Structure−Activity Relationship Studies on N^3-Substituted Willardiine Derivatives Acting as AMPA or Kainate Receptor Antagonists

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N^3-Substitution of the uracil ring of willardiine with a variety of carboxyalkyl or carboxybenzyl substituents produces AMPA and kainate receptor antagonists. In an attempt to improve the potency and selectivity of these AMPA and kainate receptor antagonists a series of analogues with different terminal acidic groups and interacidic group spacers was synthesized and pharmacologically characterized. (*S*)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxythiophene-3-ylmethyl)pyrimidine-2,4-dione (**43**, UBP304) demonstrated high potency and selectivity toward native GLU_{K5}-containing kainate receptors (K_D 0.105 ± 0.007 μM vs kainate on native GLU_{K5}; K_D 71.4 ± 8.3 μM vs (*S*)-5-fluorowillardiine on native AMPA receptors). On recombinant human GLU_{K5}, GLU_{K5}/GLU_{K6}, and GLU_{K5}/GLU_{K2}, K_B values of 0.12 ± 0.03, 0.12 ± 0.01, and 0.18 ± 0.02 μM, respectively, were obtained for **43**. However, **43** displayed no activity on homomeric GLU_{K6} or GLU_{K7} kainate receptors or homomeric GLU_{A1-4} AMPA receptors (IC_{50} values > 100 μM). Thus, **43** is a potent and selective GLU_{K5} receptor antagonist.

Introduction

(*S*)-Glutamate (**1**) can activate a range of glutamate receptor subtypes in the vertebrate central nervous system (CNS). There are two general classes of glutamate receptors, the ionotropic glutamate (iGlu) receptors and the metabotropic glutamate (mGlu) receptors.[1,2] The iGlu receptors are ligand-gated ion channels, which mediate fast synaptic responses in the CNS. The iGlu receptors were divided into three groups based on their pharmacology and are referred to as the (*S*)-2-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA, **2**), (2*S*,3*S*,4*S*)-3-carboxymethyl-4-isopropenylpyrrolidine-2-carboxylic acid (kainate, **3**), and *N*-methyl-D-aspartic acid (NMDA, **4**) receptor subtypes. AMPA receptors are tetrameric assemblies of a combination of GLU_{A1-4} subunits (IUPHAR nomenclature of the receptors that are also known as GluR1−4 or GluRA-D).[3c] Kainate receptors are tetrameric assemblies of GLU_{K5-7}, GLU_{K1}, and GLU_{K2} subunits (IUPHAR nomenclature of the receptors that are also known as GluR5−7, KA1, and KA2).[3c] Subunit-selective agonists and antagonists would facilitate the investigation of the roles of the various subunits that make up AMPA and kainate receptors in central nervous system function. Despite considerable interest in the discovery of selective AMPA and kainate receptor antagonists, very few subunit-selective compounds have emerged.[1,2] A series of decahydroisoquinolines, including LY382884 (**5**) and **6**, has been reported to selectively antagonize GLU_{K5} receptors.[4,5] The use of these antagonists has provided evidence to support the role of GLU_{K5} in hippocampal synaptic plasticity and in a number of CNS disorders such as epilepsy, chronic pain, ischaemia, and migraine.[4,5] Recent reports have highlighted the discovery of the first noncompetitive antagonists for GLU_{K5}, for example NS3763 (**7**).[6]

We have previously reported that 5-substituted analogues of the natural product willardiine (**8**−**10**) exhibit selective agonist activity at either AMPA or kainate receptors.[7] We have also demonstrated that the agonist action of the willardiines can be converted to antagonism by N^3-subtitution with carboxyalkyl or carboxybenzyl substituents.[8] In particular, the carboxyethyl and carboxybenzyl groups have been highlighted as useful N^3-substituents for obtaining AMPA and kainate antagonist activity. Thus, **11** was a moderately potent AMPA receptor antagonist with weaker activity at kainate receptors.[8b] *p*-Carboxy substitution of the phenyl ring led to a mixed AMPA/kainate receptor antagonist (**12**), while *o*-carboxy substitution resulted in a selective GLU_{K5} antagonist (**13**).[8] While **11**−**13** were moderately potent AMPA and kainate receptor antagonists there is a need for more potent and selective antagonists. Herein we report the synthesis and pharmacological characterization of a new series of N^3-substituted willardiine analogues in which the terminal acid group and the nature of the linker were varied in an attempt to enhance the potency at AMPA or kainate receptors. This novel series of compounds was pharmacologically characterized on both cloned and native AMPA and kainate receptors.

Results

Chemistry. The N^3-substituted uracil analogues **14**, **16a**, and **16b** were prepared as described previously.[8d] Conversion of these nitrile-substituted compounds into the corresponding tetrazole-substituted analogues (**15**, **17a**, and **17b**) was effected using sodium azide and ammonium chloride in DMF (Scheme 1). The N-Boc protecting group was removed using TFA and the amino acids purified using ion-exchange resin chromatography. A considerable amount of racemization occurred during the tetrazole formation with ee values of 75, 84, and 60% for **15**, **17a**, and **17b**, respectively, as adjudged by chiral HPLC analysis. The iodination of the uracil ring of **17b** was carried out using iodine monochloride in aqueous hydrochloric acid[9] to give **18** (Scheme 1).

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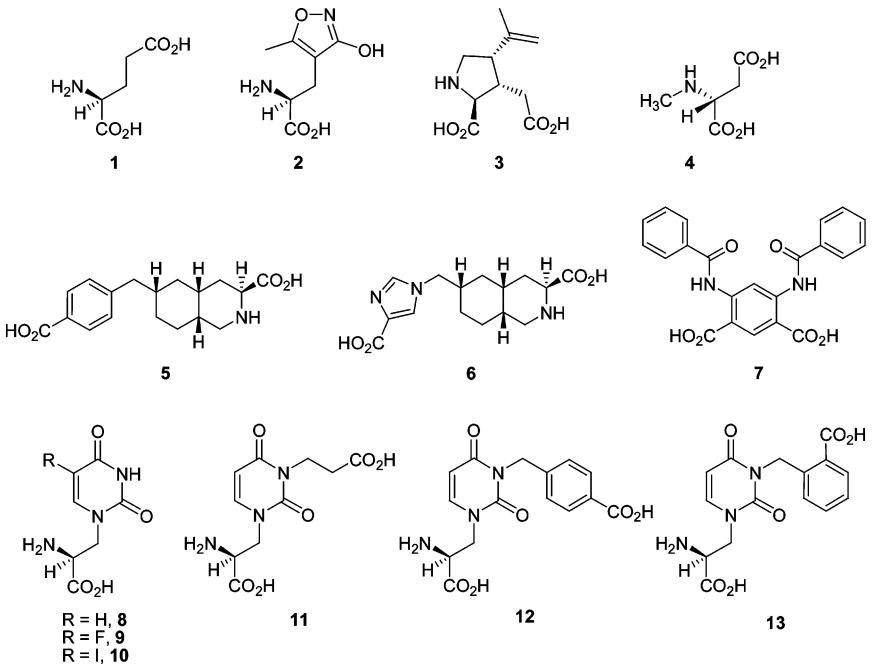
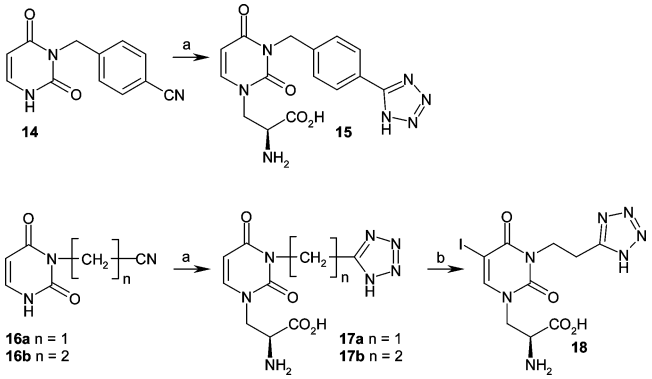


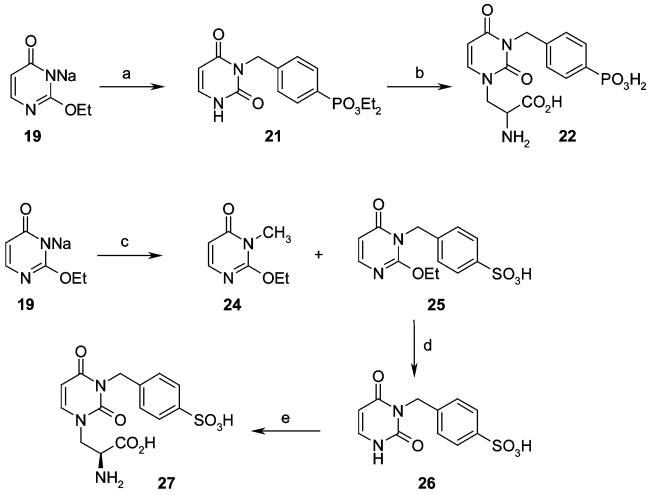
Figure 1. Structures of glutamate receptor agonists and antagonists.

Scheme 1[a]



[a] (a) (i) NaH, DMF (ii) (*S*)-*N*-Boc-serine-β-lactone (**46**) (iii) NaN_3, NH_4Cl, 90 °C, (iv) TFA; (b) 3 M ICl in 6 M HCl, 70 °C.

Scheme 2[a]

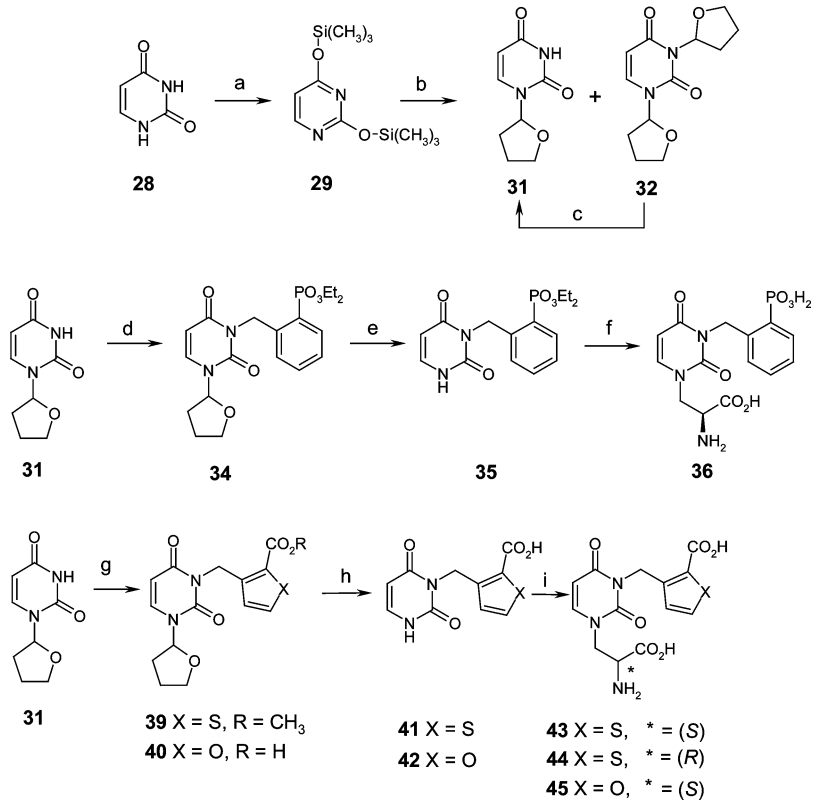


[a] (a) (i) Diethyl 4-bromomethylphenylphosphonate (**20**), DMF, 60 °C (ii) CH_3CN/6 M HCl (10:1); (b) (i) NaH, DMF (ii) (*S*)-*N*-Boc-serine-β-lactone (**46**); 6 M HCl, reflux; (c) methyl 4-bromomethylphenylsulfonate (**23**), DMF, 60 °C, silica gel chromatography; (d) concentrated HCl/H_2O (1:10), rt; (e) (i) NaH, DMF, rt (ii) (*S*)-*N*-Boc-serine-β-lactone (**46**) (iii) 1M HCl, 50 °C.

For the synthesis of the *p*- and *o*-phosphonic acid derivatives (**22** and **36**) the benzyl bromides (**20**[26] and **33**,[31] Schemes 2 and 3) were prepared from the corresponding commercially available 4- and 2-bromotoluenes. Treatment with hot triethyl phosphite in the presence of nickel bromide catalyst[10] afforded the diethyl phosphonate. The methyl group of the resultant diethoxyphosphino-substituted toluenes was reacted with NBS to give reasonable yields of the benzyl bromides **20** and **33** (typically 70% conversion). The resulting crude reaction mixture containing the substituted benzyl bromide (**20** or **33**) was used for the alkylation of the uracil derivatives. Reaction of the sodium salt (**19**) with the benzyl bromide (**20**) afforded the desired N^3-substitued phosphonate (**21**) after removal of the ethoxy group of the intermediate with aqueous HCl in acetonitrile. Treatment of the sodium salt of **21** with the (*S*)-*N*-Boc-serine-β-lactone (**46**)[11] followed by extended hydrolysis of the amino acid in refluxing 6 M HCl afforded the desired amino acid (**22**). However, it was observed that the extended hydrolysis resulted in racemization of the amino acid as confirmed by chiral HPLC and the lack of optical rotation.

The sulfonate (**27**) was also synthesized to further extend the series of compounds with different acidic groups attached to the 4-position of the phenyl ring. The substituted benzyl bromide (**23**)[27] was synthesized from commercially available *p*-methyl toluenesulfonate by treatment with NBS. Reaction of the protected uracil (**19**) with this benzyl bromide (**23**) gave a mixture of compounds (only one regioisomer of each product shown for clarity in Scheme 2). The methylated products (e.g. **24**) and the three regioisomers resulting from benzyl substitution (e.g. **25**) were separated by silica gel chromatography. The desired sulfonic acid (**25**) was obtained in 21% yield after crystallization. ^1H NMR and ESMS analysis confirmed the identity of this compound. Removal of the ethoxy group of **25** by treatment with aqueous HCl afforded the uracil intermediate **26**. Reaction of the di-sodium salt of **26** with (*S*)-*N*-Boc-serine-β-lactone (**46**) followed by removal of the Boc group with 1 M aqueous HCl afforded the desired amino acid (**27**) in 13% yield and >99% ee.

