 14%): mp 200 °C dec; IR 3450 (s), 3300–2500 (several bands, m–s), 1730 (s), 1650 (m), 1635 (m), 1545 (m), 1415 (m) cm^{-1}; UV 256 nm (log ε 3.46); ^{1}H NMR (D_2O) δ 5.82 (1 H, br s), 3.86–3.63 (1 H, dd, J = 8 and 15 Hz), 2.86 (d, J = 15 Hz), 2.60 (br d, J = 8 Hz), 2.9–2.0 (m) (a total of 6 H).

3-Methoxy-4-methyl-7,8-dihydro-6*H*-cyclohept[1,2-*d*]-isoxazole (10). Compound **10** was synthesized as described above for **6** by using **9**[27] (1.00 g, 5.5 mmol), magnesium chips (151 mg, 6.3 mmol), and methyl iodide (900 mg, 6.3 mmol). CC [silica gel: 90 g; eluent: toluene–ethyl acetate (4:1)] of crude **10** (965 mg), followed by ball-tube distillation (0.4 mm; oven temperature 165 °C) gave **10** (400 mg, 40%). Anal. ($C_{10}H_{13}NO_2$) C, H, N.

Ethyl α-(Ethoxycarbonyl)-α-acetamido-3-methoxy-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazole-4-propionate (11). Compound **11** was synthesized via 3-methoxy-4-(bromomethyl)-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazole in analogy with the synthesis of **7**, using **10** (400 mg, 2.2 mmol), NBS (600 mg, 3.4 mmol), benzoyl peroxide (10 mg), diethyl acetamidomalonate (484 mg, 2.2 mmol), and sodium (51 mg, 2.2 mmol). CC [silica gel, 35 g; eluent: dichloromethane–ethyl acetate (2:1)], followed by recrystallization (ethyl acetate) of crude **11**, gave **11** (116 mg, 19%), mp 150.5–151.5 °C. Anal. ($C_{19}H_{26}N_2O_7$) C, H, N.

(*RS*)-α-Amino-3-hydroxy-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazole-4-propionic Acid Hydrobromide (12). Crude **12** was synthesized as described above for **8** by using **11** (108 mg, 0.27 mmol) and hydrobromic acid (1.5 mL, 48%). Recrystallization (2-propanol) gave **12** (37 mg, 43%): mp 185 °C dec; UV 250 nm (log ε 3.45) nm; ^{1}H NMR (Me_2SO-d_6) δ 12 (2 H, br s), 5.82 (1 H, t, J = 6.1 Hz), 3.94 (1 H, br s), 3.6–3.1 (3 H, br signal), 3.18 (1 H, dd, J = 5.9 and 13.7 Hz), 2.97 (2 H, t, J = 6 Hz), 2.76 (1 H, dd, J = 9.5 and 13.7 Hz), 2.33 (2 H, m), 2.0–1.8 (2 H, dm). Anal. ($C_{11}H_{14}N_2O_4$·HBr) C, H, N, Br.

Ethyl α-(Ethoxycarbonyl)-α-(*N*-methylacetamido)-3-methoxy-5-methylisoxazole-4-propionate (14). To a suspension of sodium hydride (48 mg, 2.0 mmol) in dry *N*,*N*-dimethylformamide (DMF; 5 mL), kept at temperatures below 40 °C, was added a solution of **13**[28] (500 mg, 1.5 mmol) in dry DMF (5 mL) during a period of 5 min. Upon addition of methyl iodide (1.0 g, 7.0 mmol), the mixture was heated at 80 °C for 45 min and then evaporated. A mixture of this residue and water (25 mL) was extracted with ether (5 × 25 mL). The combined ether phases were dried ($MgSO_4$) and evaporated to give an oil. CC [silica gel, 50 g; eluent: toluene–ethyl acetate (1:1)] afforded **14** (322 mg, 62%) as an oil. Anal. ($C_{16}H_{24}N_2O_7$) C, H, N.

