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Perspective

Excitatory Amino Acid Neurotransmission

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In the mammalian central nervous system (CNS) there are literally billions of neuronal synapses with each employing a particular neurotransmitter. A neurotransmitter can be either excitatory or inhibitory, depending on whether it produces depolarization or hyperpolarization of the neuronal membrane, respectively. Furthermore, the duration of transmitter action either may be in the low millisecond time range or it may show a prolonged latency and duration of action spanning tenths of seconds or seconds. An attractive model for neuronal organization suggests that there is considerable specificity of the various transmitters for these differing roles.[1] While such "classical neurotransmitters" as acetylcholine and the catecholamines, dopamine and norepinephrine, may show either excitatory or inhibitory actions, they also, in most if not all cases, act in the CNS by mechanisms that are of prolonged duration and serve to modulate the intensity of millisecond excitatory or inhibitory impulses.[2] Escalating evidence suggests that the neurotransmitter receptors for these systems are coupled to gated ion channels via guanine nucleotide–binding proteins (G proteins) and second messenger intermediates. This mechanism provides great flexibility for adjusting the intensities and durations of many different stimuli. In contrast, millisecond neurotransmitters act on receptors that are coupled to a gated ion channel located on the same transmembrane protein molecule. This arrangement allows microsecond triggering of channel opening in response to binding of the effector molecule. Like the slow-acting modulatory neurotransmitters, millisecond neurotransmitters may also exhibit inhibitory or excitatory effects. For example, the neurotransmitters γ-aminobutyric acid (GABA) and glycine are thought to be millisecond inhibitory neurotransmitters. However, the most prevalent neurotransmitter class in the mammalian CNS would appear to be the millisecond excitatory neurotransmitters. Foremost among the candidates for millisecond excitatory neurotransmitters in the CNS are the acidic amino acids L-glutamic acid (**1**) and L-aspartic acid (**2**).[3-6] Glutamic acid, in particular, has been shown to satisfy many of the criteria required for a substance to be designated as a neurotransmitter.[7,8]

It has been over 30 years since Hayashi[9] first reported the convulsant effects of L-glutamic acid and over 25 years since Curtis et al.[10] showed through intracellular recording that glutamic acid was capable of depolarizing individual neurons in the mammalian CNS. Since that time a wide array of electrophysiological, biochemical, pharmacological, and anatomical studies have been carried out using excitatory amino acids. These studies and in particular the studies that have utilized various agonists and antagonists of excitatory neurotransmission have indicated that at least four receptor systems may mediate the actions of the excitatory amino acids.[11-15] Three of these receptors, the *N*-methyl-D-aspartate (NMDA) receptor, the quisqualate receptor, and the kainate receptor were named after the agonists *N*-methyl-D-aspartic acid (**3**), quisqualic acid (**4**), and α-kainic acid (**5**), respectively.[16] The fourth, the AP4 receptor, was named for a potent antagonist of specific excitatory pathways of the CNS, namely, L-2-amino-4-phosphonobutanoic acid (**6**). There is increasing evidence that these prototypic pharmacological agents, particularly

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(16) An alternative terminology, AA_1, AA_2, AA_3, and AA_4, has been proposed for these receptors by Fagg et al. in ref 13.

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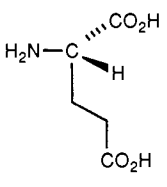
quisqualic acid and **6**, may mediate multiple functions in the CNS. Therefore, identification of additional subclasses of excitatory amino acid receptors appears likely.



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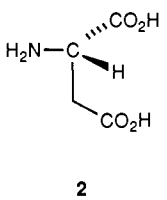

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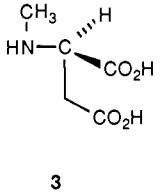

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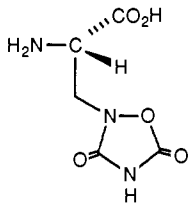

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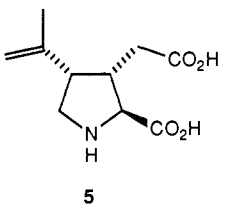

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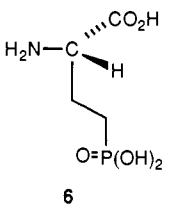

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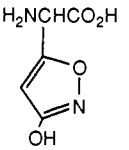
In this perspective, we will briefly summarize salient features of each of these receptor types. For each, we will describe the structural features of the pharmacological agonists and antagonists and available evidence suggesting possible natural agonists. We will also present some properties of the ligand–receptor binding sites that have been identified by radioligand binding–displacement assays using these specific acidic amino acid agonists and antagonists. We will describe neural systems in which these receptors seem to play prominent roles. We will then examine the broader question of the capabilities and limitations of correlating pharmacologically defined excitatory amino acid receptors with radioligand–receptor binding sites. Finally, we will summarize our view of the status of efforts to identify acidic amino acid receptors which mediate millisecond excitatory neurotransmission.

NMDA Receptor

The best characterized excitatory amino acid receptor is the NMDA receptor. This receptor is selectively activated by *N*-methyl-D-aspartic acid (**3**) and is highly sensitive to Mg^{2+}, which has been shown to block the NMDA ion channel in a voltage-dependent manner.[17,18] Structure–activity studies have been conducted on both agonists and antagonists of the NMDA receptor. For agonists, activity and the nature of the side-chain acidic function are generally related as follows: $CO_2H > SO_3H > PO_3H_2$.[14] However, other acidic side-chain functionalities are also consistent with agonist activity at the NMDA receptor. An example is the isoxazole moiety found in the NMDA receptor agonist ibotenic acid (**7**).[19] With respect to the number of atoms separating the side-chain acidic function and the α-acidic function, it has been shown in the spinal cord that L-monoamino dicarboxylic acids with a connecting chain of up to six carbon atoms possess agonist activity with aspartic acid and glutamic acid exhibiting the highest activity.[20]