Scheme 3[a]



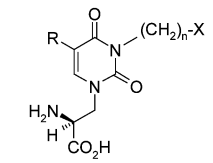
[a] (a) HMDS, TMSCl, $(NH_4)_2SO_4$, reflux; (b) 2-acetoxytetrahydrofuran (**30**), CH_3CN, 60 °C; (c) AcOH/EtOH (1:5); (d) (i) NaH, DMF (ii) Diethyl 2-bromomethylphenylphosphonate (**33**), 60 °C; (e) TFA, rt (f) (i) NaH, DMF, rt (ii) (*S*)-*N*-Boc-serine-*β*-lactone (**46**) (iii) TMSBr, reflux; (g) (i) NaH, DMF (ii) methyl 3-bromomethylthiophene-2-carboxylate (**37**) or methyl 3-bromomethylfuran-2-carboxylate (**38**), 60 °C; (h) (i) LiOH, dioxane/water (1:1), rt (ii) TFA, rt; (i) 2 equiv. NaH, DMF, rt (ii) (*S*)-*N*-Boc-serine-*β*-lactone (**46**) or (*R*)-*N*-Boc-serine-*β*-lactone (**47**) (iii) 2 M HCl, 50 °C.

We have reported previously that addition of a 2-carboxybenzyl substituent to the N^3 position of the uracil ring of willardiine produced a potent and selective GLU_{K5} receptor antagonist.[8d] Our structure−activity relationship studies on analogues of **12** suggested that a phosphonate group was the optimal replacement of the carboxylate group, and therefore we decided to synthesize **36** (Scheme 3). The phosphono-substituted benzyl bromide **33**[31] was synthesized from 2-bromotoluene by adaptation of previously reported procedures.[10] Thus 2-bromotoluene was treated with triethyl phosphite and nickel bromide to produce the phosphonate, which was reacted with NBS to form the required benzyl bromide (**33**). Attempted alkylation of **19** with this benzyl bromide (**33**) gave only poor yields of the desired N^3-substituted product, as deduced by TLC. A more efficient synthesis of the appropriately N^3-alkylated uracil was therefore sought. We have previously observed that uracils protected with a tetrahydrofuryl group at the N^1 position can be alkylated to give N^3-substituted uracils in reasonable yields (unpublished observations). The desired N^1-protected uracil starting material (**31**) was prepared in a one-pot procedure by adaptation of previously reported methods (Scheme 3).[12,14,15] Uracil (**28**) was initially silylated with hexamethyldisilazide in the presence of TMSCl and ammonium sulfate catalyst.[12] This moisture-sensitive intermediate **29** was alkylated with an excess of 2-acetoxytetrahydrofuran (**30**), prepared by modification of a previously reported method,[13] to give a mixture of mono- and di-protected uracils (**31** and **32**). The mixture was then hydrolyzed in ethanol/acetic acid[14] to afford almost exclusively the desired N^1-alkylated derivative **31**, which was purified by silica gel chromatography. Earlier attempts to effect this alkylation employed a previously reported method that used the catalyst cesium chloride in the reaction between the silylated uracil (**29**) and 2-acetoxytetrahydrofuran (**30**).[15] The authors reported very high ratios of mono- to di-protected products for a range of 5-substituted uracils. In our hands, no reaction was observed in the presence of this catalyst even when anhydrous analytical grade cesium chloride was used.

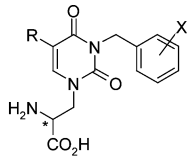
Alkylation of the protected uracil (**31**) with the phosphono-substituted benzyl bromide **33** afforded the N^3-substituted intermediate (**34**) in 39% yield. In this synthesis only the N^3-substituted isomer was produced, and therefore this method is an improvement on the original procedure that involved alkylation of **19** leading to a mixture of N^1-, N^3-, and O^4-substituted products. Removal of the protecting group of **34** was achieved with TFA, and the sodium salt of the resultant N^3-substituted uracil (**35**) was treated with (*S*)-*N*-Boc-serine-*β*-lactone (**46**). The Boc group of the resultant intermediate was removed in the presence of dilute hydrochloric acid, and the phosphonate ester groups were removed by treatment with bromotrimethylsilane followed by aqueous hydrolysis. The use of these milder conditions for hydrolysis of the phosphonate esters avoided the racemization associated with prolonged 6 M aqueous HCl hydrolysis and provided **36** from compound **35** in 14% yield over three steps.

To further extend the series of N^3-substituted willardiines, the benzene ring in the linker was replaced by five membered aromatic rings such as furan and thiophene. Alkylation of the N^1-protected uracil (**31**) with the appropriately substituted benzyl bromide (**37**[32] or **38**,[33] synthesized via standard methods as outlined above) gave the corresponding esters (**39** or **40**), which were hydrolyzed using lithium hydroxide to give the corresponding free acids. Removal of the protecting group was achieved with TFA to give the uracils **41** and **42**, the di-sodium salts of which were treated with (*S*)- or (*R*)-*N*-Boc-serine-*β*-

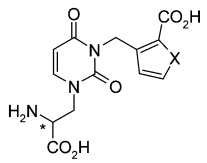
Table 1. Summary of the Activity of Novel N^3-Substituted Willardiine Analogues at AMPA and Kainate Receptors in Electrophysiological and Radioligand Binding Assays[a]



11, X = CO_2H, R = H, n = 2
17a, X = tetrazole, R = H, n = 1
17b, X = tetrazole, R = H, n = 2
18, X = tetrazole, R = I, n = 2



12, X = 4-CO_2H, R = H, * = *S*
13, X = 2-CO_2H, R = H, * = *S*
15, X = 4-tetrazole, R = H, * = *S*
22, X = 4-PO_3H_2, R = H, * = *RS*
27, X = 4-SO_3H, R = H, * = *S*
36, X = 2-PO_3H_2, R = H, * = *S*



43, X = S, * = *S*
44, X = S, * = *R*
45, X = O, * = *S*

compound	K_D (μM) vs kainate[b]	IC_{50} (μM) vs fDR−VRP[c]	GLU_{K6} K_i (μM) vs [^{3}H]kainate[d]
1, Glu			3.28 ± 1.44
49, CNQX	1.66[e]		1.05 ± 0.07
50, NBQX	1.78[e]	0.214 ± 0.043	
11	73.1 ± 4.5	23.8 ± 3.9	>1000
12	9.25 ± 0.54	10.3 ± 2.4	>100
13	0.402 ± 0.045	106 ± 13	>1000
15	ND[f]	50.9 ± 10.3	ND[f]
17a	60.2 ± 4.5	45.4 ± 10.3	>100
17b	53.7 ± 5.1	6.87 ± 1.7	>100
18	ND[f]	3.73 ± 0.62	ND[f]
22	5.80 ± 0.67	107 ± 24	>1000
27	4.68 ± 0.09	49.0 ± 10.1	>100
36	10.5 ± 1.0	>100	ND[f]
43	0.105 ± 0.007	31.2 ± 5.0[g]	>100
44	ND[h]	>100	>1000
45	1.88 ± 0.15	11.7 ± 1.3	>100

[a] All values are from three independent experiments and are expressed as the mean ± SEM. ND: not determined. [b] Apparent K_D values for antagonism of kainate induced depolarization of neonatal rat dorsal root fibers calculated using the Gaddum−Schild equation. [c] Depression of the fast component of the dorsal root evoked ventral root potential (fDR-VRP) in the neonatal rat spinal cord preparation, a measure of antagonist activity at AMPA receptors expressed on motoneurones. [d] The K_i value for the inhibition of [^{3}H]kainate binding to rat GLU_{K6} expressed on HEK293 cell membranes. [e] Values taken from ref 25. [f] Test result not obtained due to inadequate compound supply. [g] The K_D value for inhibition by **43** of the depolarization of neonatal rat motoneurones induced by **9** (a selective AMPA receptor agonist) was 71.4 ± 8.3 μM (n = 3). [h] Compound **44** at a concentration of 100 μM depressed a kainate response on isolated dorsal root by 68.4 ± 4.1%.

lactone (**46** or **47**) followed by treatment with dilute HCl to remove the Boc group to yield the willardiine derivatives **43**−**45**.

Pharmacology

In Vitro Electrophysiology. Native Kainate Receptor Antagonist Activity. To examine antagonist activity at GLU_{K5}-containing kainate receptors, the ability of compounds to depress kainate-induced depolarization of neonatal rat dorsal root fibers was assessed (see Table 1 for data).[8] This preparation has been previously shown to express a population of kainate receptors[16] containing the GLU_{K5} subunit.[17] In accordance with previous results from pharmacological characterization of carboxyalkyl-substituted willardiines, altering the chain length of the interacidic methylene spacer unit had little effect on kainate receptor antagonism by tetrazole-substituted derivatives, with **17a** and **17b** showing similar potency as antagonists. Both **17a** and **17b** were of comparable potency to the 2-carboxyethyl analogue (**11**).

We have reported previously that the 4-carboxybenzyl analogue (**12**) was a moderately potent kainate receptor antagonist. Replacement of the terminal carboxylate group with a sulfonate (**27**) or a phosphonate group (**22**) improved kainate receptor antagonist potency by approximately 2-fold. We have shown previously that the kainate receptor antagonist activity resides in the *S*-enantiomer with the *R*-enantiomer being almost inactive.[8d] Since the enantiomeric purity of **22** was not as high as that of **27**, it may be that the pure *S*-enantiomer of the phosphonate (**22**) may prove to be more potent than **12** and **27**. The effect of moving the phosphonate group from the 4-position (**22**) to the 2-position (**36**) of the benzene ring was investigated, as we have reported previously that the 2-carboxybenzyl analogue (**13**) was more potent than the 4-carboxy-susbtituted analogue (**12**). However, **36** proved to be far less potent than the carboxy-substituted analogue **13** and was also less potent than the 4-phosphono analogue (**22**).

The effect of altering the aromatic ring in the interacidic spacer unit of **13** was assessed in an attempt to improve the potency of **13**, one of the most potent kainate receptor antagonists identified in our previous studies.[8c,d] Replacement of the benzene ring of **13** with a thiophene ring produced a compound (**43**) that was approximately 4 times more potent than the parent compound as a GLU_{K5}-containing kainate receptor antagonist (K_D value 105 ± 7 nM). The stereoselectivity of this compound was confirmed by the low antagonist potency of **44**, the *R*-enantiomer. Interestingly, replacement of the benzene ring of **13** with a furan ring reduced kainate receptor antagonist potency, as **45** was less potent than either **13** or **43**.

Native AMPA Receptor Antagonist Activity. The neonatal rat spinal cord preparation was used to assess the potency of the novel N^3-substituted willardiine analogues at native AMPA receptors.[8] The ability of compounds to reduce the fast component of the dorsal root-evoked ventral root potential (fDR-VRP), which is mediated chiefly by AMPA receptors expressed on motoneurones,[8a] was used as an expeditious method of investigating the antagonist potency at AMPA receptors (see Table 1 for data).

For compounds with an alkyl chain as the interacidic group linker it was found that changing the terminal acidic group from a carboxylic acid to a tetrazole group enhanced AMPA receptor

antagonist activity. The tetrazole-5-ylethyl substituted compound (**17b**) was ~6−7-fold more potent than the tetrazole-5-ylmethyl derivative (**17a**) and ~3.5 times more potent than the carboxyethyl derivative (**11**). Iodo-substitution at the 5-position of the uracil ring of **17b** gave **18**, which was twice as potent as the parent compound and is the most potent AMPA receptor antagonist we have identified so far.

The carboxybenzyl-substituted derivative (**12**) was ~5-fold more potent as an AMPA receptor antagonist than the tetrazole-substituted derivative (**15**). The 4-phosphonobenzyl- and 4-sulfobenzyl-substituted derivatives (**22** and **27**) were approximately 10- and 5-fold less potent, respectively, than **12**. Changing the position of the phosphono group of **22** from the 4-position to the 2-position of the benzene ring, as in **36**, had little effect on AMPA receptor antagonist potency. Thus, none of the 2-substituted benzyl derivatives had AMPA receptor antagonist activity comparable to their activity at GLU_{K5} kainate receptors.

The analogues of **13** with either a thiophene (**43**) or a furan (**45**) ring, like the parent compound, were moderately potent AMPA receptor antagonists (IC_{50} values in the 11−50 μM range). Indeed substitution of the benzene ring of **13** with a thiophene or furan ring resulted in ~3- and 9-fold increase in AMPA receptor antagonist potency for compounds **43** and **45**, respectively. To characterize more accurately the activity of **43** on AMPA receptors a K_D value was obtained for the antagonism of depolarizations of neonatal rat motoneurones induced by the selective AMPA receptor agonist **9**. In this assay a K_D value of 71.4 ± 8.3 μM ($n = 3$) was calculated for **43** confirming that it is only a moderately potent AMPA receptor antagonist. Thus **43** is >650-fold selective for native GLU_{K5}-containing kainate receptors vs AMPA receptors. The much lower activity of the *R*-enantiomer (**44**) is in accordance with the previously reported finding[8d] that the *S*-enantiomer is the active isomer for AMPA receptor antagonist activity.

Characterization of 43 on NMDA and Group I Metabotropic Glutamate Receptors Expressed on Neonatal Rat Motoneurones. At a concentration of 50 μM **43** had no activity on NMDA- or (*S*)-3,5-dihydroxyphenylglycine (DHPG, **48**)-induced depolarizations of neonatal rat motoneurones ($n = 3$). Thus, **43** shows at least 500-fold selectivity for native GLU_{K5}-containing kainate receptors vs NMDA or group I metabotropic glutamate receptors (mGlu1 and mGlu5 expressed on motoneurones are activated by DHPG).

