Synthesis and pharmacology of glutamate receptor ligands: new isothiazole analogues of ibotenic acid†

Charlotte G. Jørgensen,[a] Rasmus P. Clausen,[a] Kasper B. Hansen,[b] Hans Bräuner-Osborne,[a] Birgitte Nielsen,[a] Bjørn Metzler,[a] Jan Kehler,[b] Povl Krogsgaard-Larsen[a] and Ulf Madsen*[a]

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The naturally occurring heterocyclic amino acid ibotenic acid (Ibo) and the synthetic analogue thioibotenic acid (Thio-Ibo) possess interesting but dissimilar pharmacological activity at ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs). Therefore, a series of Thio-Ibo analogues was synthesized. The synthesis included introduction of substituents by Suzuki and Grignard reactions on 4-halogenated 3-benzyloxyisothiazolols, reduction of the obtained alcohols, followed by introduction of the amino acid moiety by use of 2-(*N*-*tert*-butoxycarbonylimino)malonic acid diethyl ester. The obtained Thio-Ibo analogues (**1**, **2a–g**) were characterized in functional assays on recombinant mGluRs and in receptor binding assays on native iGluRs. At mGluRs, the activity at Group II was retained for compounds with small substituents (**2a–2d**), whereas the Group I and Group III receptor activities for all new compounds were lost. Detection of NMDA receptor affinity prompted further characterization, and two-electrode voltage-clamp recordings at recombinant NMDA receptor subtypes NR1/NR2A-D expressed in *Xenopus* oocytes were carried out for compounds with small substituents (chloro, bromo, methyl or ethyl, compounds **2a–d**). This series of Thio-Ibo analogues defines a structural threshold for NMDA receptor activation and reveals that the individual subtypes have different steric requirements for receptor activation. The compounds **2a** and **2c** are the first examples of agonists discriminating individual NMDA subtypes.

Introduction

(*S*)-Glutamic acid (Glu) is the main excitatory neurotransmitter in the central nervous system and activates ionotropic glutamate receptors (iGluRs) as well as metabotropic glutamate receptors (mGluRs). The mGluRs and iGluRs are both implicated in fundamental physiological processes and are involved in the development of several neurological diseases.[1–4] Within the last decade many selective compounds have emerged providing important pharmacological tools, but the pharmacological coverage of the Glu receptors is still incomplete.[2]

According to activation by the selective agonists, the iGluRs are divided into *N*-methyl-D-aspartic acid (NMDA), (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid (KA) receptors. Seven NMDA (NR1, NR2A-D, NR3A-B), four AMPA (GluR1-4) and five KA (GluR5-7 and KA1-2) receptor subunits have been identified.[1] The native iGluRs are tetrameric combinations of the respective subunits, of which NMDA and AMPA receptors are likely heteromeric and KA receptors may be either homomeric or heteromeric in composition.[1,5,6] Functional NMDA receptors are heteromeric assemblies typically of two NR1 subunits and two NR2 subunits, that are activated by simultaneous binding of glycine and Glu. The NR1 subunits provide the glycine binding sites and the NR2 subunits form the Glu binding sites.[5]

Eight subtypes of mGluRs have been characterized and these are divided into three groups according to protein sequence identity and signal transduction pathways: Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8).[2,4]

Ibotenic acid (Ibo) is a naturally occurring amino acid, which was isolated from the mushroom *Amanita muscaria.*[7] Ibo is a conformationally 3-isoxazolol analogue of Glu (Fig. 1), and interacts with KA receptors (low affinity), NMDA receptors and Group I and Group II mGluRs.[8,9] The thio-analogue of Ibo, thioibotenic acid (Thio-Ibo) synthesized by Bunch *et al.*[10] shows a pharmacology different from that of Ibo.[8] Both Ibo and Thio-Ibo activate Group I and Group II mGluRs with similar potency, but interestingly the agonist potency of Thio-Ibo at Group III mGluRs is increased more than 500-fold compared to Ibo. This difference in the activity profile of Thio-Ibo as compared to Ibo

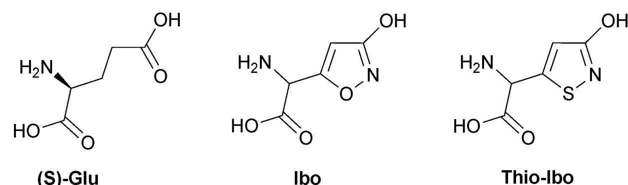


Fig. 1 Chemical structures of (*S*)-Glu, Ibo, and Thio-Ibo. The depicted structures are the uncharged forms of the ligands.