Conformationally constrained analogues of glutamic acid such as *trans*-2,3-piperidinedicarboxylic acid (**8**)[21] and *cis*-1(*R*)-amino-1,3(*R*)-dicarboxycyclopentane (**9**)[22,23] have been found to be NMDA receptor agonists, thus suggesting that the bioactive conformation of glutamic acid at the NMDA receptor may be a folded one. Quinolinic acid (**10**), a planar heteroaromatic metabolite of tryptophan, also has been shown to act as an agonist at NMDA receptors.[24,25]



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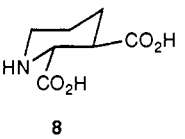

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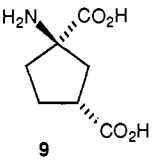

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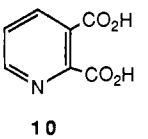

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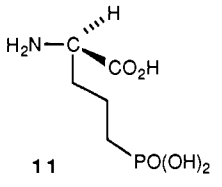
Since 1981, the identification and study of NMDA receptors has been greatly facilitated by the discovery of selective antagonists with potencies at micromolar concentrations. In general there are three structural features seen in such antagonists of the NMDA receptor.[14] First of all, they are α-amino acids that possess the D configuration. Secondly, the chain length that separates the α-acidic function and the acidic side-chain moiety is either four or six atoms. Finally, antagonist potency varies according to the following side-chain acidic functions: $PO_3H_2 > CO_2H > SO_3H$. Examples of some of the most potent NMDA receptor antagonists are D-2-amino-5-phosphonopentanoic acid (**11**),[26] D-2-amino-7-phosphonoheptanoic acid (**12**),[27] the dipeptide (*β*-D-aspartylamino)methylphosphonic acid (**13**),[28] and, most potent of all, the conformationally constrained antagonist 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (**14**).[29,30] Compounds **11**–**14** are all competitive antagonists of the NMDA receptor and potent antagonists of NMDA-induced depolarization of spinal motoneurons, but they have little effect on kainic acid or quisqualic acid induced depolarizations.[29-32]



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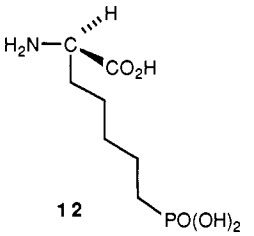

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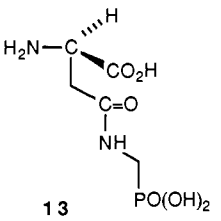

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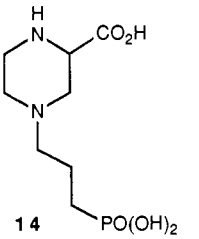

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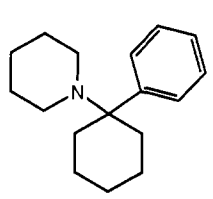
More recently, noncompetitive antagonists of the NMDA receptor have been discovered.[33] Examples of such antagonists are phencyclidine (**15**),[34] *N*-allylnormetazocine (**16**),[35] and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (**17**, MK-801).[36,37] The results of both electrophysiological and binding studies indicate that these noncompetitive antagonists interact with the activated state of the cation channel that is associated with the NMDA receptor and that these antagonists are use-dependent since their antagonism of the receptor is increased by the presence of NMDA receptor agonists.[37-40] Recently, the messenger RNA for the NMDA receptor from rat brain has been injected into *Xenopus* oocytes and has been shown to be expressed as active NMDA channels in the oocyte membrane.[41] The channel protein expressed in oocytes has been shown to be blocked by phencyclidine derivatives and by **17**.[42]



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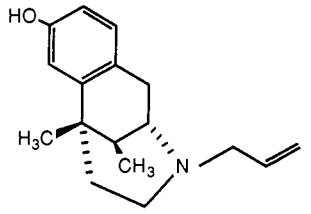

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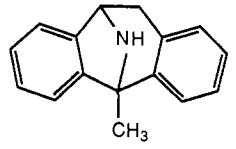

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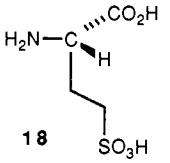
The amino acid glycine appears to also modulate NMDA receptors, since it potentiates NMDA responses by increasing the frequency of channel opening.[43] Although this modulatory glycine binding site has yet to be fully explored, it may very well offer another avenue for the development of agents that can affect the NMDA receptor.

Several radioligand binding assays for the NMDA receptor have been developed utilizing either NMDA receptor agonists or antagonists as the radioligands.[12,14] Among the agonists, [^{3}H]glutamic acid, despite its lack of selectivity, appears to be the most useful radioligand because of its high affinity.[44-46] Several of the competitive NMDA receptor antagonists have proven to be very good radioligands for the NMDA receptor because of their relatively high affinity and their excellent selectivity (these antagonists reduce glutamate binding but have little effect on either kainic acid or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [**21**, AMPA] binding). Examples of two antagonists that have been successfully employed as NMDA receptor radioligands are [^{3}H]-**11**[47] and [^{3}H]-**14**.[48] In these binding studies a strong correlation between electrophysiological activity at NMDA receptors and binding affinity to NMDA binding sites in membrane preparations has been observed.[14]

The natural neurotransmitter at the NMDA receptor remains unknown. Early hypotheses proposed that aspartic acid was the NMDA receptor neurotransmitter. However, the high affinity exhibited by glutamic acid in the NMDA binding assays has led many to suggest that it is the neurotransmitter at the NMDA receptor. The presence of L-homocysteic acid (**18**) in the brain and its relatively good affinity for NMDA receptors have led some investigators to hypothesize that this material may be the natural neurotransmitter.[49,50]