Binding Studies. Recombinant Homomeric Rat GLU_{K6} Receptors. None of the compounds tested inhibited [^{3}H]kainate binding to rat GLU_{K6} expressed on HEK293 cell membranes (see Table 1 for data). Two standard compounds, glutamate (**1**) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, **49**), displaced [^{3}H]kainate binding with K_i values of 3.28 ± 1.44 μM ($n = 3$; mean ± sem) and 1.05 ± 0.07 μM ($n = 3$; mean ± SEM), respectively. As the IC_{50} value for inhibition of [^{3}H]-kainate binding to rat GLU_{K6} expressed on HEK293 cell membranes by **43** was >100 μM, we can conclude that this compound is highly selective for GLU_{K5} vs GLU_{K6}.

Recombinant Homomeric Human GLU_{K7} Receptors. Compound **43** inhibited [^{3}H]kainate binding from HEK293 cell membranes expressing human GLU_{K7} receptors with an IC_{50} value of 264 ± 91 μM. Using the Cheng−Prusoff equation, a K_i value of 111 ± 38 μM was calculated. In a similar assay 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX, **50**), which was used as a positive control, displaced [^{3}H]kainate binding with an IC_{50} value of 44 ± 20 μM and a K_i value of 18 ± 8 μM, which was similar to the previously reported K_i value of 24 μM.[18]

Calcium Fluorescence Assays. Recombinant Human AMPA and Kainate Receptor Subtypes. As **43** was found to be a potent and selective antagonist of native GLU_{K5}-containing kainate receptors, it was also tested on a range of recombinant human AMPA and kainate receptor subtypes to further characterize the selectivity of the compound. Glutamate (**1**) induced calcium influx in cells expressing human GLU_{K5}, GLU_{K5}/GLU_{K6}, and GLU_{K5}/GLU_{K2} with EC_{50} values of 22 ± 5, 27 ± 9, and 35 ± 41 μM, respectively, and this was inhibited by **43** with IC_{50} values of 0.68 ± 0.02, 0.62 ± 0.21, and 1.21 ± 0.68 μM, respectively ($n = 3$, mean ± SEM). Using the Cheng−Prusoff equation K_b values of 0.12 ± 0.03, 0.12 ± 0.01 and 0.18 ± 0.02 μM ($n = 3$, mean ± SEM) were calculated for the antagonism by **43** of glutamate-induced calcium influx in cells expressing GLU_{K5}, GLU_{K5}/GLU_{K6} and GLU_{K5}/GLU_{K2}, respectively. A representative trace showing the antagonist activity of **43** in the glutamate-induced calcium influx fluorescence assay in HEK293 cells expressing GLU_{K5} is shown in Figure 2.

Compound **43**, up to a concentration of 100 μM, had no measurable effect on calcium influx in cells expressing GLU_{K6} or GLU_{K6}/GLU_{K2}. Compound **43** up to a concentration of 100 μM failed to block glutamate induced Ca^{2+} influx in HEK293 cells individually expressing human homomeric GLU_{A1}, GLU_{A2}, GLU_{A3}, or GLU_{A4} in either the flip or flop forms. The non-NMDA receptor antagonist NBQX (**50**) was used as a control in these studies and had IC_{50} values of 6.0 ± 2.2, 2.5 ± 0.3, 1.9 ± 0.8, 1.1 ± 0.5, 37 ± 9, 28 ± 8, 50 ± 10, 29 ± 14, and 135 ± 91 μM (mean ± SEM) on GLU_{A1flop}, GLU_{A2flop}, GLU_{A3flop}, GLU_{A4flop}, GLU_{K5}, GLU_{K5}/GLU_{K6}, GLU_{K5}/GLU_{K2}, GLU_{K6}, and GLU_{K6}/GLU_{K2}, respectively. These IC_{50} values for NBQX are similar to those that we have reported previously.[24] It is apparent from the results of these assays that **43** is a selective antagonist of GLU_{K5} with little or no activity on homomeric GLU_{K6}, GLU_{K7} or AMPA receptors.

Discussion and Conclusion

Previous structure−activity studies have indicated that adding substituents bearing a terminal carboxylic acidic group to the N^3-position of the uracil ring of willardiine can generate antagonists of AMPA and/or kainate receptors.[8] The primary aim of the present study was to investigate the effect of changing either the terminal acidic group or the interacidic group linker on AMPA and kainate receptor antagonist activity.

Most of the compounds tested were weak antagonists when characterized on AMPA receptors expressed on neonatal rat motoneurones. The exceptions were the tetrazole-alkyl-substituted willardiines, in particular **17b** and **18**, which showed moderate potency as AMPA receptor antagonists. However, **17b** was less than 10-fold selective for native AMPA vs GLU_{K5}-containing kainate receptors. The improvement in GLU_{K5} kainate receptor antagonist potency observed by replacing the benzene ring of **13** with a thiophene ring may partially be explained by the different size and shape of the thiophene ring. Differences in electrostatic surface profile and/or ring electronics of the aromatic residues may also explain the difference in potency of **13**, **43**, and **45**. The latter explanation could explain why replacing the benzene ring of **13** with either a thiophene or a furan ring resulted in an increase in AMPA receptor antagonist potency.

The best antagonist to be identified in the native GLU_{K5} kainate receptor assay, **43** (apparent K_D 105 nM), showed >500-

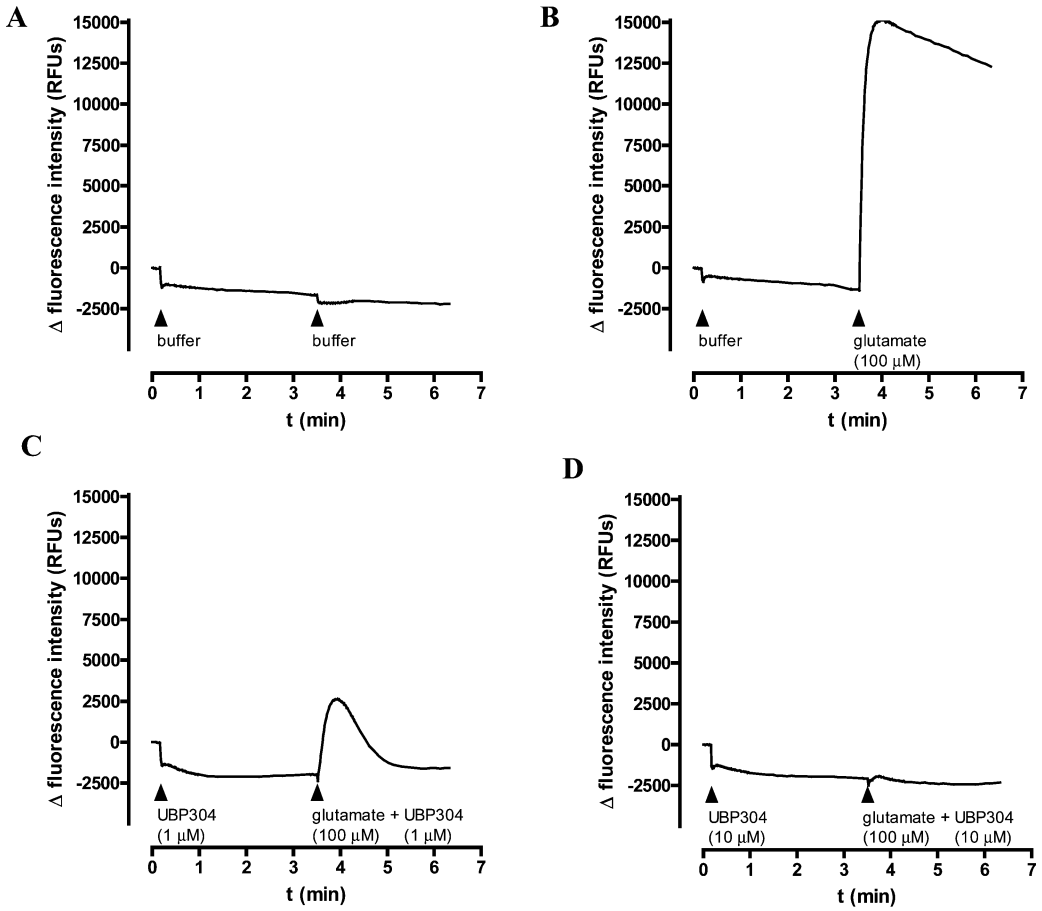


Figure 2. Representative FLIPR traces showing calcium influx in HEK293 cells stably expressing recombinant human homomeric GLU_{K5} receptors. Ordinate: change in fluorescence intensity (relative fluorescence units). Abscissa: time (min). Arrowheads indicate the time of the first and second buffer additions, respectively. Panel A shows control buffer additions. The relatively small dilution effect of the buffer additions on fluorescence intensity can be observed. Panel B shows the calcium signal evoked by glutamate (100 μM). Panels C and D show the effect of **43** (UBP304, 1 and 10 μM) on glutamate-induced calcium influx.

fold selectivity for these receptors vs AMPA, NMDA, and group I mGlu receptors expressed on neonatal rat motoneurones. When tested on cloned human kainate receptor subunits, **43** was found to antagonize only those receptors containing GLU_{K5} receptor subunits and had no effect on homomeric GLU_{K6} or GLU_{K7}. Furthermore **43** had no activity on cloned human homomeric GLU_{A1-4} AMPA receptors. The potency and selectivity of **43** compares favorably with one of the most potent decahydroisoquinoline GLU_{K5} receptor antagonists (**6**) (K_i values 0.156 $\pm$ 0.075 and 48.5 $\pm$ 1.9 μM for inhibition of [^{3}H]kainate binding to human GLU_{K5} and GLU_{K7}, respectively) described[4d] to date. Thus **43** is likely to be a useful pharmacological tool for characterizing the physiological roles of GLU_{K5}-containing kainate receptors.

Experimental Section

Chemistry. General Procedures. Proton NMR spectra were measured on a JEOL NMR spectrometer at either 300.40 or 270.18 MHz. Carbon NMR spectra were run on a 300.40 MHz JEOL NMR spectrometer at 75.45 MHz or a 270.18 MHz JEOL spectrometer at 67.94 MHz. 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt in D_2O, or tetramethylsilane in $CDCl_3$, DMSO-d_6, or TFA-*d* were used as internal standards. Elemental analyses were performed in the microanalytical laboratory in the Department of Chemistry, University of Bristol, Bristol, UK. Melting points were determined in capillary tubes on Electrothermal IA9100 electronic melting point equipment. Determinations of optical rotation were carried out in 6 M HCl at room temperature with a wavelength of 589 nm at Tocris Cookson Ltd. Thin-layer chromatography was performed on Merck silica gel 60 F_{254} plastic sheets. Silica gel for flash chromatography was silica gel 60 (220−440 mesh) from Fluka chemicals, U.K. The eluents for thin-layer chromatography of amino acids included [2 (pyridine:acetic acid:water (3:8:11)): 3 (*n*-butanol)] and [propan-2-ol:35% aqueous ammonia (70: 30)]. Amino acids were detected by spraying plates with a 2% solution of ninhydrin in 70% ethanol. Purity of amino acids was also determined by high voltage paper electrophoresis, using pH 4 buffer and an applied voltage of 4 Kv. Ion-exchange resin chromatography was carried out using Dowex 50WX8−400 acid form resin and the acetate or hydroxide form of Dowex X18−400 obtained from Aldrich Chemical Co., UK or Biorad AG1X8 from Bio-Rad, UK. Chiral HPLC was carried out using a RSTech, Chirosil RCA+, 150 $\times$ 4.6 column, flow rate 1.0 mL/min, detection 220 nM with a mobile phase consisting of methanol/5 mM perchloric acid (85:15). All solids were dried over P_2O_5 in vacuo for 3 days prior to reaction. With the exception of compounds **22** and **36** all of the amino acids synthesized were washed with anhydrous ethanol followed by diethyl ether to remove surface water. All anhydrous reactions were conducted under argon. All reagents and dry solvents were obtained from the Aldrich Chemical Co., UK unless otherwise stated.

(*S*)-1-(2-Amino-2-carboxyethyl)-3-(4-tetrazol-5-ylbenzyl)pyrimidine-2,4-dione (15). A 60% suspension of sodium hydride in mineral oil (0.23 g, 5.81 mmol) was added portionwise to a stirred solution of 3-(4-cyanobenzyl)pyrimidine-2,4-dione (**14**)[8d] (1.25 g, 5.81 mmol) in dry DMF (40 mL) under a dry nitrogen atmosphere. The resulting reaction mixture was stirred for 18 h. To this was added (*S*)-3-(*t*-butoxycarbonylamino)oxetan-2-one (**46**) (1.09 g, 5.81 mmol), and the resulting mixture was stirred at room temperature for 18 h. To this was added sodium azide (0.6 g, 9.23 mmol) and

ammonium chloride (0.49 g, 9.16 mmol), and the mixture was heated to 90 °C until the reaction had gone to completion as deduced by TLC. The mixture was concentrated under reduced pressure (1 mmHg, 60 °C), and TFA (30 mL) was added. The acid was immediately removed under reduced pressure and the residue was dissolved in the minimum quantity of water and poured onto a column containing Dowex 50WX8−400 (0,25 mmol of cation/mL resin; 120 mL). The column was eluted with water and then 1 M aqueous pyridine. The ninhydrin-positive fractions of the 1 M aqueous pyridine eluate were combined and evaporated to dryness under reduced pressure. The residue was dissolved in the minimum quantity of water, and Dowex X18−400 (hydroxide form) was added with stirring until alkaline to pH paper. This mixture was then poured onto a column containing Dowex X18−400 (acetate form) resin (45 mL). The column was eluted with water and then acetic acid of ascending concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2, and 3 M. The ninhydrin-positive fractions of the 3 M aqueous acetic acid eluate were combined and evaporated to dryness under reduced pressure. Crystallization of the residue from water gave **15** (0.05 g, 25%) as a white solid. mp 188.3−189.2 °C (dec); $[\alpha]_D^{20} = -4.0$ (*c* 0.5, 6 M HCl); ee 75% as determined by chiral HPLC; ^{1}H NMR (D_2O + NaOD [pH = 12], 270.17 MHz) δ 4.46−4.68 (ABX system, J = 15.3 Hz, 4.4 Hz, 6.9 Hz, 3H, C*H*CH_2 and CHC*H*$_2$), 5.20 (s, 2H C*H*$_2$Ph), 6.0 (d, J = 8.1 Hz, 1H, HCC*H*CO), 7.58 (d, J = 8.3 Hz, 2H, Ph), 7.79 (d, J = 8.1 Hz, 1H, *H*CCHCO), 7.95 (d, J = 8.3 Hz, 2H, Ph); ^{13}C NMR (D_2O + NaOD [pH = 12], 67.94 MHz) δ 47.2, 51.6, 54.4, 104.6, 129.9, 130.8, 130.9, 143.8, 147.9, 155.3, 156.2, 167.6, 170.7. Anal. ($C_{15}H_{15}N_7O_4 \cdot 1.9\ H_2O \cdot 0.1$ AcOH) C, H, N.