[a]*Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, 2100, Copenhagen, Denmark. E-mail: um@dfuni.dk; Fax: +45 35306040*
[b]*H. Lundbeck A/S, 2500, Valby, Copenhagen, Denmark*

† Electronic supplementary information (ESI) available: Detailed experimental procedures and compound characterization data of compounds **1**, **2b–g**, **3**, **5**, **6**, **7b–c**, **f–g**, **9c**,**f–g**, **10**, **11**, **12**, **13b–g**, **14b–g**. See DOI: 10.1039/b615162k

was ascribed to a lowering of the relative energy for the preferred mGluR4 active conformation.[8]

Based on the unique pharmacology of Thio-Ibo, a selection of analogues was chosen as model compounds in order to further investigate the structure–activity relationship at both iGluRs and mGluRs.

Eight different substituents were chosen from three categories: (1) α-substituents (compound **1**). Some mGluR agonists have successfully been converted into antagonists by introducing substituents in the α-position of the amino acid.[4,11] (2) Halogen and alkyl substituents (compounds **2a–d**), in analogy with other series of subtype selective Glu receptor active compounds.[12,13] (3) Aromatic substituents (compounds **2e–g**) (Fig. 2).

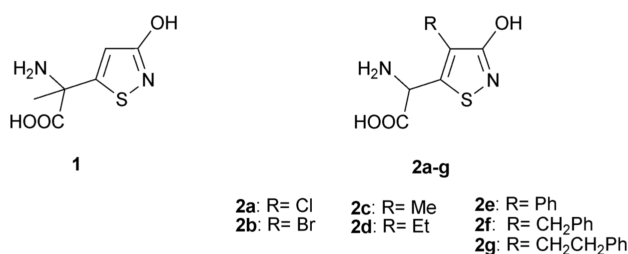


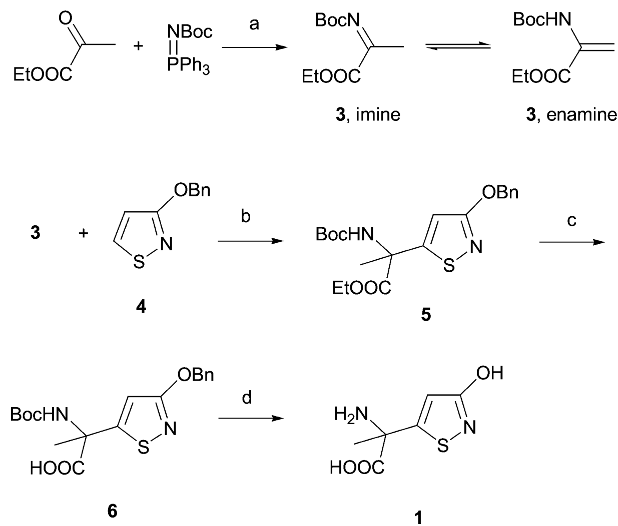
Fig. 2 Chemical structures of the new Thio-Ibo analogues **1** and **2a–g**.

Results

Chemistry

Direct functionalization of a protected Thio-Ibo derivative was not feasible and instead the amino acid analogues were prepared *via* 3-benzyloxyisothiazoles.

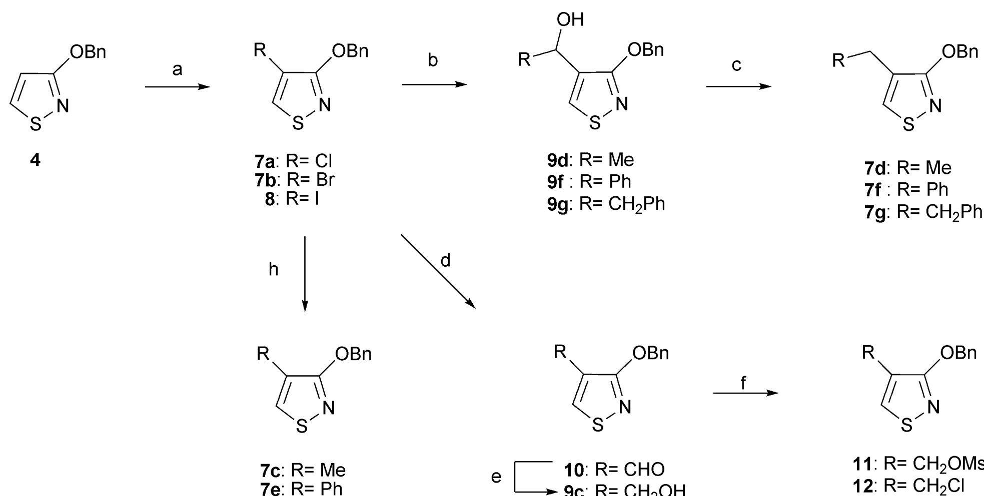
An amino acid synthon, ketimine **3**, for the preparation of α-methyl amino acids, was prepared by an aza-Wittig reaction (Scheme 1).[14] Regioselective lithiation of the 5-position of **4** was accomplished with LDA,[10] and further reaction with ketimine **3** resulted in the protected amino acid **5**. Ketimine **3** exists as a tautomeric mixture (Scheme 1) as observed by NMR, and this may explain the moderate yield (36%) of the protected amino acid **5**. The ester **5** was hydrolyzed under basic conditions yielding **6**. Subsequent treatment with hydrogen bromide in glacial acetic acid removed the Boc and benzyl protecting groups to give compound **1**. This two-step deprotection procedure was found to be more efficient than acidic deprotection of **5** in one step.