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NMDA receptors play an important role in long-term potentiation[51] and this may have implications for learning and memory and synaptic plasticity.[52,53] NMDA receptor agonists thus might have potential as nootropic agents, providing the excitotoxicity of such agents can be eliminated. Since both the competitive and noncompetitive NMDA receptor antagonists have been shown to be effective anticonvulsants in various animal models of epilepsy,[54-57] such agents have the potential of being useful in treating such disorders. In the case of the competitive antagonists a major drawback is the generally low activity of these compounds after peripheral administration. Their polar nature not only presents problems for oral bioa-

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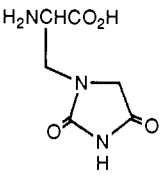
vailability but also for access to the CNS. This particular problem may possibly be overcome by the design of more lipophilic agents or of appropriate prodrugs. Although the noncompetitive antagonists are readily able to enter the CNS, they suffer from a potentially more serious problem in that they produce such undesirable side effects as confusional states, amnesia, and muscle relaxation. Evidence suggests, however, that the competitive antagonists also are capable of producing such side effects.[58] This suggests that such side effects may in fact be tied to the drug's effect on NMDA receptors. If this proves to be the case, this could severely limit the potential usefulness of these types of agents.

The observations over the years that glutamic acid causes neuronal degeneration led Olney to propose the excitotoxic hypothesis.[59] He postulated that the neurotoxicity of glutamic acid was the result of this excitatory amino acid depolarizing its postsynaptic receptor, thereby bringing about detrimental changes in the ion balance in the cell. This has led to speculation that the excitatory amino acids may be involved in neurodegenerative diseases such as Alzheimer's disease, Huntington's chorea, and ischemic brain damage.[56,60-62] The NMDA receptor antagonists, in particular **17**, have been shown to be effective in blocking neurodegeneration brought about by hypoxia.[63]

Quisqualate Receptor

This excitatory amino acid receptor system was initially identified through the actions of the relatively selective agonist, quisqualic acid (**4**). This receptor, along with the kainate receptor, is thought to mediate fast excitatory synaptic transmission.

Structure–activity studies with quisqualic acid have been very limited. Bycroft et al.[64] have reported a general synthetic procedure for the synthesis of quisqualic acid and related analogues. They have synthesized the D-isomer of quisqualic acid as well as the hydantoin (**19**) and triazolidinedione (**20**) analogues. These analogues have been tested on glutamatergic nerve–muscle junctions of the locust.[65] Surprisingly, D-quisqualic acid showed significant activity, while the hydantoin and triazolidinedione analogues were inactive. The lack of activity of **19** may be due to its higher pK_a (8.2) compared to that seen in quisqualic acid (4.2). This would not explain the inactivity of **20**, however, since its pK_a value of 4.7 is comparable to that of quisqualic acid. To explain the activity of D-quisqualic acid and the inactivity of **19** and **20**, Boden et al.[65] have postulated that a pyramidal configuration of the β-heterocyclic nitrogen atom of quisqualic acid is capable of interconversion and that this may account for the potency of the L-isomer and the activity of the D-isomer of quisqualic acid. The corresponding nitrogen atom in analogues **19** and **20** is planar.



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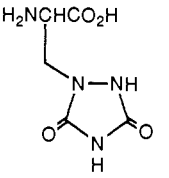
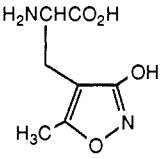


20

In an attempt to prepare analogues of ibotenic acid, an excitatory amino acid analogue isolated from *Amanita* mushrooms, Krogsgaard-Larsen et al.[66] synthesized **21**. This analogue was found to be a selective agonist at the quisqualate receptor with very little affinity for either the NMDA or kainate receptors. It is presently the ligand of choice in identifying the quisqualate receptor, since it induces depolarization analogous to quisqualic acid, but as pointed out below, it does not antagonize chloride-dependent sequestration and *N*-acetyl-L-aspartyl-L-glutamyl peptidase, or sensitize neurons to **6** as quisqualic acid does. Resolution of **21** and the evaluation of the enantiomers in binding studies indicate that the L-isomer is the more active isomer.[67]



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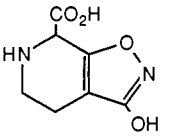

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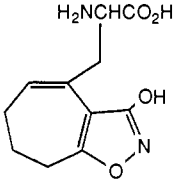

23

Two conformationally constrained analogues of **21**, compounds **22** and **23**, have been synthesized and found to be highly selective agonists at quisqualate receptors with potencies comparable to that of **21**.[68] From these studies it has been proposed that **21** and glutamic acid interact with the quisqualate receptor in a folded conformation. Interestingly, as pointed out above, studies with conformationally constrained NMDA receptor agonists also point to a folded conformation of glutamic acid at the NMDA receptor. These results, thus, would seem to suggest that different folded conformations of glutamic acid are involved in its interaction with either NMDA or quisqualate receptors.

One of the major drawbacks to the characterization of this class of receptors has been the lack of good selective antagonists. Early work relied on the diethyl ester of glutamic acid, but this agent is a very weak antagonist and is easily prone to hydrolysis. Many of the present antagonists of quisqualate receptors are rather nonselective, in the sense that they are also capable of blocking NMDA and kainate receptors. Examples of antagonists that fall into this category are kynurenic acid (**24**)[69-71] and the piperazine-2,3-dicarboxylates **25** and **26**.[72] The sulfonate peptide (γ-glutamylamino)methanesulfonic acid (**27**) also shows slight selectivity for quisqualate and kainate re-

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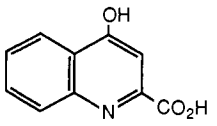
ceptors.[28] Recently, novel and potent inhibitors of kainic acid and quisqualic acid, but not NMDA responses, have been described. The most potent of these compounds is 6-cyano-7-nitro-2,3-dihydroxyquinoxaline (**28**).[73] Preliminary studies on **28** show potent inhibition of non-NMDA excitation in cortical and hippocampal neurons.[74-76] The continued development of such selective antagonists should facilitate the characterization of the quisqualate receptor.