(*S*)-1-(2-Amino-2-carboxyethyl)-3-(tetrazol-5-ylmethyl)pyrimidine-2,4-dione (17a). Following the procedure by which **15** was synthesized, treating 3-cyanomethylpyrimidine-2,4-dione (**16a**)[8d] (1.03 g, 6.8 mmol) in dry DMF (40 mL) with a 60% suspension of sodium hydride in mineral oil (0.27 g, 6.8 mmol), then with (*S*)-3-(*t*-butoxycarbonylamino)oxetan-2-one (**46**) (1.27 g, 6.8 mmol), gave an intermediate that was treated with sodium azide (0.49 g, 7.48 mmol) and ammonium chloride (0.4 g, 7.48 mmol). This gave **17a** (0.46 g, 24%), as a white solid, following the same workup and purification procedures described for **15**. mp 197.5−198.6 °C (dec); $[\alpha]_D^{20} = -25.3$ (*c* 0.3, 6 M HCl); ee 84% as determined by chiral HPLC; ^{1}H NMR (D_2O + NaOD [pH = 12], 270.17 MHz) δ 4.43−4.65 (ABX system, J = 15.23, 5.23, 6.59 Hz, 3H, C*H*CH_2 and CHC*H*$_2$), 5.49 (s, 2H, CH_2), 6.01 (d, J = 8.08 Hz, 1H, HCC*H*CO), 7.74 (d, J = 8.08 Hz, 1H, *H*CCHCO); ^{13}C NMR (D_2O + NaOD [pH = 12], 67.94 MHz) δ 34.0, 48.7, 51.8, 101.5, 101.7, 145.3, 152.2, 163.9, 167.9. Anal. ($C_9H_{11}N_7O_4 \cdot 0.7\ H_2O$) C, H, N.

(*S*)-1-(2-Amino-2-carboxyethyl)-3-(2-tetrazol-5-ylethyl)pyrimidine-2,4-dione (17b). Following the procedure by which **15** was synthesized, treating 3-(2-cyanoethyl)pyrimidine-2,4-dione (**16b**)[8d] (2.22 g, 13 mmol) in dry DMF (60 mL) with a 60% suspension of sodium hydride in mineral oil (0.54 g, 13 mmol), then with (*S*)-3-(*t*-butoxycarbonylamino)oxetan-2-one (**46**) (2.43 g, 13 mmol), gave an intermediate that was treated with sodium azide (0.93 g, 14 mmol) and ammonium chloride (0.76 g, 14 mmol). This gave **17b** (0.55 g, 14%), as a white solid, following the same workup and purification procedures described for **15**. mp 176.6−178.6 °C (dec); $[\alpha]_D^{20} = -10$ (*c* 1.0, 6 M HCl); ee 60% as determined by chiral HPLC; ^{1}H NMR (D_2O + NaOD [pH = 12], 270.17 MHz) δ 3.49 (t, J = 6.6 Hz, 2H, C*H*$_2$CH$_2$), 4.32 (t, J = 6.6 Hz, 2H, CH$_2$C*H*$_2$), 4.4−4.62 (ABX system, J = 15.23, 4.58, 6.7 Hz, 3H, C*H*CH_2 and CHC*H*$_2$), 5.89 (d, J = 7.9 Hz, 1H, HCC*H*CO), 7.69 (d, J = 7.9 Hz, 1H, *H*CCHCO); ^{13}C NMR (D_2O + NaOD [pH = 12], 67.94 MHz) δ 23.73, 41.77, 51.66, 54.65, 104.32, 148.0, 155.21, 156.5, 167.5, 170.9. Anal. ($C_{10}H_{13}N_7O_4 \cdot 1.1\ H_2O$) C, H, N.

(*S*)-1-(2-Amino-2-carboxyethyl)-5-iodo-3-(2-tetrazol-5-ylethyl)pyrimidine-2,4-dione (18). To a stirred suspension of (*S*)-1-(2-amino-2-carboxyethyl)-3-(2-tetrazol-5-ylethyl)-pyrimidine-2,4-dione (**17b**) (0.1 g, 0.34 mmol) in a mixture of water (0.7 mL) and 2 M hydrochloric acid (0.7 mL) at room temperature was added 3 M iodine monochloride in 6 M hydrochloric acid (0.12 mL, 0.36 mmol). The mixture was heated to 70 °C for 1 h, a further aliquot of 3 M iodine monochloride (0.12 mL, 0.36 mmol) was added and the mixture heated at 70 °C for 1 h. The solvent was then evaporated under reduced pressure. The residue was applied to a column of Dowex 50WX8−400 resin (0.25 mmol of cation/mL resin; 6 mL). The column was eluted with water and then 1 M aqueous pyridine. The ninhydrin-positive fractions of the 1 M aqueous pyridine eluate were combined and evaporated to dryness under reduced pressure. The solid was crystallized from water to give **18** (0.095 g, 67%) as a white solid. mp 192.6−194.0 °C (dec); $[\alpha]_D^{20} = +11.0$ (*c* 1.0, 6 M HCl); ee 79% as determined by chiral HPLC; ^{1}H NMR (D_2O + NaOD [pH = 12], 270.17 MHz) δ 3.54 (t, J = 6.6 Hz, 2H, C*H*$_2$-CH$_2$), 4.4 (t, J = 6.6 Hz, 2H, CH$_2$C*H*$_2$), 4.44−4.68 (m, 3H, C*H*CH$_2$), 8.23 (s, 1H, ICC*H*); ^{13}C NMR (D_2O + NaOD [pH = 12], 67.94 MHz) δ 23.5, 43.0, 51.4, 54.4, 70.6, 151.7, 154.6, 156.0, 164.5, 170.4. Anal. ($C_{10}H_{12}N_7O_4I \cdot 2.8\ H_2O$) C, H, N.

Diethyl 4-Bromomethylphenylphosphonate (20). Benzoyl peroxide (0.02 g) was added to a solution of diethyl 4-methylphenylphosphonate[26] (15.0 g, 65.8 mmol) in carbon tetrachloride (700 mL). A 100 W lamp was used to irradiate the mixture, which subsequently heated the mixture to reflux. NBS (10.5 g, 59.2 mmol) was then added to the mixture in four equal portions over 4 h. The mixture was allowed to react for a further 2 h. The mixture was cooled to room temperature and then filtered. The filtrate was concentrated under reduced pressure to a quarter of its original volume and then extracted with water (3 × 100 mL) and once with saturated brine solution (50 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **20** as a golden oil (14.2 g, ∼70% by ^{1}H NMR), which was used without purification in the next step; ^{1}H NMR ($CDCl_3$, 399.78 MHz) of the crude product revealed a signal at 4.49 ppm (s, 2H, PhC*H*$_2$Br) corresponding to the product and a signal at 2.40 ppm (s, 3H, PhC*H*$_3$) corresponding to the starting material (∼2:1 ratio). The ^{1}H NMR data for compound **20** is consistent with that reported previously.[26]

3-(4-Diethoxyphosphinobenzyl)pyrimidine-2,4-dione (21). A solution of diethyl 4-bromomethylphenylphosphonate (**20**) (14.0 g, 32.0 mmol) was added to a stirred suspension of 2-ethoxypyrimidin-4-one sodium salt[8d] (**19**) (4.5 g, 32.0 mmol) in dry DMF (50 mL) under a dry nitrogen atmosphere. The resulting reaction mixture was stirred for 2 days at 60 °C. The mixture was concentrated under reduced pressure (1 mmHg, 50 °C), and the products were separated from the insoluble salts by repeated washings with ethyl acetate (3 × 100 mL), which were subsequently combined, filtered, and evaporated under reduced pressure. The resulting mixture was purified using silica gel chromatography, eluting with petroleum ether/ethyl acetate (3:7). Elution from the column gave 2-ethoxy-3-(4-diethoxyphosphinobenzyl)pyrimidin-4-one (1.9 g, 16%) as an oil. ^{1}H NMR ($CDCl_3$, 270.17 MHz) δ 1.33 (m, 9H, POCH$_2$C*H*$_3$ and COCH$_2$C*H*$_3$), 4.10 (m, 4H, POC*H*$_2$CH$_3$), 4.41 (q, J = 7.3 Hz, 2H, COC*H*$_2$CH$_3$), 5.22 (s, 2H, NC*H*$_2$Ph), 6.17 (d, J = 6.6 Hz, 1H, HCC*H*CO), 7.45 (dd, J = 3.8 Hz, J = 8.3 Hz, 2H, Ph), 7.64 (d, J = 6.6 Hz, 1H, *H*CCHCO), 7.76 (dd, J = 13.2 Hz, J = 8.3 Hz, 2H, Ph), ^{13}C NMR ($CDCl_3$, 67.94 MHz) δ 14.1, 16.3 (d, J = 6.2 Hz), 44.0, 62.2 (d, J = 5.7 Hz), 65.3, 108.7, 127.9 (d, J = 188.9 Hz), 128.3 (d, J = 15.1 Hz), 132.0, (d, J = 10.4 Hz), 140.8 (d, J = 3.6 Hz), 152.3, 156.6, 162.8; MS (EI) m/z = 366 (M^+), 338(*), 282; HRMS m/z (M^+) calculated for $C_{17}H_{23}N_2O_5P$: 366.134461. Found: 366.134789.

2-Ethoxy-3-(4-diethoxyphosphinobenzyl)pyrimidin-4-one (1.3 g, 3.9 mmol) was dissolved in acetonitrile/6 M aqueous HCl (10:1, 50 mL) and stirred for 18 h at room temperature.The mixture was concentrated under reduced pressure and cyclohexane was added, which was subsequently removed under reduced pressure. The resulting residue was purified by column chromatography eluting with 10% methanol in dichloromethane to give **21** (1.1 g, 92%) as an oil. ^{1}H NMR (DMSO-d_6, 270.17 MHz,) δ 1.31 (t, J = 7.1 Hz, 6H, CH$_2$C*H*$_3$), 4.10 (m, 4H, C*H*$_2$CH$_3$), 5.14 (s, 2H, C*H*$_2$Ph), 5.73 (d, J = 7.6 Hz, 1H HCC*H*CO), 7.17 (dd, J = 7.6 Hz, J = 1.7 Hz, 1H, *H*CCHCO), 7.53 (dd, J = 7.9 Hz, J = 4.0 Hz, 2H, Ph), 7.75 (dd, J = 8.3 Hz, J = 7.9 Hz, 2H, Ph), 10.76 (br d, J = 1.7 Hz, 1H, N*H*); ^{13}C NMR (DMSO-d_6, 67.94 MHz) δ 16.3 (d, J = 6.7 Hz),

43.3, 62.3 (d, $J = 5.2$ Hz), 101.4, 127.3 (d, $J = 101.9$ Hz), 128.7 (d, $J = 15.0$ Hz), 131.9 (d, $J = 10.4$ Hz), 139.3, 141.5 (d, $J = 3.1$ Hz), 152.5, 163.5, MS (EI) $m/z = 338$ (M^+)(*); HRMS m/z (M^+) calculated for $C_{15}H_{19}N_2O_5P$: 338.103160. Found: 338.103188. Anal. ($C_{15}H_{19}N_2O_5P$) C, H, N.