Scheme 1 *Reagents and conditions*: a) THF, reflux, 30%; b) LDA, Et_2O, −78 °C, then imine **3**, −78 °C, 36%; c) LiOH (aq), THF, rt, 75%; d) HBr, AcOH, rt, 21%.

The 4-substituted Thio-Ibo derivatives (**2a–g**) were prepared from the corresponding 4-substituted 3-benzyloxyisothiazoles (**7a–7g**) (Scheme 2).

Earlier reports by Lewis *et al.* on the synthesis of 3-isothiazolol and 4-methylisothiazolol have involved cyclization reactions of 3,3′-dithiodipropionamides.[15] In 1994, Beeley *et al.* reported very low yields using this method to synthesize 3-isothiazolol.[16] Moreover, the acyclic starting materials for the synthesis of 4-substituted



Scheme 2 *Reagents and conditions*: a) **7a**: NCS, MeCN, 58%; **7b**: NBS, MeCN, 88%; **8**: ICl, K_2CO_3, $CHCl_3$, 82%; b) *i*PrMgCl, RCHO, THF; **9d**: 84% (from **8**); **9f**: 86% (from **8**); **9g**: 26% (from **8**) or 43% (from **7b**); c) TFA, Et_3SiH, CH_2Cl_2; **7d**: 70%; **7f**: 54%; **7g**: 65%; d) *i*PrMgCl, DMF, THF, 82% (from **8**); e) $NaBH_4$, MeOH, 98%; f) **11**: MsCl, Et_3N, THF; **12**: $SOCl_2$; h) **7c**: $MeB(OH)_2$, $PdCl_2(PPh_3)_2$, K_2CO_3, DMF, MW 160 °C, 5 min, 32% (from **7b**); **7e**: $PhB(OH)_2$, $PdCl_2(PPh_3)_2$, K_2CO_3 (aq), DMF, 94% (from **8**).

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compounds are difficult to obtain.[16,17] The synthetic difficulties and low yields described by Beeley *et al.* are in accordance with our own experience concerning the synthesis of 3-isothiazolol and 4-substituted analogues. We found that introduction of the substituents on O-protected 3-isothiazolol after the cyclization represented a more convenient approach. Halogenations of alkyl protected 3-isothiazolol with iodo monochloride or bromine in acetic acid occurs selectively in the 4-position of the isothiazole.[18] In contrast, when chlorinating agents such as sulfuryl chloride or chlorine are used on 3-isothiazolol, additional chlorination at C-5 follows rapidly.[15] The benzyl protecting group in compound **4** did not withstand the acidic halogenation conditions previously described and the syntheses of **7a**, **7b** and **8** were accomplished employing non-acidic conditions (Scheme 2).

The 4-iodo (**8**) or 4-bromo (**7b**) compounds were suitable starting materials for magnesium–halogen exchange followed by Grignard reactions with various aldehydes leading to alcohols **9d**, **f** and **g**. Treatment of **9d**, **f** and **g** with trifluoroacetic acid and triethylsilane gave the respective isothiazoles **7d**, **f** and **g**. However, the final step was not applicable for the synthesis of methyl compound **7c**.

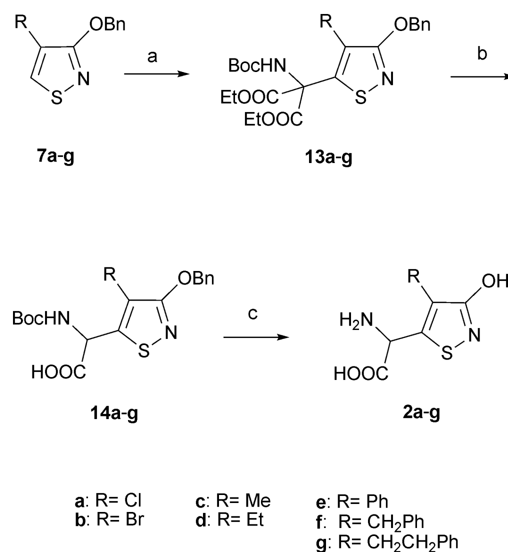
Alcohol **9c** was effectively obtained *via* the aldehyde **10** and various other reductive methods were tried for the synthesis of **7c** from **9c**. Treatment of **9c** with sulfur trioxide in pyridine, followed by $LiAlH_4$,[19] conversion of **9c** to mesylate **11** followed by reduction with $LiAlH_4$ or $NaBH_4$,[20] conversion of **9c** to chloride **12** with subsequent reduction using $NaBH_4$[21] and treatment of **10** with tosylhydrazide followed by catecholborane and NaOAc[22] were all unsuccessful.