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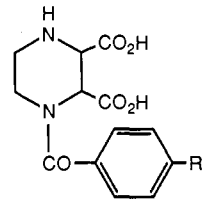
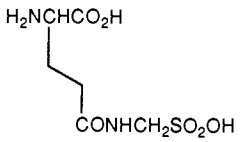


25: R = Cl
26: R = Br



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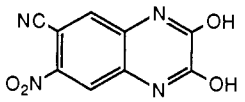

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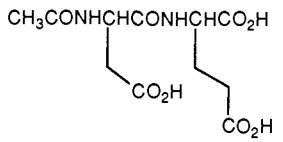

29

At present, [^{3}H]-**21** is the ligand used to label the quisqualate receptor. Although there is significant overlap in the distribution of NMDA and quisqualate receptors, **21** possesses a distinct distribution. The pharmacological profile of **21** binding is also distinct and there is a reasonably good correlation between displacement of [^{3}H]-**21** binding and excitatory potency.

Recent studies have clearly shown that quisqualic acid is not as selective as originally thought since it has been found to affect a number of other processes within the CNS. For example, it has been shown to block a chloride-dependent glutamic acid neuronal sequestration process.[77] It also has been found to be a very good inhibitor of a peptidase that cleaves the excitatory neuropeptide *N*-acetyl-L-aspartyl-L-glutamic acid (**29**)[78] and to be able to sensitize hippocampally evoked responses to blockade by **6**.[79] With regard to this latter phenomenon it has been found that the exposure of hippocampal slices to quisqualic acid sensitized the synaptic responses to the agonist effects of **6**. Concentrations of quisqualic acid that depressed extracellular field potentials by 50% led to an 33-fold increase in the potency of **6**. The sensitization began to occur within 4 min of exposure and lasted more than 90 min.[79,80] Interestingly, the induced depolarizations by **6** of cortical neurons sensitized by quisqualic acid were blocked by **28**.[81]

Since quisqualic acid has been shown to possess a variety of biological effects, the development of selective antagonists of these effects would be an important advance. Such specific antagonists could serve as valuable tools in helping to delineate the role that the various processes affected by quisqualic acid play in excitatory neurotransmission.

Kainate Receptor

The prototypic agonist at this receptor is α-kainic acid (**5**), which was originally isolated from the seaweed *Digenea simplex*. Domoic acid (**30**) from the seaweed *Chondria armata* is an even more potent agonist than kainic acid at this receptor.[82] The unsaturated isopropylidene side chain of **5** appears essential for high activity since dihydrokainic acid has less agonist activity than the weak agonist glutamic acid.[83] Various stereoisomers of **5** have been evaluated as kainate receptor agonists. These include α-allokainic acid (**31**) and β-kainic acid (**32**). Both of these stereoisomers however show very little activity.[84,85] Interestingly, the *cis*- and *trans*-4-carboxyl analogues **33** and **34**, as well as the *cis*- and *trans*-4-methyl ketone analogues **35** and **36**, have been reported to possess neuroexcitatory activity similar to that of kainic acid.[86] Recently, Curry et al.[23] have reported that the cis-(1*S*,3*S*),trans-(1*S*,3*R*,), and trans-(1*R*,3*S*) isomers of 1-amino-1,3-cyclopentanedicarboxylic acid resemble kainic acid on the CA1 pyramidal neurons of rat hippocampal slices. As has been mentioned above, the cis-(1*R*,3*R*) isomer, compound **9** behaves as an NMDA receptor agonist.

Recently, two amino acids that are structurally related to **5** and **30** have been isolated from the poisonous mushroom *Clitocybe acromelalga*.[87] These two novel neuroexcitatory amino acids have been named acromelic acid A (**37**) and B (**38**). Both **37** and **38** are reported to be 100 times more potent than kainic acid in depolarizing the crayfish neuromuscular junction.[88] Whether this dramatic difference will also be seen in mammalian systems has yet to be determined.

As in the case of quisqualate receptors, potent and specific antagonists of kainate receptors are lacking and this has hindered further characterization of this class of excitatory amino acid receptors. The sulfonate peptide **27** and the piperazine-2,3-dicarboxylates **25** and **26** have been shown to antagonize the effects of kainic acid and to be effective as anticonvulsant agents.[89-92] Lactones like

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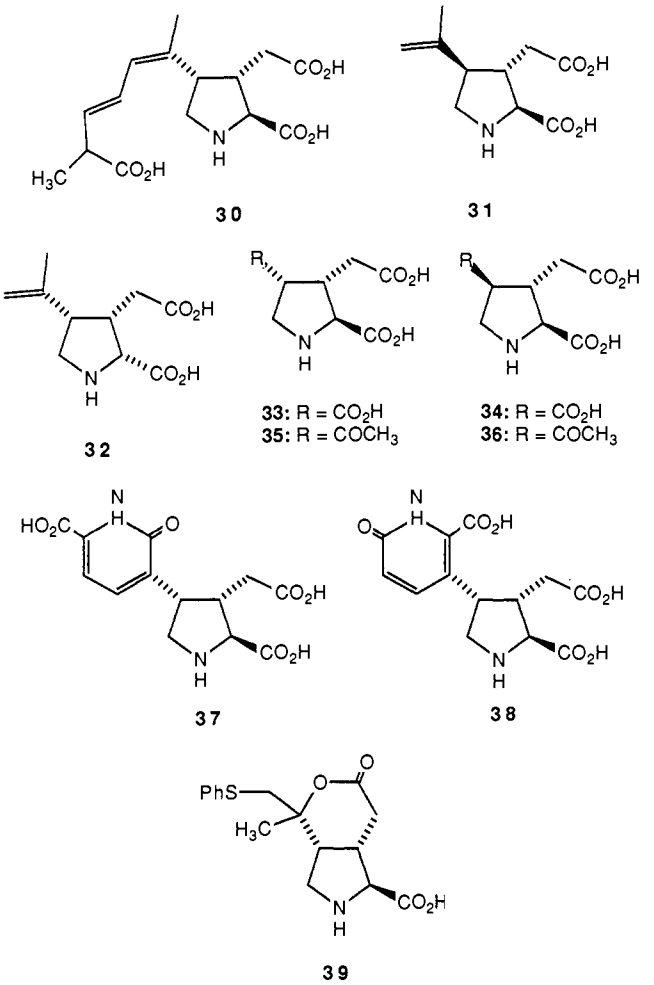