(*RS*)-1-(2-Amino-2-carboxyethyl)-3-(4-phosphonobenzyl)pyrimidine-2,4-dione (22). A 60% suspension of sodium hydride in mineral oil (2.32 g, 7.5 mmol) was added portionwise to a stirred solution of 3-(4-diethoxyphosphinobenzyl)pyrimidine-2,4-dione (**21**) (0.30 g, 7.5 mmol) in dry DMF (50 mL) under a dry argon atmosphere. The resulting solution was stirred for 2 h. (*S*)-3-(*t*-Butoxycarbonylamino)oxetan-2-one (**46**) (1.41 g, 7.5 mmol) was added portionwise, and the resulting mixture was stirred at room temperature for 2 d. The mixture was evaporated under reduced pressure (1 mmHg, 60 °C), and the resulting residue was washed by repeatedly grinding under diethyl ether and decanting to leave a white solid, which was dissolved in 6 M aqueous HCl (100 mL). The mixture was heated under reflux for 18 h, but contained a small amount of the mono ethyl ester as determined by ^{1}H NMR. Complete hydrolysis was achieved with a further 18 h of reflux. The mixture was evaporated under reduced pressure to leave an off-white solid, which was dissolved in water (5 mL) and applied to Dowex 50WX8−400 ion-exchange resin (H^+ form) (0.25 mmol of cation/mL resin; 30 mL) with stirring for 20 min. The mixture was applied to a column of the same type of resin (10 mL). The column was eluted with water until no ninhydrin-positive fractions were observed and then elution continued with 1 M aqueous pyridine. The ninhydrin-positive fractions of the 1 M aqueous pyridine fractions were combined and evaporated to dryness under reduced pressure. The residue was dissolved in the minimum amount of water and applied to Biorad AG-1 × 8 ion-exchange resin (acetate form) (0.5 mmol of anion/mL of resin; 30 mL) with stirring for 30 min. The mixture was applied to a column containing 5 mL of the same type of resin. The column was eluted with water and then aqueous acetic acid of increasing concentrations of 0.01, 0.05, 0.1, 0.5, and 1 M. The ninhydrin-positive fractions of the 0.5 M and the 1 M acetic acid eluates were combined and evaporated under reduced pressure. The excess moisture remaining on the resulting solid was removed by drying in a stream of argon overnight, giving **22** (1.36 g, 43%) as a white solid. mp 187.4−191.5 °C (dec); racemic as determined by chiral HPLC; ^{1}H NMR (D_2O, 300.40 MHz) δ 4.28−4.36 (m, 1H, CH_2CH), 4.41−4.48 (m, 2H, CH_2CH), 5.15, (s, 2H, CH_2Ph), 5.97 (d, $J = 7.9$ Hz, 1H, HCC*H*CO), 7.42 (d, $J = 8.1$ Hz, $J = 3.3$ Hz, 2H, Ph), 7.65 (d, $J = 7.9$ Hz, 1H, *H*CCHCO), 7.73 (dd, $J = 8.1$ Hz, $J = 13.1$ Hz, 2H, Ph); ^{13}C NMR (D_2O, 62.90 MHz) δ 44.9, 49.7, 52.9, 102.2, 127.5 (d, $J = 14.72$), 131.1 (d, $J = 10.6$), 132.2 (d, $J = 181.5$), 139.7, 145.6, 153.5, 165.6, 170.0; MS (electrospray) 368.2 [M − H]$^-$, 369.2 [M], 392.1 [M + Na]$^+$. Anal. ($C_{14}H_{16}N_3O_7P\cdot 2.7\ H_2O$) C, H, N.

Methyl 4-Bromomethylphenylsulfonate (23). Benzoyl peroxide (0.02 g) was added to a solution of methyl *p*-toluenesulfonate (100 g, 540 mmol) in carbon tetrachloride (1.0 L). A 100 W lamp was used to irradiate the mixture, which subsequently heated the mixture to reflux, and NBS (67.2 g, 378 mmol) was added to the mixture in four equal portions over 5 h. The mixture was allowed to react for a further 1.5 h. The mixture was cooled to room temperature and then extracted with water (3 × 330 mL) and once with a saturated brine solution (100 mL). The organic layer was separated, dried ($MgSO_4$), and concentrated under reduced pressure to give a pale yellow oil (126 g), which solidified upon cooling. The mixture was crystallized from ethyl acetate/petroleum ether and gave **23** (19.1 g, 13%) as a white solid. mp 63.6−64.6 °C; lit. mp 72−73 °C;[27] ^{1}H NMR ($CDCl_3$, 270.17 M Hz) δ 3.78 (s, 3H, $PhSO_3CH_3$), 4.51 (s, 2H, $PhCH_2Br$), 7.89 (d, $J = 8.6$ Hz, 2H, Ph), 7.90 (d, $J = 8.6$ Hz, 2H, Ph); ^{13}C NMR ($CDCl_3$, 75.45 MHz) δ 31.2, 56.5, 128.6, 129.9, 135.0, 143.9.

2-Ethoxy-3-(4-sulfobenzyl)pyrimidin-4-one (25). To a solution of 2-ethoxypyrimidin-4-one sodium salt (**19**)[8d] (5.0 g, 35.7 mmol) in dry DMF (100 mL) was added methyl 4-bromomethylphenylsulfonate (**23**) (7.89 g, 29.8 mmol), and the resulting mixture was stirred at 60 °C for 48 h. The mixture was concentrated under reduced pressure (1 mmHg, 60 °C), and the resulting white solid analyzed by TLC, which revealed the presence of six compounds. The mixture was separated using silica gel chromatography, eluting with ethyl acetate/petroleum ether (1:1), which eluted the first three compounds with very little separation between them. The column was further eluted with ethyl acetate followed by ethyl acetate/acetonitrile (1:1). Finally the column was eluted with methanol, and the second set of three compounds eluted as a crude mixture. The mixture containing the second set of compounds was evaporated under reduced pressure, and the resulting residue was separated using silica gel chromatography, eluting with methanol/dichloromethane (15:85). The major fraction eluted as a pure compound and gave **25** as a white solid (2.5 g, 21%). mp >300 °C; ^{1}H NMR (DMSO-d_6, 300.4 MHz) δ 1.26 (t, $J = 7.15$ Hz, 3H, OCH_2CH_3), 4.36 (q, $J = 7.15$ Hz, 2H, OCH_2CH_3), 5.08 (s, 2H, CH_2Ph), 6.13 (d, $J = 6.6$ Hz, 1H, HCC*H*CO), 7.22 (d, $J = 8.3$ Hz, 2H, Ph), 7.56 (d, $J = 8.3$ Hz, 2H, Ph), 7.75 (d, $J = 6.6$ Hz, 1H, C*H*CHCO); ^{13}C NMR (d^6 DMSO, 75.45 MHz) δ 13.8, 43.2, 64.8, 107.8, 125.6, 126.9, 136.5, 147.4, 152.4, 156.34, 161.7. MS (ES) analysis of the first set of three compounds to be eluted from the column revealed a peak at 155.1 [M + H]$^+$ pertaining to 2-ethoxy-3-methylpyrimidin-4-one (**24**) and the corresponding N^1-methyl and O^4-methyl analogues. This was confirmed by the ^{1}H NMR analysis ($CDCl_3$, 270.17 MHz) of a crude mixture of all three isomers. Singlets pertaining to the three methyl signals (O^4-, N^3-, and N^1-substituted) were observed at 3.42, 3.49, and 3.34 ppm, respectively. Also the presence of six sets of dd's pertaining to the CH protons of the uracil ring of these compounds, and the absence of signals in the aromatic region expected for the benzenesulfonic acid group corroborated the structural assignment of these products.

3-(4-Sulfobenzyl)pyrimidine-2,4-dione (26). 2-Ethoxy-3-(4-sulfobenzyl)pyrimidin-4-one (**25**) (2.20 g, 7.09 mmol) was dissolved in water/HCl (concentrated)(10:1; 100 mL) with warming. The mixture was stirred at room temperature for 2 days until the reaction had gone to completion, as deduced by TLC. The mixture was concentrated under reduced pressure, and the resulting residue was crystallized from boiling water to give **26** (1.37 g, 69%) as a white solid. mp >300 °C; ^{1}H NMR (DMSO-d_6, 300.40 MHz) δ 4.93 (s, 2H, CH_2Ph), 5.65 (d, $J = 7.6$ Hz, 1H, HCC*H*CO), 7.20 (d, $J = 8.4$ Hz, 2H, Ph), 7.48 (dd, $J = 5.9$ Hz, $J = 7.6$ Hz, *H*CCHCO), 7.53 (d, $J = 8.4$ Hz, 2H, Ph), 11.24(br d, $J = 5.9$ Hz, 1H, NH); ^{13}C NMR (DMSO-d_6, 75.45 MHz) δ 42.2, 99.7, 125.4, 126.7, 137.4, 140.9, 147.0, 151.3, 162.9. Anal. ($C_{11}H_{10}N_2O_5S$) C, H, N.

(*S*)-1-(2-Amino-2-carboxyethyl)-3-(4-sulfobenzyl)pyrimidine-2,4-dione (27). A 60% suspension of sodium hydride in mineral oil (0.39 g, 9.7 mmol) was added portionwise to a stirred solution of 3-(4-sulfobenzyl)pyrimidine-2,4-dione (**26**) (1.37 g, 4.9 mmol) in dry DMF (50 mL) under a dry argon atmosphere. The resulting reaction mixture was stirred for 3 h. To this was added (*S*)-3-(*t*-butoxycarbonylamino)oxetan-2-one (**46**) (0.91 g, 4.9 mmol), and the resulting mixture was stirred at room temperature for 2 days. 1 M aqueous HCl (20 mL) was added, and the mixture was evaporated under reduced pressure (1 mmHg, 60 °C) to leave an off white oil, which was subsequently redissolved in water and evaporated under reduced pressure. The residue was dissolved in water (2 mL) and applied to Dowex 50WX8−400 ion-exchange resin (H^+ form) (0.25 mmol of cation/mL of resin; 20 mL) with stirring for 30 min. The mixture was applied to a column containing an equivalent volume of the same type of resin, eluting with water, until no ninhydrin-positive fractions were observed, then 1 M aqueous pyridine. The combined aqueous washes were evaporated under reduced pressure. The residue was dissolved in the minimum amount of water and bound onto Biorad AG1 × 8 ion-exchange resin (acetate form) (0.5 mmol of anion/mL of resin; 14 mL) with mixing. The resulting resin was applied to an equivalent amount of the same type of resin in a column. The column was washed with water and then THF/water (1:1) before being eluted with aqueous acetic acid of increasing concentrations of 0.1, 1, and 2 M. The column was finally eluted with aqueous formic acid (4 M). The ninhydrin-positive fractions of the 4 M aqueous formic

acid eluate were combined and evaporated under reduced pressure to leave an off-white solid which was subsequently crystallized from water to give **27** (0.24 g, 13%) as a white solid. mp 264.2−264.6 °C (dec); $[\alpha]_D^{20} = -13.0$ (*c* 1.0, 6 M HCl); ee >99% as determined by chiral HPLC; ^{1}H NMR (D_2O, 300.40 MHz) δ 4.26−4.47 (m, 3H, C*H*$_2$CH and CH$_2$C*H*), 5.15 (s, 2H, C*H*$_2$Ph), 5.96 (d, $J = 7.9$ Hz, 1H, HCC*H*CO), 7.45 (d, $J = 8.3$ Hz, 2H, Ph), 7.63 (d, $J = 7.9$ Hz, 1H, *H*CCHCO), 7.76 (d, $J = 8.3$ Hz, 2H, Ph); ^{13}C NMR (D_2O, 75.45 MHz) δ 47.1, 52.1, 55.4, 104.5, 128.5, 130.4, 142.1, 144.3, 147.9, 155.8, 167.9, 172.4; MS (electrospray) 392.1 $[M + Na]^+$, 369.2 [M], 368.2 $[M - H]^-$. Anal. ($C_{14}H_{15}N_3O_7S\cdot 2H_2O$) C, H, N.

2-Acetoxytetrahydrofuran (30). To 2,3-dihydrofuran (100 mL, 1.32 mol) was added acetic acid (60.5 mL, 1.1 mol) followed by *p*-toluenesulfonic acid (0.02 g). An exothermic reaction was noted after 5 min of mixing. The reaction mixture was stirred for 18 h at room temperature, then taken up in diethyl ether (500 mL) and washed with a saturated solution of sodium bicarbonate (3 × 50 mL) followed by brine (50 mL). The organic layer was then dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was distilled under reduced pressure (10 mmHg), and the fraction collected at 78−82 °C (lit. bp 80 °C, 20 mmHg[28]) was **30** (81.5 g, 60%). ^{1}H NMR ($CDCl_3$, 399.78 MHz) δ 2.01−2.04 (m, 7H, OCH$_2$C*H*$_2$CH$_2$ and OCH$_2$CH$_2$C*H*$_2$ and COC*H*$_3$), 3.89−3.95 (m, 1H, OC*H*$_2$CH$_2$), 4.03−4.08 (m, 1H, OC*H*$_2$CH$_2$), 6.28 (t, $J = 1.5$ Hz, 1H, OC*H*O); ^{13}C NMR ($CDCl_3$, 399.78 MHz) 21.3, 23.0, 32.1, 68.9, 99.0, 170.4.

1-(Tetrahydrofuran-2-yl)uracil (31). To a suspension of uracil (7.0 g, 62.5 mmol) in hexamethyldisilazane (48.5 mL, 230 mmol) was added chlorotrimethylsilane (3.6 mL, 28.1 mmol) under a dry argon atmosphere with rigorous exclusion of moisture. The mixture was heated to reflux for 3 h. The mixture was concentrated under reduced pressure (1 mmHg, 60 °C) to leave a pale green oil, which was dissolved in dry acetonitrile (50 mL) and concentrated under reduced pressure. The resulting oil was dissolved in dry acetonitrile (100 mL) under a dry argon atmosphere. To this was added 2-acetoxytetrahydrofuran (**30**) (17.9 g, 137.5 mmol), and the mixture was heated to 60 °C for 18 h. The mixture was concentrated under reduced pressure and dissolved in ethanol/acetic acid (1:5, 100 mL), and the mixture was heated under reflux for 2 h. The mixture was concentrated, and the remaining residue was purified using silica gel chromatography, eluting with ethyl acetate to give **31** as a white solid (10.6 g, 93.2%). mp 114.6−115.5 °C; lit. mp 103−104 °C;[29] ^{1}H NMR ($CDCl_3$, 270.17 MHz) δ 1.88−2.12 (m, 3H, C*H*$_2$C*H*$_2$), 2.35−2.44 (m, 1H, C*H*$_2$CH$_2$), 3.94−4.03 (m, 1H, CHOC*H*$_2$), 4.16−4.24 (m, 1H, CHOC*H*$_2$), 5.73 (d, $J = 8.3$ Hz, 1H, HCC*H*CO), 5.99−6.03 (m, 1H, C*H*OCH$_2$), 7.35 (d, $J = 8.3$ Hz, 1H, *H*CCHCO), 9.51 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75.45 MHz) δ 24.0, 33.0, 70.1, 87.5, 101.8, 139.3, 150.4, 163.6, MS (EI) $m/z = 182$ (*); HRMS (M^+) calculated for $C_8H_{10}N_2O_3$: 182.069142. Found: 182.069355. Anal. ($C_8H_{10}N_2O_3$) C, H, N.