(*RS*)-α-(Methylamino)-3-hydroxy-5-methylisoxazole-4-propionic Acid Hydrobromide Hemihydrate (15). A solution of **14** (320 mg, 0.9 mmol) in hydrobromic acid (1.5 mL; 48%) was refluxed for 15 min and then evaporated. Crude **15** was recrystallized [methanol–ether (1:1)] to give **15** (167 mg, 64%), mp 185.5–186.5 °C; UV 219 nm (log ε 3.71); ^{1}H NMR (D_2O) δ 3.92 (1 H, t, J = 6 Hz), 2.91 (2 H, d, J = 6 Hz), 2.72 (3 H, s), 2.20 (3 H, s). Anal. ($C_8H_{12}N_2O_4$·HBr·$^1/_2H_2O$) C, H, N, Br.

Ethyl α-(Ethoxycarbonyl)-α-acetamido-β-[2-(methoxycarbonyl)phenyl]propionate (17). To a suspension of sodium hydride (24 mg, 1.0 mmol) in dry *N*,*N*-dimethylacetamide (DMA; 1 mL), kept at 10–15 °C, was added dropwise a solution of diethyl acetamidomalonate (217 mg, 1.0 mmol) in dry DMA, followed by the dropwise addition of a solution of **16**[29] (229 mg, 1.0 mmol) in dry DMA (2 mL). The mixture was heated at 100 °C for 4 h, and then evaporated. Upon addition of water (10 mL) the mixture was extracted with ether (2 × 10 mL), and the combined ether phases were evaporated to give an oil. CC [silica gel, 40 g; eluent: toluene–ethyl acetate (3:1)] gave **17** (187 mg, 51%), mp 63.5–64.5 °C (toluene). Anal. ($C_{18}H_{23}NO_7$) C, H, N.

(*RS*)-1-Oxo-1,2,3,4-tetrahydroisoquinoline-3-carboxylic Acid (18) and (*RS*)-β-(2-Carboxyphenyl)alanine Hydrobromide Hydrate (19). A solution of **17** (150 mg, 0.41 mmol) in hydrobromic acid (4 mL, 48%) was refluxed for 3 h. TLC [eluent: 1-butanol–glacial acetic acid–water (4:1:1)] of the evaporated reaction mixture revealed the presence of two components with R_f 0.57 (visualizer: UV light; iodine vapor) and 0.31 (visualizer: UV light; ninhydrin). Upon addition of water (10 mL), the mixture was extracted with ethyl acetate (4 × 10 mL). The combined, dried ($MgSO_4$), and evaporated organic phases afforded **18** (52 mg, 66%), mp 238.0–240.0 °C (water) (lit.[30] mp 235–237 °C).

The aqueous phase was evaporated, and the residue was recrystallized (glacial acetic acid) to give **19** (28 mg, 23%): mp 156.0–157.5 °C; IR 3600–2700 (several bands, s–m), 1750 (s), 1675 (s), 1595 (m) cm^{-1}; ^{1}H NMR (D_2O) δ 7.9–7.3 (4 H, m), 4.29 (1 H, dd, J = 6.4 and 7.9 Hz), 3.47 and 3.55 (2 H, m, J = 6.4, 7.9, and 13.7 Hz). Anal. ($C_{10}H_{11}NO_4$·HBr·H_2O) C, H, N, Br.

Microelectrophoretic Studies. Experiments were performed on lumber dorsal horn interneurons and Renshaw cells of cats anesthetized with pentobarbitone sodium (35 mg/kg intraperitoneally initially, supplemented intravenously when required). Extracellular action potentials were recorded by means of the center barrel of seven-barrel micropipets, which contained 3.6 M NaCl. The compounds were administered electrophoretically from the outer barrels of the micropipets,[21] which contained aqueous solutions of the following compounds: GDEE (0.2 M, pH 3.5), 2APV (0.05 M in 0.15 M NaCl, pH 7.6), NMDA (0.05 M in 0.15 M NaCl, pH 7.5), QUIS (0.005 M in 0.15 M NaCl, pH 7.5), ibotenic acid (0.05 M in 0.15 M NaCl, pH 7.4), AMPA (0.1 M, pH 8), **4** (0.1 M, pH 7.3), **8** (0.05 M in 0.15 M NaCl, pH 7.5), **12** (0.05 M in 0.15 M NaCl, pH 7.5), **15** (0.1 M, pH 7.5), **19** (0.1 M, pH 7.6), and (*RS*)-2-(3-carboxyphenyl)glycine (0.1 M, pH 7.6). The excitatory amino acids were administered for times sufficient to obtain maximal effects at the particular rate of ejection. The relative potencies of the compounds were determined from a comparison of electrophoretic currents required to produce equal and submaximal excitation of the cells, making allowance for the dilution of some amino acids in 0.15 M NaCl. Antagonism was apparent from a slower onset and reduced degree of excitation.