39 derived from 5 have been found to antagonize kainic acid, but these compounds also appear to block NMDA receptors.[93]

Saturable, Na^+-independent binding of [^{3}H]kainic acid to membrane preparations has been described by many investigators.[12] The pharmacological characterization of these binding sites indicate that they are distinct from the binding sites of the other acidic amino acids. Autoradiographic studies with [^{3}H]kainic acid have also demonstrated that these binding sites have a unique distribution in the brain.[94,95] Furthermore, there is a good correlation between the neurotoxic and neuroexcitatory potency of kainic acid analogues and their affinity for the [^{3}H]kainic acid binding sites.[96]

AP4 Receptor

In the early 1980s, several independent groups of investigators reported that the glutamic acid analogue 6 inhibited synaptic responses at micromolar concentrations in three different excitatory neuronal systems. These were the lateral perforant path projection pathway to dentate granule cells of the rat hippocampus,[97] the lateral olfactory tract projections to pyramidal cells in the prepyriform cortex,[98-100] and photoreceptor transmission to ON bipolar cells of the mudpuppy retina.[101-103] Later reports demonstrated inhibition of projections to dorsal horn neurons in the spinal cord,[31,104] and mossy fiber projections from dentate granule cells to CA3 pyramidal cells in the guinea pig hippocampus.[105,106] The inhibition was selective for 6, since the compound's enantiomer, D-2-amino-4-phosphonobutanoic acid, and its lower and higher homologues, L-2-amino-3-phosphonopropanoic acid and L-2-amino-5-phosphonopentanoic acid, showed little activity for these systems.

Consistent with the identification of the AP4 class as a distinct receptor type are the findings that, in the synaptic fields sensitive to inhibition by 6, NMDA receptor antagonists are poor inhibitors and the depolarizations induced by the iontophoretic application of *N*-methyl-D-aspartic acid, quisqualic acid, and kainic acid are not inhibited by 6.[31,104,107] For example, DL-2-amino-4-phosphonobutanoic acid is only moderately effective against *N*-methyl-D-aspartic acid and kainic acid focal potentials while 6 is a poor blocker of glutamic acid and quisqualic acid depolarizations. The order of potency of antagonist compounds against lateral perforant path synaptic responses (6 > L-*O*-phosphoserine > L-2-amino-5-phosphonopentanoic acid) is not matched by the order of potency of these compounds against any of the applied excitatory compounds. The data thus do not fit the simple three receptor scheme of NMDA, quisqualate, and kainate.[107]

The mechanism by which 6 blocks excitatory pathways may differ among the known examples. Thus intracellular recording in the mudpuppy retina demonstrated that 6 selectively activates postsynaptic receptors on the ON bipolar cells.[101,102,108] This affects hyperpolarization in a manner analogous to the natural transmitter by a mechanism involving closure of ion channels. For the lateral perforant path and mossy fiber projections, there is evidence that 6 operates presynaptically to block neurotransmitter release.[109,110] Since the presynaptic boutons are inaccessible to intracellular recording, the mechanism for inhibition of release is unknown.

In structure–activity studies it was found that the nature of the side-chain acidic function was an important determinant in whether a given compound inhibited excitatory transmission in the lateral perforant path in a manner analogous to 6 or acted as an agonist at receptors of undetermined localization to depolarize the postsynaptic cell. Compounds with either a carboxyl (1), sulfonate (18), or tetrazole (40) moiety were agonists, while those having a phosphonate (6) or phosphinate (41) moiety were antag-

(92) Davies, J.; Watkins, J. C. *Brain Res.* **1985**, *327*, 113.
(93) Goldberg, O.; Luini, A.; Teichberg, V. I. *Neurosci. Lett.* **1981**, *23*, 187.
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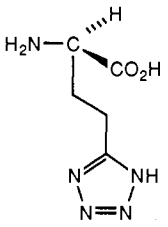
onist.[111-113] In studies aimed at elucidating the bioactive conformation of **6**, the syntheses of cyclopentyl (**42** and **43**) and cyclohexyl (**44** and **45**) analogues of **6** were carried out.[114] The trans isomer **42** was found to most resemble **6** in its spectrum of biological activity. Since the amino and phosphonate moieties are cis to one another in this isomer, the possibility of an ionic interaction between these two moieties exists. This would give rise to a highly folded conformation.



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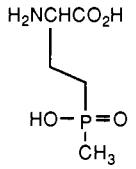

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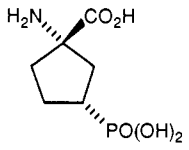

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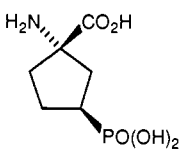

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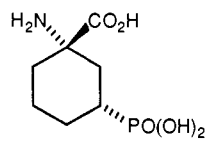

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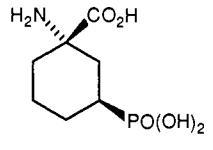