Diethyl 2-Bromomethylphenylphosphonate (33). To a suspension of nickel(II) bromide (5.1 g, 23 mmol) in 2-bromotoluene (40.0 g, 233.0 mmol) was added triethyl phosphite (10 mL, 58.0 mmol), and the mixture was heated to 180 °C until the onset of a vigorous reaction. When the vigor of the reaction had subsided, the remainder of the triethyl phosphite (34.1 mL, 199 mmol) was added dropwise over the course of 1 h. After this time, the reaction mixture was heated under reflux for a further 2.5 h. The resulting mixture was taken up in ethyl acetate (100 mL) and filtered through Celite, and the filtrate was evaporated under reduced pressure to give a green oil which was separated using silica gel chromatography, eluting with ethyl acetate/petroleum ether (3:2). Evaporation of the solvent under reduced pressure gave diethyl 2-methylphenylphosphonate (41.8 g, 79%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 399.78 MHz) δ 1.33 (t, $J = 7.1$ Hz, CH$_2$C*H*$_3$), 2.58 (s, 3H, PhC*H*$_3$), 4.03−4.20 (m, 4H, C*H*$_2$CH$_3$), 7.24−7.28 (m, 1H, Ph), 7.41−7.44 (m, 1H, Ph), 7.41−7.44 (m, 1H, Ph), 7.88−7.94 (m, 1H, Ph), ^{13}C NMR ($CDCl_3$, 67.94 MHz) δ 16.3 (d, $J = 6.2$ Hz), 21.2 (d, $J = 3.6$ Hz), 61.9 (d, $J = 5.7$ Hz), 125.4 (d, $J = 14.5$ Hz), 127.0 (d, $J = 184.2$ Hz), 131.2 (d, $J = 15.1$ Hz), 132.4 (d, $J = 3.1$ Hz), 133.9 (d, $J = 9.9$ Hz), 141.8 (d, $J = 9.9$ Hz); MS (EI) $m/z = 228$ $(M)^+$. ^{1}H NMR data is consistent with that reported previously.[30]

Benzoyl peroxide (0.02 g) was added to a solution of diethyl 2-methylphenylphosphonate (17.2 g, 75.4 mmol) in carbon tetrachloride (600 mL). A 100 W lamp was used to irradiate the mixture, which subsequently heated the mixture to reflux, and NBS (12.1 g, 67.9 mmol) was added in four equal portions over 8 h. The mixture was irradiated for 18 h, then filtered and concentrated to one-third of the original volume under reduced pressure. The mixture was then washed with water (2 × 50 mL) and brine (1 × 100 mL) and dried ($MgSO_4$) and the solvent removed under reduced pressure to leave **33** (20.0 g, ∼70% by ^{1}H NMR) as a golden oil, which was used without further purification in the next step. ^{1}H NMR of the crude product revealed a signal at 4.90 ppm (s, 2H, PhC*H*$_2$Br) corresponding to the product and a signal at 2.58 ppm (s, 3H, PhC*H*$_3$) (2:1 ratio); MS (EI) $m/z = 306$ $(M)^+$, 308 $(M)^+$, 171(*). ^{1}H NMR data for compound **33** is consistent with that reported previously.[31]

3-(2-Diethoxyphosphinylbenzyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (34). A 60% suspension of sodium hydride in mineral oil (0.48 g, 12.1 mmol) was added to a solution of 1-(tetrahydrofuran-2-yl)uracil (**31**) (2.20 g, 12.1 mmol) in DMF (50 mL). The resulting reaction mixture was stirred for 2 h at room temperature. To this was added diethyl 2-bromomethylphenylphosphonate (**33**) (6.36 g, ∼14.5 mmol), and the mixture was heated to 60 °C for 2 days. The mixture was evaporated under reduced pressure (1 mmHg, 60 °C), and the residue was taken up in ethyl acetate (50 mL) and washed with water (2 × 20 mL) and brine (20 mL), dried ($MgSO_4$), and evaporated under reduced pressure to give a brown oil. The oil was separated using silica gel chromatography, eluting with ethyl acetate, which gave **34** (2.17 g, 39%) as a golden oil. ^{1}H NMR ($CDCl_3$, 270.17 MHz) δ 1.37 (t, $J = 6.9$ Hz, 6H, CH$_2$C*H*$_3$), 1.97−2.12 (m, 3H, C*H*$_2$CH$_2$ and CH$_2$C*H*$_2$), 2.35−2.45 (m, 1H, CH$_2$C*H*$_2$), 4.08−4.27 (m, 6H, OC*H*$_2$-CH$_2$ and OC*H*$_2$CH$_3$), 5.48 (s, 2H, C*H*$_2$Ph), 5.84 (d, $J = 8.1$ Hz, 1H, HCC*H*CO), 6.03 (dd, $J = 6.4$ Hz, $J = 2.8$ Hz, C*H*OCH$_2$), 6.91 (t, $J = 6.6$ Hz, 1H, Ph), 7.28−7.47 (m, 2H, Ph), 7.42 (d, $J = 8.1$ Hz, 1H, *H*CCHCO), 7.98−8.07 (m, 1H, Ph), ^{13}C NMR ($CDCl_3$, 67.94 MHz) δ 14.2, 16.3 (d, $J = 4.7$ Hz), 24.0, 33.1, 62.3 (d, $J = 5.2$ Hz), 70.2, 88.1, 101.1, 124.4 (d, $J = 13.5$ Hz), 126.5 (d, $J = 14.5$ Hz), 126.6 (d, $J = 188.4$ Hz), 132.8 (d, $J = 2.6$ Hz), 134.8 (d, $J = 9.3$ Hz), 137.5, 139.2 (d, $J = 95.0$ Hz), 150.8, 162.8; MS (electrospray) 409.3 $[M + H]^+$.

3-(2-Diethoxyphosphinylbenzyl)pyrimidine-2,4-dione (35). A solution of 3-(2-diethoxyphosphinylbenzyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (**34**) (2.0 g, 4.9 mmol) in TFA (50 mL) was stirred at room temperature for 18 h. Analysis by TLC revealed the presence of a small quantity of starting material. The mixture was thus heated to 50 °C for a further 3 h, until the starting material was no longer detectable. The mixture was concentrated under reduced pressure, and the residue was separated using silica gel chromatography, eluting with methanol/ethyl acetate (1:9). The resulting solid was crystallized from methanol and gave **35** as a white solid (1.3 g, 78%). mp 175.7−177.9 °C (dec); ^{1}H NMR (DMSO-d_6, 270.17 MHz) δ 1.27 (t, $J = 6.9$ Hz, 6H, CH$_2$C*H*$_3$), 4.01−4.12 (m, 4H, C*H*$_2$CH$_3$), 5.26 (s, 2H, C*H*$_2$Ph), 5.70 (d, $J = 7.6$ Hz, 1H, HCC*H*CO), 6.86−6.91 (m, 1H, Ph), 7.36−7.42 (m, 1H, Ph), 7.51−7.60 (m, 2H, Ph + *H*CCHCO), 7.84 (dd, $J = 14.2$ Hz, $J = 10.2$ Hz, 1H, Ph), 11.31 (br s, 1H, N*H*); ^{13}C NMR ($CDCl_3$, 67.94 MHz) δ 16.0 (d, $J = 6.2$ Hz), 61.7 (d, $J = 5.2$ Hz), 99.6, 124.1 (d, $J = 13.5$ Hz), 125.3 (d, $J = 180.6$ Hz), 126.4 (d, $J = 14.5$ Hz), 133.4 (d, $J = 9.9$ Hz), 139.9 (d, $J = 10.4$ Hz), 141.1, 149.0, 151.4, 163.0, MS (electrospray) 339 $[M + H]^+$, 361 $[M + Na]^+$. Anal. ($C_{15}H_{19}N_2O_5P$) C, H, N.

(*S*)-1-(2-Amino-2-carboxyethyl)-3-(2-phosphonobenzyl)pyrimidine-2,4-dione (36). A 60% suspension of sodium hydride in mineral oil (0.24 g, 5.92 mmol) was added to a solution of 3-(2-diethoxyphosphinylbenzyl)pyrimidine-2,4-dione (**35**) (2.00 g, 5.92 mmol) in dry DMF (50 mL) under a dry argon atmosphere. The resulting reaction mixture was stirred for 12 h at room temperature. To this was added (*S*)-3-(*t*-butoxycarbonylamino)oxetan-2-one

(**46**)[11] (1.11 g, 5.92 mmol), and the mixture was stirred at room temperature for 48 h. The mixture was concentrated under reduced pressure (1 mmHg, 60 °C), and the residue was dissolved in 2 M aqueous HCl and concentrated under reduced pressure. This resulting residue was dissolved in water (5 mL) and applied to Dowex 50WX8−400 ion-exchange resin (H^+ form) (0.25 mmol of cation/mL of resin, 30 mL) with stirring for 30 min. The mixture was then applied to a column containing 30 mL of the same type of resin, eluting with water and THF/water (1:1) until the eluate was colorless and then 1 M aqueous pyridine. The ninhydrin-positive fractions of the 1 M aqueous pyridine eluate were combined and evaporated to dryness under reduced pressure. The residue was dissolved in water (5 mL) and applied to a column containing Biorad AG1 × 8 ion-exchange resin (acetate form) (0.5 mmol of anion/mL of resin, 30 mL), and the column was then eluted with water. The major ninhydrin-positive fraction was evaporated to dryness, and the residue was dried in a desiccator over phosphorus pentoxide at room temperature for 24 h. The glassy solid was suspended in bromotrimethylsilane and heated under reflux under a dry argon atmosphere for 18 h. The mixture was concentrated under reduced pressure (1 mmHg, 60 °C) to leave a white solid, which was dissolved in water (2 mL) and applied to Dowex 50WX8−400 ion-exchange resin (20 mL) with stirring for 30 min. The mixture was then applied to a column containing 20 mL of the same type of resin, eluting with water and then 1 M aqueous pyridine. The ninhydrin-positive fractions of the 1 M aqueous pyridine eluate were combined and evaporated to dryness under reduced pressure. The residue was dissolved in water (2 mL) and applied to Biorad AG1 × 8 ion-exchange resin (15 mL) and stirred at room temperature for 30 min. The mixture was then applied to a column containing 15 mL of the same type of resin and washed with increasing concentrations of aqueous acetic acid of 0.2 M, 0.5, 1, and 2 M. The product was eluted with aqueous formic acid (4 M), which was concentrated under reduced pressure. The residue was dissolved in water (10 mL) and concentrated under reduced pressure, and this process was repeated. The resulting white solid was dried under a stream of argon at room temperature for 18 h to give **36** as a white glassy solid (0.3 g, 14%). mp 270.1−275.5 °C (dec); $[\alpha]^{20}_D = -5.14°$ (*c* 0.175, 6 M HCl); ee 98% as determined by chiral HPLC; ^{1}H NMR (D_2O, 399.78 MHz) δ 4.27−4.33 (m, 2H, CHCH_2); 4.41−4.47 (m, 1H, C*H*CH_2), 5.47 (s, 2H, CH_2Ph), 5.99 (d, J = 7.8 Hz,1H, HCC*H*CO), 6.96−6.99 (m, 1H, Ph), 7.37−7.47 (m, 2H, Ph), 7.68 (d, J = 7.8 Hz, 1H, *H*CCHCO), 7.88 (dd, J = 14.2 Hz, J = 13.7 Hz, 1H, Ph); MS (electrospray) 370.2 [M + H]$^+$, 369.2 [M + Na]$^+$. Anal. ($C_{14}H_{16}N_3O_7P$ 2 H_2O) C, H, N.

Methyl 3-Bromomethylthiophene-2-carboxylate (37). Benzoyl peroxide (0.02 g) was added to a solution of methyl 3-methylthiophene-2-carboxylate (5.8 g, 37.2 mmol) in carbon tetrachloride (370 mL). A 100 W lamp was used to irradiate the mixture, which subsequently heated the mixture to reflux, and NBS (6.0 g, 33.5 mmol) was added in four equal portions at 30 min intervals. The mixture was allowed to react for a further 1.5 h. The mixture was filtered and concentrated to half of the original volume under reduced pressure. The mixture was then washed with water (2 × 100 mL) and brine (1 × 100 mL) and dried ($MgSO_4$), and the solvent was removed under reduced pressure to leave **37** (20.0 g, ∼70% by ^{1}H NMR) as a golden oil which was used without further purification in the next step. ^{1}H NMR ($CDCl_3$, 270.17 MHz) δ 3.89 (s, 3H, CO_2CH_3), 4.91 (s, 2H, CH_2Br), 7.17 (d, J = 5.0 Hz, 1H, SHCC*H*), 7.46 (d, J = 5.0 Hz, 1H, S*H*CCH); MS (EI)m/z = 234/236 [M]$^+$, 155 (*). ^{1}H NMR data for compound **37** was consistent with that reported previously.[32]

3-(2-Methoxycarbonylthiophene-3-ylmethyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (39). A 60% suspension of sodium hydride in mineral oil (0.66 g, 16.5 mmol) was added to a solution of 1-(tetrahydrofuran-2-yl)uracil (**31**) (3.0 g, 16.5 mmol) in dry DMF (30 mL) under a dry argon atmosphere. The resulting reaction mixture was stirred at room temperature for 2 h. To this was added methyl 3-bromomethylthiophene-2-carboxylate (**37**) (6.00 g, ∼18 mmol), and the mixture was stirred at room temperature for 18 h. After this time, the reaction had not gone to completion (as deduced by TLC) and the mixture was heated to 60 °C for 48 h. The mixture was concentrated under reduced pressure (1 mmHg, 60 °C), and the residue was separated using silica gel chromatography, eluting with ethyl acetate/petroleum ether (3:2) to give **39** as a golden oil (5.1 g, 91%). ^{1}H NMR ($CDCl_3$, 270.17 MHz) δ 1.87−2.10 (m, 3H, CH_2CH_2); 2.33−2.43 (m, 1H, CH_2CH_2), 3.90 (s, 3H, CO_2CH_3), 3.94−4.03 (m, 1H, CHOCH_2), 4.08−4.25 (m, 1H, CHOCH_2), 5.52 (s, 2H, CH_2Ar), 5.81 (d, J = 6.6 Hz, 1H, HCC*H*CO), 5.98−6.01 (m, 1H, C*H*OCH_2), 6.76 (d, J = 6.6 Hz, 1H, *H*CCHCO), 7.37 (d, J = 2.3 Hz, C*H*CHS), 7.39 (d, J = 2.3 Hz, 1H, CHC*H*S); ^{13}C NMR ($CDCl_3$, 67.94 MHz) δ 14.2; 24.0, 33.1, 40.0, 52.0, 60.3, 70.1, 88.1, 101.1, 127.7, 130.6, 137.5, 144.8, 150.8, 162.7; MS (EI) m/z = 336 (M$^+$); HRMS m/z (M$^+$) calculated for $C_{15}H_{16}N_2O_5S$: 336.077944. Found 336.078224.