Kainic Acid Binding Studies. The effects of the compounds on KA binding were studied as described earlier[17] on the basis of a published procedure.[20] The membrane preparation was frozen rapidly at −70 °C and kept at −20 °C for at least 18 h before use in the receptor-binding assay. For the [^{3}H]KA binding assay procedures, aliquots of synaptic membranes (0.8–1.2 mg of protein) were incubated in triplicate at 4 °C for 20 min in 2 mL of 0.05 M Tris–citrate buffer (pH 7.1) containing 0.005 μM [^{3}H]KA, and the IC_{50} values of the agents tested were determined by conventional procedures.

Acknowledgment. This work was supported by grants from the Danish Medical Research Council and by the Australian National University. The collaboration with Dr. M. J. Peet, Dr. J. D. Leah, R. Malik (Canberra), and Dr. K. Schaumburg (Copenhagen), the technical assistance

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of P. Searle, J. S. Johansen, and S. Stilling, and the secretarial assistance of B. Hare are gratefully acknowledged. Dr. J. C. Watkins kindly supplied 2APV. Part of this work was performed at the Department of Medicinal Chemistry, University of Kansas, Lawrence, KS, and Drs. Mertes and L. A. Mitscher are acknowledged for fruitful discussions.

Registry No. 1, 82988-61-2; 2, 89017-61-8; 3, 89017-62-9; 4, 89017-63-0; 5, 65202-90-6; 6, 89017-64-1; 7, 89017-65-2; 8, 89017-66-3; 9, 65202-91-7; 10, 89017-67-4; 11, 89017-68-5; 12, 89017-69-6; 13, 75989-23-0; 14, 89017-70-9; 15, 89017-71-0; 16, 2417-73-4; 17, 89017-72-1; 18, 89017-73-2; 19, 89017-74-3; diethyl acetamidomalonate, 1068-90-2; 3-methoxy-8-(bromomethyl)-5,6-dihydro-4*H*-cyclohept[1,2-*d*]isoxazole, 89017-75-4; 3-methoxy-4-(bromomethyl)-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazole, 89017-76-5; (±)-ibotenic acid, 31758-99-3.

2-Substituted Thiazolidine-4(*R*)-carboxylic Acids as Prodrugs of L-Cysteine. Protection of Mice against Acetaminophen Hepatotoxicity