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Since all known analogues with pharmacological actions analogous to **6** have close structural homology to L-glutamic acid, this compound was initially assumed to be the natural agonist. Neurophysiological studies, however, disclosed discrepancies not only in the ability of antagonists to block the excitatory effects of exogenously applied glutamic acid or aspartic acid in comparison with the effects these antagonists have on the endogenous transmitter released by excitatory neurons, but also in the effects of iontophoretically applied glutamic acid and aspartic acid in comparison to the synaptic actions of endogenous excitatory neurotransmitter.[99,100,102] This led to the suggestion that molecules other than glutamic acid or aspartic acid may be serving as excitatory neurotransmitters within the CNS. Recent work on such alternative neurotransmitters has focused on small acidic peptides. For example, the acidic tripeptide, (β-hydroxybutyryl)aspartylaspartylglutamic acid has been isolated from the spinal cord and found to have excitatory effects when iontophoretically applied to cat spinal neurons. Furthermore, these studies have shown that transsection of the spinal cord results in a selective depletion of this acidic tripeptide in the spinal cord distal to the lesion.[115,116]

Most recent work on new excitatory neurotransmitter candidates has centered on the acidic dipeptide **29**. This dipeptide derivative was isolated first from rabbit brain in 1965.[117] Since that time this peptide has been found in other mammalian brain tissue, including human brain tissue.[118-120] The distribution of **29** within the CNS is uneven. The highest concentrations of **29** are found in the spinal cord and medulla, while low levels are found in the cortex.[119,121] This dipeptide also has been found in the olfactory bulb, septal nuclear area, lateral geniculate nucleus, superior colliculus, and the entorhinal cortex/hippocampal formation.[121]

The effects of lesions of specific neuronal pathways on the levels of **29** support the neuronal localization of this dipeptide. Decortication, as well as kainic acid striatal and hippocampal lesions, has been shown to cause significant reductions in the levels of **29**.[122] Concentrations of **29** in the entorhinal cortex of rats with kindled seizures was found to be significantly elevated; no significant changes were seen in the hippocampus or spinal cord.[123]

Neurophysiological studies indicate that **29** is a neuroexcitant. When **29** is injected into the hippocampus, it induces convulsant activity similar to that produced by quisqualic acid.[124] Dipeptide **29** also was found to be excitatory when iontophoresed onto dendrites of rat CA1 hippocampal pyramidal cells[125] and onto rat pyriform cortex pyramidal cells.[126] Depolarizations produced by **29** were similar to the endogenous excitatory postsynaptic potentials (EPSP). Furthermore, the rise and decay times produced by **29** more closely approximated those of the EPSP than those produced by glutamic acid.[125] Excitation of the pyramidal cells elicited by stimulation of the lateral olfactory tract and the response to **29** were blocked by **6** and not by other excitatory amino acid antagonists such as **11**. Responses to aspartic acid or glutamic acid were not antagonized by **11**.[99,100,126]

In early studies a class of [^{3}H]glutamic acid binding sites was found to be displaced by **6**.[127] These binding sites were also identified by using [^{3}H]-**6** as the radioligand,[128,129] and were characterized by their Cl^- and Ca^{2+} dependence. Since these early studies it has become apparent in the numerous binding studies that have been conducted on **6** that there are many compounds that can block [^{3}H]-**6** binding, but not inhibit synaptic responses in a manner

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similar to that of **6**.[114,130-132] These results strongly suggest that the Cl^-/Ca^{2+}-dependent binding site of **6** in brain synaptosomal membrane preparations does not correspond to the same receptor that mediates this compound's inhibition of synaptic transmission. In fact it is now fairly widely accepted that much of what was thought to be the binding of **6** measured by these early procedures is probably attributable to uptake and/or sequestration by membrane vesicles.[12,77,133] Thus the isolation and characterization of the binding site of **6** that corresponds to the receptor through which **6** exerts its neuropharmacological actions remain to be accomplished. The development of more selective and potent analogues of **6** would certainly facilitate this process.

It was originally thought that **29** showed high affinity (K_i = 0.42 μM) and specificity for a subpopulation of Cl^--dependent binding sites labeled with [^{3}H]glutamic acid.[124,134] In this binding system, glutamic acid, **6**, and **29** exhibited parallel displacement curves while *N*-methyl-D-aspartic acid and kainic acid had negligible affinity. Recently, a brain peptidase that avidly cleaves **29** to acetyl-L-aspartic acid and L-glutamic acid has been described.[135-137] Quisqualic acid has been found to be a specific inhibitor of this peptidase with an IC_{50} = 480 nM. The inhibition by quisqualic acid is quite specific; even **21** is ineffective. It is now thought that the quisqualate-sensitive glutamic acid uptake system is not involved directly in uptake of **29**, but that the assay for **29** uptake actually measures uptake of the glutamic acid derived from the cleavage of **29**.

The biological role of the presynaptic AP4 receptors of the hippocampal perforant path and mossy fiber systems are unknown. There is good electrophysiological evidence that the ON bipolar retinal cell AP4 receptor is the postsynaptic receptor for the transmitter released by retinal photoreceptor cells.[101,102,108] The distribution and electrophysiological profile of **29** has led to the suggestion that it may possibly be the neurotransmitter of not only the olfactory pathway[121,126] but also the neurotransmitter which is used to communicate retinal signals to the brain.[121,138] Data also argue for the involvement of **29** in efferent motor pathways.[139] The development of receptor antagonists to **29** are needed to help further elucidate the role that this acidic dipeptide plays in the CNS and the relationship of *N*-acetyl-L-aspartyl-L-glutamic acid receptors to AP4 receptors.