3-(2-Carboxythiophene-3-ylmethyl)pyrimidine-2,4-dione (41). A solution of lithium hydroxide monohydrate (0.38 g, 9.1 mmol) in water (20 mL) was added to a solution of 3-(2-methoxycarbonylthiophene-3-yl-methyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (**39**) (2.78 g, 8.3 mmol) in dioxane (20 mL), and the mixture was stirred at room temperature for 18 h. The solution was acidified to pH 1 with 2 M aqueous HCl and then evaporated under reduced pressure. The residue was suspended in water (20 mL) and stirred for 30 min at room temperature. The solid was collected by filtration, washed with water (2 × 20 mL), and air-dried to a constant mass to give 3-(2-carboxythiophene-3-ylmethyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (2.7 g, 96%); mp 170.4−171.7 °C; ^{1}H NMR ($CDCl_3$, 399.78 MHz) δ 1.88−1.97 (m, 2H, CH_2CH_2), 1.99−2.07 (m, 1H, CH_2CH_2), 2.22−2.31 (m, 1H, CH_2-CH_2), 3.82−3.87 (m, 1H, CHOCH_2), 4.18−4.23 (m, 1H, CHOCH_2), 5.26 (s, 2H, CH_2Ar), 5.78 (d, J = 8.1 Hz, 1H, *H*CCHCO), 5.94−5.96 (m, 1H, C*H*OCH_2), 6.70 (d, J = 5.1 Hz, 1H, Ar), 7.69 (d, J = 8.1 Hz, 1H, HCC*H*CO), 7.71 (d, J = 5.1 Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$, 100.54 MHz) δ 23.5, 31.7, 69.4, 87.2, 100.1, 127.4, 127.5, 131.4, 139.4, 144.7, 150.3, 162.0, 163.2,185.3; MS (electrospray) 345 [M + Na]$^+$.

A solution of 3-(2-carboxythiophene-3-ylmethyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (1.80 g, 5.12 mmol) in trifluoroacetic acid (20 mL) was stirred at room temperature for 2 days, at which point analysis by TLC revealed a small quantity of the starting material. The mixture was therefore heated to 80 °C for 4 h and concentrated under reduced pressure to leave a dark brown solid, which was triturated under diethyl ether and collected by filtration. The solid was washed with ethyl acetate (2 × 5 mL) and air-dried. The resulting solid was crystallized from hot methanol to give **41** (1.35 g, 96%) as a white solid. mp 275−276.4 °C (dec); ^{1}H NMR (DMSO-d_6, 270.17 MHz) δ 5.23 (s, 2H, CH_2Ph), 5.67 (d, J = 7.6 Hz, 1H, CHC*H*O), 6.68 (d, J = 5.1 Hz, 1H, C*H*CHS), 7.54 (d, J = 7.6 Hz, 1H, C*H*CHCO), 7.70 (d, J = 5.1 Hz, 1H, CHC*H*S), 11.27, (br s, N*H*); ^{13}C NMR (DMSO-d_6, 67.94 MHz) δ 99.6, 127.3, 131.3, 141.0, 144.9, 151.4, 163.0, 163.2, 163.8; MS (EI) m/z = 252 (M$^+$); HRMS m/z (M$^+$) calculated for $C_{10}H_8N_2O_4S$: 252.020479. Found 252.019596. Anal. ($C_{10}H_8N_2O_4S$) C, H, N.

(*S*)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxythiophene-3-ylmethyl)pyrimidine-2,4-dione (43). A 60% suspension of sodium hydride in mineral oil (0.19 g, 4.76 mmol) was added to a solution of 3-(2-carboxythiophene-3-ylmethyl)pyrimidine-2,4-dione (**41**) (0.60 g, 2.38 mmol) in dry DMF (70 mL) under a dry argon atmosphere. The resulting reaction mixture was stirred at room temperature for 18 h. (*S*)-3-(*t*-Butoxycarbonylamino)oxetan-2-one (**46**) (0.45 g, 2.38 mmol) was added, and the mixture was stirred at room temperature for 2 days. The mixture was evaporated under reduced pressure (1 mmHg, 60 °C), and 1 M aqueous HCl was added (20 mL). The mixture was evaporated under reduced pressure, and the residue was suspended in water (5 mL) and applied to Dowex 50WX8−400 ion-exchange resin (H^+ form) (0.25 mmol of cation/mL of resin; 20 mL) with stirring for 30 min. The mixture was then applied to a column containing an equivalent volume of the same type of resin, eluting with water until no ninhydrin-positive fractions were observed, THF/water (1:1) until the eluate was colorless and then 1 M aqueous pyridine. The ninhydrin-positive

fractions of the 1 M aqueous pyridine eluate were combined and evaporated to dryness under reduced pressure. Crystallization of the residue from water yielded **43** (0.41 g, 51%) as a white solid. mp 234.4−235.1 °C (dec); $[\alpha]^{25}_D = -8.52°$ (*c* 0.132, 6 M HCl); ee >99% as determined by chiral HPLC;^{1}H NMR (TFA-*d*, 270.17 MHz) δ 4.68 (dd, $J_{BA} = 15.8$ Hz, $J_{BX} = 6.1$ Hz, 1H, C*H*$_2$CH); 4.83 (dd, $J_{AB} = 15.8$ Hz, $J_{AX} = 3.3$ Hz, 1H, C*H*$_2$CH), 5.02 (dd, $J_{AX} + J_{BX} = 9.0$ Hz, 1H, CH$_2$C*H*), 5.72 (s, 2H, C*H*$_2$Ph), 6.32 (d, $J = 8.1$ Hz, 1H, CHC*H*CO), 6.90 (d, $J = 5.1$ Hz, 1H, C*H*CHS), 7.66 (d, $J = 5.1$ Hz, CHC*H*S), 7.75 (d, $J = 8.1$ Hz, 1H, *H*CCHCO), ^{13}C NMR (TFA-*d*, 67.94 MHz) δ 43.6, 52.2, 56.0, 105.3, 128.7, 129.7, 136.3, 146.4, 147.4, 156.5, 168.0, 170.1, 171.2, MS (electrospray) 340.2 $[M + H]^+$, 362.1 $[M + H]^+$. Anal. ($C_{13}H_{13}N_3O_6S$) C, H, N.

***(R)*-1-(2-Amino-2-carboxyethyl)-3-(2-carboxythiophene-3-ylmethyl)pyrimidine-2,4-dione (44).** Following the procedure by which **43** was synthesized, treating 3-(2-carboxythiophene-3-ylmethyl)pyrimidine-2,4-dione (**41**) (1.0 g, 3.96 mmol) in dry DMF (20 mL) with a 60% suspension of sodium hydride in mineral oil (0.32 g, 7.93 mmol), then with (*R*)-3-(*t*-butoxycarbonylamino)-oxetan-2-one (**47**) (0.74 g, 3.96 mmol), gave (**44**) (0.99 g, 74%) as a white solid. mp 226.1−227.6 °C (dec); $[\alpha]^{25}_D = +14.78°$ (*c* 0.558, 6M HCl); ee >99% as determined by chiral HPLC; ^{1}H NMR (TFA-*d*, 399.78 MHz) δ 4.69 (dd, $J_{BA} = 15.8$ Hz, $J_{BX} = 6.0$ Hz, 1H C*H*$_2$CH), 4.82 (dd, $J_{AB} = 15.6$ Hz, $J_{AX} = 3.2$ Hz, 1H, C*H*$_2$CH), 5.01 (dd, $J_{AX} + J_{BX} = 9.2$ Hz, 1H, CH$_2$C*H*), 5.72 (s, 2H, C*H*$_2$Ph), 6.31 (d, $J = 8.1$ Hz, 1H, HCC*H*CO), 6.90 (dd, $J = 5.1$ Hz, 1H C*H*CHS), 7.67 (d, $J = 5.1$ Hz, 1H, CHC*H*S), 7.74 (dd, $J = 8.1$ Hz, 1H, *H*CCHCO); MS (electrospray) 340.2 $[M + H]^+$, 362.1 $[M + Na]^+$. Anal. ($C_{13}H_{13}N_3O_6S$) C, H, N.

Methyl 3-Bromomethylfuran-2-carboxylate (38). Benzoyl peroxide (0.04 g) was added to a solution of methyl 3-methylfuran-2-carboxylate (7.0 g, 49.95 mmol) in carbon tetrachloride (500 mL). A 100 W lamp was used to irradiate the mixture, which subsequently heated to reflux, and NBS (8.0 g, 44.95 mmol) was added in four equal portions at 30 min intervals. The mixture was allowed to react for a further 1.5 h. The mixture was filtered and concentrated to half of the original volume under reduced pressure. The mixture was then washed with water (2 × 100 mL) and brine (100 mL) and dried ($MgSO_4$), and the solvent was removed under reduced pressure to leave **38** (8.06 g, ∼80% by ^{1}H NMR) as a golden oil, which was used without further purification in the next step. ^{1}H NMR ($CDCl_3$, 399.78 MHz) δ 3.94 (s, 3H, CO_2CH_3), 4.71 (s, 2H, C*H*$_2$Br), 6.62 (d, $J = 2.0$ Hz, 1H, Ar), 7.51 (d, $J = 2.0$ Hz, 1H, Ar). ^{1}H NMR data for compound **38** was consistent with that reported previously.[33]

3-(2-Methoxycarbonylfuran-3-ylmethyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (40). A 60% suspension of sodium hydride in mineral oil (0.44 g, 11.0 mmol) was added to a solution of 1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (**31**) (2.00 g, 11.0 mmol) in anhydrous DMF (20 mL) under a dry argon atmosphere. The resulting reaction mixture was stirred at room temperature for 3 h. A crude mixture containing methyl 3-bromomethylfuran-2-carboxylate (**38**) (7.30 g, ∼15.3 mmol) was added, and the reaction mixture was heated to 60 °C for 48 h. The mixture was concentrated under reduced pressure (1 mmHg, 60 °C), and the resulting residue was suspended in ethyl acetate (100 mL), washed with water (2 × 50 mL) and saturated brine solution (50 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was separated using silica gel chromatography, eluting with ethyl acetate/petroleum ether (3:1) to give **40** (1.8 g, 52%) as a light brown solid, which was crystallized from ethyl acetate. mp 112.6−113.8 °C; ^{1}H NMR ($CDCl_3$, 270.17 MHz) δ 2.12−1.87 (m, 3H, C*H*$_2$CH$_2$ + CH$_2$C*H*$_2$), 2.34−2.44 (m, 1H, C*H*$_2$CH$_2$), 3.94 (s, 3H, CO_2CH_3), 3.97−4.04 (m, 1H, CHOC*H*$_2$), 4.18−4.26 (m, 1H, CHOC*H*$_2$), 5.37 (s, 2H, C*H*$_2$Ph), 5.81 (d, $J = 7.9$, 1H, HCC*H*CO), 5.98−6.02 (m, 1H, CHOC*H*$_2$), 6.32 (d, $J = 2.0$ Hz, 1H, Ar), 7.38 (d, $J = 7.9$ Hz, 1H, Ar), 7.44 (d, $J = 2.0$ Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$, 67.94 MHz) δ 23.8, 33.0, 36.3, 51.8, 70.1, 87.9, 100.9, 112.2 130.4, 137.5, 140.0, 145.2, 150.5, 159.3, 162.6; MS (electrospray) 343 $[M + Na]^+$.

3-(2-Carboxyfuran-3-ylmethyl)pyrimidine-2,4-dione (42). A solution of lithium hydroxide monohydrate (0.47 g, 11.2 mmol) in water (30 mL) was added to a solution of 3-(2-methoxycarbonylfuran-3-ylmethyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (**40**) (3.0 g, 9.4 mmol) in dioxane (30 mL), and the mixture was stirred at room temperature for 18 h. The solution was acidified to pH 1 with 2 M aqueous HCl and then evaporated under reduced pressure. The residue was dissolved in water (20 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic fractions were dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was suspended in diethyl ether (100 mL) and stirred overnight at room temperature. The solid was collected by filtration and air-dried to a constant weight to give 3-(2-carboxyfuran-3-ylmethyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (2.2 g, 77%) as a white solid; mp 161.9−162.7 °C (dec); ^{1}H NMR (DMSO-d_6, 270.17 MHz) δ 1.87−2.07 (m, 3H, C*H*$_2$CH$_2$ + CH$_2$C*H*$_2$), 2.20−2.30 (m, 1H, C*H*$_2$CH$_2$), 3.81−3.89 (m, 1H, CHOC*H*$_2$), 4.17−4.25 (m, 1H, CHOC*H*$_2$), 5.15 (s, 2H, C*H*$_2$Ph), 5.77 (d, $J = 8.2$ Hz, 1H, HCC*H*CO), 5.93−5.97 (m, 1H, C*H*OCH$_2$), 6.30 (d, $J = 1.7$ Hz, 1H, Ar), 7.68 (d, $J = 8.2$ Hz, 1H, *H*CCHCO), 7.75 (d, $J = 1.7$ Hz, 1H, Ar); ^{13}C NMR (DMSO-d_6, 67.94 MHz) δ 23.5, 31.7, 36.3, 69.4, 87.2, 100.1, 111.7, 130.1, 139.4, 140.1, 145.6, 150.3, 159.9, 161.9; MS (electrospray) 329 $[M + Na]^+$.