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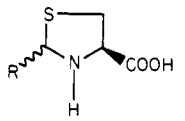
A number of 2-alkyl- and 2-aryl-substituted thiazolidine-4(*R*)-carboxylic acids were evaluated for their protective effect against hepatotoxic deaths produced in mice by LD_{90} doses of acetaminophen. 2(*RS*)-Methyl-, 2(*RS*)-*n*-propyl-, and 2(*RS*)-*n*-pentylthiazolidine-4(*R*)-carboxylic acids (compounds **1b,d,e**, respectively) were nearly equipotent in their protective effect based on the number of surviving animals at 48 h as well as by histological criteria. 2(*RS*)-Ethyl-, 2(*RS*)-phenyl-, and 2(*RS*)-(4-pyridyl)thiazolidine-4(*R*)-carboxylic acids (compounds **1c,f,g**) were less protective. The enantiomer of **1b**, viz., 2(*RS*)-methylthiazolidine-4(*S*)-carboxylic acid (**2b**), was totally ineffective in this regard. Thiazolidine-4(*R*)-carboxylic acid (**1a**), but not its enantiomer, **2a**, was a good substrate for a solubilized preparation of rat liver mitochondrial proline oxidase [$K_m = 1.1 \times 10^{-4}$ M; $V_{max} = 5.4$ μmol min^{-1} (mg of protein)$^{-1}$]. Compound **1b** was not a substrate for proline oxidase but dissociated to L-cysteine in this system. At physiological pH and temperature, the hydrogens on the methyl group of **1b** underwent deuterium exchange with solvent D_2O ($k_1 = 2.5 \times 10^{-5}$ s), suggesting that opening of the thiazolidine ring must have taken place. Indeed, **1b** labeled with ^{14}C in the 2 and methyl positions was rapidly metabolized by the rat to produce $^{14}CO_2$, 80% of the dose being excreted in this form in the expired air after 24 h. It is suggested that these 2-substituted thiazolidine-4(*R*)-carboxylic acids are *prodrugs of* L-*cysteine* that liberate this sulfhydryl amino acid in vivo by nonenzymatic ring opening, followed by solvolysis.

In an earlier communication,[1] we advanced the hypothesis that thiazolidine-4-carboxylic acids, in particular, the 2-substituted thiazolidine-4-carboxylic acids exemplified by 2(*RS*)-methylthiazolidine-4(*R*)-carboxylic acid (**1b**), can be considered to be prodrug forms of L-cysteine



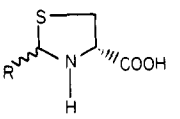
1a, R = H
b, R = CH_3
c, R = C_2H_5
d, R = *n*-C_3H_7
e, R = *n*-C_5H_{11}
f, R = C_6H_4
g, R = 4-C_5H_4N

and provided evidence that **1b** can protect mice against acetaminophen-induced hepatic necrosis and death. In contrast, the parent thiazolidine-4(*R*)-carboxylic acid (**1a**) without alkyl substitution at C-2 was shown to be much less protective than **1b**; indeed, **1a** exhibited CNS toxicity at one-third the molar dose of **1b**.[1]

We now present evidence that other 2-alkyl- and 2-aryl-substituted thiazolidine-4(*R*)-carboxylic acids prepared from L-cysteine and the corresponding aldehydes are equally or nearly equally as protective as **1b** against acetaminophen-induced toxic deaths. We also show that (a) **1b** is not a substrate for rat liver mitochondrial proline oxidase; (b) the methyl group of **1b** undergoes deuterium exchange at physiological pH and temperature, a result possible only through opening of the thiazolidine ring; and (c) **1b** labeled with ^{14}C at the 2 and the methyl positions gives rise to the excretion of $^{14}CO_2$ in the expired air of mice administered the labeled compound, thereby supporting our premise that L-cysteine and acetaldehyde are liberated nonenzymatically in vivo from **1b**.

Results

Protection against Acetaminophen Toxicity by 2-Substituted Thiazolidine-4(*R*)-carboxylic Acids. The results shown in Table I suggest that, in general, 2-alkyl- and 2-phenylthiazolidine-4(*R*)-carboxylic acids (**1b** through **1f**) can protect mice against lethality by LD_{90} doses of acetaminophen even at half the molar dose of the latter. The 2-(4-pyridyl) analogue (**1g**) was not particularly effective in this regard, although no deaths were observed with this compound administered alone. The 2-ethyl analogue (**1c**) was less protective than **1b** but was comparable to the 2-phenyl analogue **1f** based on toxic deaths. However, the latter was much more protective than **1c** by histological criteria (Table I). Of mechanistic significance, whereas **1b** was protective, the enantiomeric 2(*RS*)-methylthiazolidine-4(*S*)-carboxylic acid (**2b**) derived from D-cysteine and acetaldehyde did not afford any protection.



2a, R = H
b, R = CH_3

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‡ Hennepin County Medical Center.

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0022-2623/84/1827-0591$01.50/0