Millisecond Transmitter Receptors vs Ligand Binding Sites

A major goal of neuroscience is to identify and classify specific receptors which mediate behavioral or physiological responses and to isolate and define these receptors at the molecular level. Radioligand–receptor interactions provide a powerful probe for achieving this goal. However, the task of equating a binding site identified by specific binding and displacement of radioligands with a receptor characterized by pharmacological assays is constrained by the chemical principles governing ligand–binding site interactions. Thus, the basic ligand–receptor interaction involves the chemical equilibrium characterized by the reaction where ligand (L) and receptor (R) interact to form a ligand–receptor complex (LR) by association with a second-order rate constant k_1 and dissociation with a first-order rate constant k_{-1} (eq 1). The equilibrium dissociation constant $K_d = k_{-1}/k_1$.[140]

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LR \quad (1)$$

For millisecond excitatory transmission, k_{-1} must be approximately 10^3 s^{-1} or greater. This arises from the experimental observation that these synapses are capable of repetitively firing and "resetting" at rates of 200 Hz or higher. Thus the transmitter must dissociate from its receptor and be removed from the synaptic cleft by diffusion and/or uptake within a few milliseconds. These circumstances preclude use of a natural agonist of millisecond excitatory transmission as a radioligand binding probe for a receptor protein in its native state if the free ligand is separated from the bound ligand by filtration or centrifugation and washing. The washing step requires seconds or minutes during which time the bound ligand would dissociate and be removed.

An alternative approach is to separate the bulk of the free ligand from the membrane-bound ligand–receptor complex by centrifugation, leaving a small volume of supernatant entrapped in the unwashed precipitate to maintain equilibrium conditions. For this method to be useful, the concentration of bound ligand must approximate or be greater than the concentration of free ligand entrapped in the precipitate. Otherwise, most of the measured radioactivity will arise from free ligand, and it will not be possible to detect bound ligand above this background of "nonspecific binding". For this reason, the sensitivity for detecting binding sites by equilibrium centrifugation (or by equilibrium dialysis) is inversely proportional to the K_d. It has been noted that many ligand–receptor systems have values of $k_1 \sim 10^6$–10^7 M^{-1} s^{-1}.[141] Furthermore, there is a theoretical diffusion limited maximum for $k_1 \sim 10^{10}$ M^{-1} s^{-1}.[142,143] Therefore a native millisecond neurotransmitter receptor complex can be expected to exhibit a $K_d = k_{-1}/k_1 \sim 10^{-3}$–$10^{-4}$ M. It is easy to show that a ligand–receptor protein complex with a $K_d \sim 10^{-4}$ M can only be measured by equilibrium binding or equilibrium dialysis experiments if first purified to homogeneity. It certainly cannot be detected if the receptor protein represents less than 10^{-3} of the total protein in a membrane preparation.

Although neurotransmitter K_ds $\sim 10^{-4}$ M seem astonishingly high in comparison to the K_ds for hormone receptors, where extremely high dilutions and long time scales for action are the rule, it is easy to calculate that the vesicular release of a few tens of thousands of trans-

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mitter molecules will achieve adequate concentration for occupancy of most such receptors within a synaptic cleft of typical dimensions.

Structural analogues of the natural agonist may exhibit markedly different properties including slower dissociation rates and lower K_ds. However, at present, only a very few excitatory amino acids analogues such as agonists **3**, **4**, **5**, and **21** and antagonists such as **6**, **11**, and **14** show pharmacological potencies (apparent K_ds) in the low micromolar range. Their activities as agonists or antagonists terminate in seconds or less after cessation of iontophoresis. Yet, in ligand–receptor binding studies, they show K_ds in the micromolar to nanomolar range and dissociation times of many minutes.

Although phenomena such as "desensitization" are often cited to justify unacceptably slow kinetics or small K_ds for suspected or hoped-for physiological functions, there is little evidence that such changes commonly occur or that desensitized molecules necessarily retain the pharmacology of native molecules. Indeed, for the nicotinic cholinergic receptor, there is now evidence that what once were thought to be desensitized channel gating sites are actually distinctive binding sites located on separate subunits of the receptor molecule.[144,145] Since the generality of coexistence of high- and low-affinity sites is unknown, the possible lack of such sites or failure to desensitize to high-affinity states in millisecond glutamic acid receptors would preclude their detection and localization by ligand–receptor binding.

Status of Millisecond Excitatory Signaling

Much of the impetus for research on acidic amino acids has focused on their possible roles as neurotransmitters for the millisecond, rapidly repeating, excitatory signaling of projection pathways. Without doubt, the accelerating research in this field has strengthened the hypothesis that acidic amino acids mediate neuronal signaling. However, we must emphasize the paucity of data supporting a specific role for aspartic acid, glutamic acid, and other acidic amino acids, or a glutamic acid containing dipeptide as the primary messenger for millisecond excitatory signaling for any neuronal system in the vertebrate brain. The goal has remained even more elusive for correlating any pharmacologically defined receptor or binding site with the explicit role of a postsynaptic receptor coupled to an ion channel which mediates millisecond signaling. We will illustrate this dilemma by summarizing what we believe to be the present "bottom line" for the status of the four receptor types previously discussed.

Of these receptors, evidence is strongest that the NMDA receptors observed in different systems comprise either a unique or a very closely related group of molecular entities. There is also excellent correlation of pharmacological properties of this receptor with NMDA binding sites,[14] although their kinetic properties of binding are too slow for their observed physiological actions. Nevertheless, we are optimistic that autoradiographic methods may adequately define the regional densities of this receptor within the CNS and that ligand–receptor binding data offer a reasonable assay for future purification of this molecular species. With regard to biological roles, the evidence increasingly supports regulatory or neuromodulatory functions, such as long-term potentiation, for the NMDA receptor. Many investigators have also reported that functions of NMDA receptors impinge on the level of activity of millisecond excitatory transmission,[146,147] but to our knowledge there is no system for which millisecond activity can be primarily attributed to the NMDA receptor.