To trifluoroacetic acetic acid (20 mL) was added 3-(2-carboxyfuran-3-ylmethyl)-1-(tetrahydrofuran-2-yl)pyrimidine-2,4-dione (2.10 g, 6.86 mmol), and the solution was stirred at room temperature for 3 days. The mixture was concentrated under reduced pressure, and diethyl ether (50 mL) was added. The solid was collected by filtration, washed with diethyl ether (2 × 30 mL), and air-dried to give **42** (1.11 g, 69%) as a white solid. mp 236.5−241.1 °C (dec); ^{1}H NMR (DMSO-d_6, 270.17 MHz) δ 5.12 (s, 2H, C*H*$_2$Ph), 5.66 (d, $J = 7.6$ Hz, 1H, HCC*H*CO), 6.28 (d, $J = 1.6$ Hz, 1H, Ar), 7.51 (dd, $J = 7.6$ Hz, $J = 5.6$ Hz, 1H, *H*CCHCO), 7.77 (d, $J = 1.7$ Hz, 1H, Ar), 11.25 (d, $J = 5.6$ Hz, 1H, N*H*), 13.2 (bs, 1H, 1H, CO_2H); ^{13}C NMR (DMSO-d_6, 67.94 MHz) δ 35.7 99.7, 111.7, 130.9, 139.7, 141.1, 146.0, 151.4 159.8, 163.0; MS (electrospray) 235 $[M - H]^-$. Anal. ($C_{10}H_8N_2O_5$) C, H, N.

***(S)*-1-(2-Amino-2-carboxyethyl)-3-(2-carboxyfuran-3-ylmethyl)pyrimidine-2,4-dione (45).** A 60% suspension of sodium hydride in mineral oil (0.34 g, 8.47 mmol) was added to a solution of 3-(2-carboxyfuran-3-ylmethyl)pyrimidine-2,4-dione (**42**) (1.0 g, 4.23 mmol) in dry DMF (60 mL) under a dry argon atmosphere. The resulting reaction mixture was stirred at room temperature for 18 h. (*S*)-3-(*t*-Butoxycarbonylamino)oxetan-2-one (**46**) (0.79 g, 4.23 mmol) was added, and the mixture was stirred at room temperature for 2 days. The mixture was evaporated under reduced pressure (1 mm Hg, 60 °C), and 2 M aqueous HCl was added (40 mL). The mixture was evaporated under reduced pressure and the residue was suspended in water (10 mL) and applied to Dowex 50WX8−400 ion-exchange resin (H^+ form) (0.25 mmol of cation/mL of resin; 40 mL) with stirring for 30 min. The mixture was then applied to a column containing an equivalent volume of identical resin, eluting with water until no ninhydrin-positive fractions were observed, THF/water (1:1) until the eluate was colorless, and then 1 M aqueous pyridine. The ninhydrin-positive fractions of the 1 M aqueous pyridine eluate were combined and evaporated to dryness under reduced pressure. The residue was suspended in water (10 mL) and applied to Biorad AG1 × 8 ion-exchange resin (acetate form) (0.5 mmol of anion/mL of resin; 10 mL) and stirred for 30 min, then poured onto a column containing an equivalent volume of the same type of resin. The column was then eluted with water and then aqueous acetic acid of increasing concentrations of 0.01, 0.05, 0.1, 0.5, and 1 M. The ninhydrin-positive fractions of the 1 M aqueous acetic acid eluate were combined and evaporated under reduced pressure. Crystallization of the residue from water yielded **45** (0.55 g, 40%) as a white solid. mp 193.8−196.2 °C (dec); $[\alpha]^{25}_D = -16.54°$ (*c* 0.847 6 M HCl); ee >99% as determined by chiral HPLC;^{1}H NMR (TFA-*d*, 399.78 MHz) δ 4.68 (dd, $J_{BA} = 15.6$ Hz, $J_{BX} = 6.4$ Hz, 1H, C*H*$_2$CH), 4.81 (dd, $J_{AB} = 15.6$ Hz, $J_{AX} = 2.9$ Hz, 1H, C*H*$_2$CH), 5.01 (dd, $J_{AX} + J_{BX} = 9.3$ Hz, 1H, CH$_2$C*H*), 5.59 (s, 2H, C*H*$_2$Ar), 6.29 (d, $J = 8.3$ Hz, 1H, HCC*H*CO), 6.53 (d,

J = 1.5 Hz, 1H, *H*CCHS), 7.65 (d, J = 1.5 Hz, 1H, HCC*H*S), 7.72 (d, J = 8.3 1H, *H*CCHCO); MS (electrospray) 324 $[M + H]^+$, 346.1 $[M + Na]^+$. Anal. ($C_{13}H_{13}N_3O_7$ 1.2 H_2O) C, H, N.

Electrophysiology. Antagonism of Kainate Responses on Dorsal Root C-Fibers by Novel Willardiine Derivatives. Experiments to test the antagonist effect of the novel compounds at GLU_{K5}-containing kainate receptors were conveniently carried out on kainate-induced responses on isolated dorsal roots, as described in detail previously.[8] To prevent desensitization of kainate receptors, the dorsal root was superfused with 1 mg mL^{-1} Concanavalin A for 20 min after a 20 min exposure to glucose-free superfusion medium. Standard superfusion medium was then applied throughout the experiments. This allowed measurement of depolarizations evoked by the exogenously applied agonist, kainate (1 min applications). Noncumulative, nonsequential concentration−response curves were constructed for kainate in the absence and presence of the antagonist (30 min preincubation). The EC_{50} values (10−15 μM) for kainate-induced depolarization of the dorsal root determined in assays of antagonists did not differ significantly from previously reported values.[16,25]

Characterization of AMPA Receptor Antagonists. Hemisected spinal cords from nonanaesthetized 1- to 5-day-old rats killed by cervical dislocation were prepared and used according to the reported method.[19] To assess AMPA receptor antagonist activity the ability of the compounds to block the fast component of the dorsal root-evoked ventral root potential (fDR-VRP) in the neonatal rat hemisected spinal cord preparation was measured, as described in detail previously.[8] Concentration−response curves were constructed for test antagonists (5 min applications), in the presence of 2 mM $MgSO_4$/50 μM (*R*)-2-amino-5-phosphonopentanoic acid ((*R*)-AP5) (30 min preincubation) to block NMDA receptors. Results are expressed as mean ± SEM, n = 3.

To further characterize the AMPA receptor antagonist activity of **43**, noncumulative concentration−response curves were obtained for the selective AMPA receptor agonist **9** (1 min applications) in the absence and presence of 200 μM **43** (30 min preincubation).[8a]

Characterization of 43 on NMDA and Group I Metabotropic Glutamate Receptors Expressed on Neonatal Rat Motoneurones. Experiments performed to investigate the effect of **43** on receptors expressed on motoneurones in the hemisected neonatal rat spinal cord preparation were carried out in the presence of tetrodotoxin (TTX, 10 μM for 2 min, then 0.1 μM continuously) to block action-potential-dependent release. No electrical stimulation was applied, thus allowing measurement of depolarizations evoked by exogenously applied agonists.[8a] In experiments to determine the antagonist selectivity of **43**, medium containing approximately equieffective concentrations of either (*S*)-AMPA (0.7 μM), NMDA (10 μM), or (*S*)-3,5-dihydroxyphenylglycine (DHPG, **48**; 20 μM) was applied for 1 min, in the absence and presence of **43** (50 μM; 30 min preincubation).

Recombinantly Expressed Rat GLU_{K6} Kainate Receptor Binding Assay in HEK293 Cells. For radioligand binding studies, HEK293 cells were transfected with GLU_{K6} DNA using Lipofectamine 2000 then membranes harvested 2 days later as described in detail previously.[8c] Displacement radioligand binding studies were carried out in the presence of 10 nM [^{3}H]kainate, with nonspecific binding defined as that not displaced by 100 μM kainate. The novel compounds were tested at concentrations of 10 μM, 100 μM, and 1 mM to give an initial indication of their affinity. Competition binding curves were generated for the standard kainate receptor ligands **1** and **48** and analyzed by iterative nonlinear regression using GraphPAD Prism.[8c]

[^{3}H]kainate Displacement Assay for GLU_{K7}. Membrane Preparation. Adherent HEK293 cells stably transfected with human GLU_{K7} kainate receptors were thawed and lysed in 10 volumes of ice-cold distilled water and centrifuged for 30 min at 40000*g*. The resulting pellets were resuspended in >100 volumes of assay buffer (50mM Tris-HCl, pH 7.4) and centrifuged at 40000*g* again to remove endogenous glutamate. The resulting pellets were resuspended in 4 mL assay buffer and subjected to [^{3}H]kainate binding experiments.

[^{3}H]Kainate Displacement Assay. Displacement of [^{3}H]kainate binding by **43** or NBQX (50) was carried out in borosilicate tubes containing 125 μg of membrane protein, 7 nM [^{3}H]kainate (PerkinElmer, Boston, MA), test compounds in a range of concentrations, and assay buffer to a final volume of 200 μL. Nonspecific binding was defined by 10 mM glutamate. Incubation was carried out at 4 °C for 2 h and terminated by rapid filtration (Millipore 12 port vacuum manifold) through Whatman GF/B filters presoaked in 0.03% polyethylenimine. Filters were washed three times with 2 mL cold assay buffer, and the retained radioactivity on the filters was measured using a liquid scintillation counter (Beckman LS6000TA Instrument). Protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Competition binding curves were analyzed using GraphPad Prism 3.02 (San Diego, CA) with slope factor set at 1 and top and bottom fixed at 100% and 0% of control [^{3}H]kainate binding, respectively. The dissociation constant (K_i) for test compounds was calculated according to the Cheng−Prusoff equation.[20]

[^{3}H]Kainate binds to membranes from these cells with K_D = 5.3 ± 0.8 nM and B_{max} = 3.0 ± 0.1 pmol/mg, determined from saturation binding experiments performed under the same conditions.

Calcium Fluorescence Assays Using Recombinant Human AMPA and Kainate Receptor Subtypes. AMPA Receptor Assays. HEK293 cells stably expressing human AMPA receptors were seeded into poly-D-lysine-coated 96-well plates (Becton Dickinson Labware, Bedford, MA) 1 or 2 days prior to experiments at 60000 cells/well (1 day) or 30000 cells/well (2 day). Cells were washed three times with 100 μL assay buffer composed of Hank's Balanced Salt Solution without phenol red (Invitrogen) with 20 mM HEPES and 3.7 mM $CaCl_2$ added (final $[CaCl_2]$ = 5 mM). Plates were then incubated for 2−3 h at room temperature in 40 μL assay buffer with 8 μM Fluo3-AM dye (Molecular Probes Inc., Eugene, OR). Following dye incubation, cells were rinsed once with 100 μL of assay buffer. Finally, 50 μL of assay buffer, which included the AMPA receptor potentiator LY392098 (10 μM; to prevent desensitization of AMPA receptors), was added to wells and fluorescence measured using a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). The FLIPR added a first addition of 50 μL of LY392098-containing assay buffer, followed by a second addition of 100 μL of LY392098-containing buffer three minutes later. **43** was added in the absence of agonist during the first addition, and in the presence of 100 μM glutamate during the second addition.

Kainate Receptor Assays. All receptor clones were stably expressed in HEK293 cells. The GLU_{K5}(Q)/GLU_{K2} cell line was created by retroviral infection of cDNA coding for the human GLU_{K2} subunit (EAA2; Allelix Biopharmaceuticals) into the GLU_{K5}(Q)-expressing cell line using the pMNLZRS/IB retroviral expression vector. HEK293 cell lines stably expressing a cloned GLU_{K5}(Q)[21] or GLU_{K6}(Q) receptor subunit,[22] or coexpressing GLU_{K5}(R) and GLU_{K6}(Q),[4c] or GLU_{K6}(Q) and GLU_{K2}[23] have been previously described. Kainate receptor expression levels for all transfected cell lines have been previously determined in our laboratory by saturation binding of [^{3}H]kainate to intact cells. B_{max} values for specific [^{3}H]kainate binding are GLU_{K5}(Q): 1.7 ± 0.5 pmol/mg, GLU_{K5}(R)/6(Q): 8 ± 2 pmol/mg, GLU_{K5}(Q)/GLU_{K2}: 0.6 ± 0.1 pmol/mg, GLU_{K6}(Q): 2.7 ± 0.3 pmol/mg, GLU_{K6}(Q)/GLU_{K2}: 1.7 ± 0.3 pmol/mg.[24] To address the possibility that changes in receptor expression levels over time could influence experimentally determined IC_{50} values, the EC_{50} value for glutamate and the IC_{50} values for routinely used control antagonists such as NBQX were continuously monitored, and no significant drift in these values was ever observed. Additionally, cells were never passaged more than 20 times.

Cell growth and ion influx studies using a FLIPR were carried out exactly as described previously, in the presence of concanavalin A.[8] The antagonist **43** was added in the absence of agonist during the first addition, and in the presence of 100 μM glutamate (**1**) during the second addition. Concentration−response curves for **43** were analyzed using GraphPad Prism 3.02 software (San Diego,

CA), with slope factor fixed at 1, and top and bottom fixed at 100% and 0% inhibition, respectively. The dissociation constant (K_b) was calculated according to the Cheng−Prusoff equation[20] from the IC_{50} value for inhibiting 100 μM glutamate-induced calcium influx.

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Supporting Information Available: Elemental analyses for intermediates and final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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