The AP4 receptor or receptors seem to subserve diverse roles in the limited number of excitatory pathways for which they have been detected. In the most clearly defined physiological role, as the ON-bipolar cell receptor for neuronal signals from photoreceptor cells, it operates at a synapse which is well-documented as modulating the level of tonic excitation, not evoking discrete millisecond neuronal firing.[108] At two projection pathways in the hippocampus, evidence for presynaptic sites of action again suggest a neuromodulatory role.[109,110] At olfactory cortical neurons, where **6** inhibits both **29**-induced excitation and excitation induced by stimulating the lateral olfactory tract, a direct role as postsynaptic receptors for millisecond excitation seems possible.[99,100,126] However, the stimulus evoked excitation is only partially inhibited by **6**,[148] again raising the alternative possibility of a slower neuromodulatory role. The nature of the inhibition of spinal pathways by **6** is even less documented.

There remain the quisqualate and kainate receptors, which now seem to often be assigned millisecond excitatory roles by default. We emphasize that the recently described compound **28** is the only antagonist with micromolar potency for excitations evoked by these compounds. All other antagonists have low potency and specificity. We suggest that these receptors be considered kainate-preferring and quisqualate-preferring subtypes and that questions of possible heterogeneity be addressed by seeking more specific antagonists.

Among the other known antagonists which may block postsynaptic receptors of projection pathways, only **24**, **25**, and **26** inhibit at 0.1–1 mM concentrations. Others which inhibit at greater than 1 mM also tend to show weak agonist activity, which depolarizes the neurons by undefined receptors and obscures possible antagonism of specific synaptic pathways. Lack of potent pathway-specific antagonists is the major obstacle to further progress in studies of millisecond excitatory transmission.

The final paragraph of a review inevitably invokes the need for further work of similar nature! We feel justified in reiterating this well-worn appeal. Specifically, novel kainic acid, quisqualic acid, *N*-acetyl-L-aspartyl-L-glutamic acid, and kynurenic acid analogues seem to us to be particularly needed if further advances are to be made in the area of excitatory amino acid neurotransmission. We also wish to point out that alternative, less explored approaches may result in revolutionary progress in this area. One potentially fruitful approach would be to seek specific reagents which might covalently label binding sites by photoaffinity or alkylating mechanisms. Although these procedures may be severely limited by their propensity for nonspecific labeling, they have the potential for selectively labeling the very millisecond transmitter binding sites with kinetic and equilibrium properties that preclude experimental demonstration by conventional binding experiments. Another is to seek naturally occurring venoms and toxins which might have been crafted by evolution for exquisite selectivity and potency. Several toxins and venoms have already been isolated from various species

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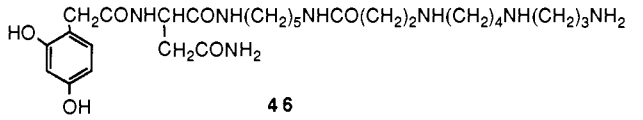
and found to have effects on glutamatergic receptors, particularly those of invertebrates.[149] Examples include the Joro spider toxin (46)[150] from *Nephila clavata* and the argiotoxins (47)[151] from the orb-web spider, *Argiope fasciata*. Both of these toxins contain the (2,4-dihydroxyphenylacetyl)asparagine moiety within their structures and act as noncompetitive glutamic acid receptor inhibitors, possibly by blocking the open cation channels.[151-153] These toxins as well as others that have been isolated, but have yet to be characterized, have the potential to serve as valuable pharmacological tools in the study of excitatory amino acid neurotransmission.



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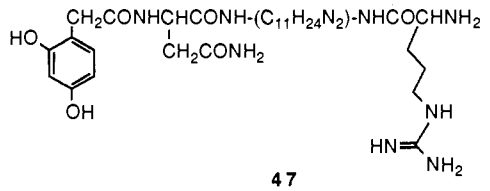

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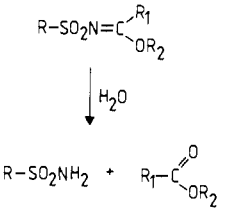
Communications to the Editor

N-Sulfonyl Imidates as a Novel Prodrug Form for an Ester Function or a Sulfonamide Group

Sir:

In recent years, chemical transformation of drug substances into per se inactive derivatives (prodrugs) that convert to the parent compounds by virtue of enzymic or chemical lability within the body system has become a useful approach to improve drug delivery.[1-3] A basal requisite for this prodrug approach is the ready availability of chemical derivative types satisfying the prodrug requirements, the most prominent of these being reconversion of the prodrug to the parent drug in vivo. Although several types of bioreversible derivatives have been exploited for utilization in designing prodrugs of various functional groups or chemical entities occurring in a variety of drug molecules,[4,5] no bioreversible derivatives for the ester group have been explored. In contrast, esters are probably the best known prodrug derivatives for drugs containing carboxyl or hydroxyl groups because of the ready availability of enzymes in the organism capable of hydrolyzing most esters.[3,4] However, numerous drugs contain an ester group as an essential part of their structure, e.g. various calcium antagonists like nifedipine and nicardipine, steroid derivatives, and anticholinergic agents. To solve delivery problems associated with some of such drugs due to e.g. unfavorable solubility and lipophilicity characteristics or extensive first-pass metabolism, by the prodrug concept, the availability of a prodrug type for the ester functionality is desirable. In this paper we report that *N*-sulfonyl imidate esters may be a prodrug type for drugs containing an ester function. In addition, the derivatives may serve as prodrug forms for primary sulfonamides (Scheme I).

Scheme I



A series of *N*-sulfonyl imidate esters (1-19) (Table I) were synthesized, by reacting *p*-toluenesulfonamide, used as a model sulfonamide, with the appropriate ortho ester (compounds 1-6) or orthocarbonate ester (18, 19) according to literature methods,[6] or by reacting *N*-(*p*-tolylsulfonyl)benzimidoyl chloride, obtained from *p*-toluenesulfonamide and phenyltrichloromethane as described previously,[7] with the appropriate alcohol (compounds 7-12), phenol (13), or amino alcohol (14-17) in acetone solutions in the presence (7-13)[8] or absence (14-17) of pyridine.[9] Compounds 14-17 were isolated as water-

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