Due to the difficulties with reducing the alcohol functionality in **9c**, we investigated the possibility of introducing the methyl group by a Suzuki cross-coupling reaction. The conditions reported by Molander[23] resulted in very low yields, but microwave heating and the use of methyl boronic acid and $PdCl_2(PPh_3)_2$ limited by-product formation sufficiently to give an isolated yield of 32% of **7c**. Standard aryl–aryl Suzuki reaction was applied to give the phenyl compound **7e** in excellent yield (Scheme 2).

The 4-subsituted isothiazoles (**7a–7g**) underwent lithiation followed by addition of 2-(*N*-*tert*-butoxycarbonylimino)malonic acid diethyl ester (Scheme 3). With substituents in the 4-position, we found that prolonged lithiation times compared to the lithiation time used for the unsubstituted analogue (**4**)[10] improved the yields, and the protected amino acids **13a–g** were isolated in 38–62% yield. Basic hydrolysis of the diesters followed by acid decarboxylation yielded **14a–g**. Acids **14a–g** were deprotected with hydrogen bromide in glacial acetic acid giving **2a–g** as zwitterions upon treatment of the corresponding hydrobromides with propylene oxide. Compound **1c** was isolated as the hydrobromide salt due to instability of the zwitterion.

pK_a Values

The pK_a values of Thio-Ibo and derivatives **2a–2d** were determined by potentiometric titration (Table 1). However, due to instability of the compounds at low pH in aqueous solution, the determination of pK_a of the carboxyl group was not reliable. The pK_a value of the 3-hydroxy group in Thio-Ibo was approximately one unit higher than that of Ibo.[24] When aliphatic substituents were introduced in the 4-position of Thio-Ibo a slight decrease in acidity was observed. Halogen substituents, on the other hand, significantly increased the acidity of the 3-isothiazolol group (Table 1).



Scheme 3 *Reagents and conditions*: a) LDA, 2-(*N*-*tert*-butoxycarbonylimino)malonic acid diethyl ester, Et_2O, −78 °C, b) LiOH (aq), THF, c) HBr, AcOH, rt.

Table 1 pK_a values for heterocyclic 3-hydroxy moiety and amino groups determined by potentiometric titration

	OH	NH_2
3-Isoxazolol[a]	5.85	–
3-Isothiazolol[a]	7.54	–
Ibo[b]	5.04	8.16
Thio-Ibo	5.9	7.8
2a	4.7	7.8
2b	4.5	7.6
2c	6.3	8.3
2d	6.3	8.2

[a] Ref. 25. [b] Ref. 24.

Pharmacology

The Thio-Ibo analogues **1**, **2a–2g** were characterized pharmacologically in receptor binding assays at native AMPA, KA and NMDA receptors and in functional assays using recombinant NMDA receptors and mGluRs.

None of the compounds (**1**, **2a–2g**) showed affinity for AMPA and KA receptor binding sites (IC_{50} > 100 μM). However, compounds **2a–2c** containing chloro, bromo and methyl substituents displayed NMDA receptor affinities comparable with that of Thio-Ibo, using [^{3}H]CGP39653 as the NMDA receptor radioligand (Table 2). The α-methyl analogue **1** was inactive (IC_{50} > 100 μM) at NMDA receptors. Introduction of larger substituents, such as ethyl (**2d**), phenyl (**2e**) and benzyl (**2f**), lead to a decrease in affinity at NMDA receptors, and the 2-phenylethyl analogue **2g** was inactive.

The observed NMDA receptor affinity of **2a–2d** prompted a functional characterization of these compounds at NMDA receptor subtypes. Glu, Ibo, Thio-Ibo and **2a–2d** were characterized by two-electrode voltage-clamp recordings on *Xenopus* oocytes expressing diheteromeric combinations of the recombinant NMDA

Table 2 Receptor binding affinities at iGluRs in rat cortical membranes[a]

	[^{3}H]AMPA IC_{50}/μM	[^{3}H]KA IC_{50}/μM	[^{3}H]CGP39653 K_i/μM
Glu	0.34[b]	0.38[b]	0.20[c]
Ibo	>100[d]	22[d]	5.3[d]
Thio-Ibo	>100[d]	>100[d]	13[d]
1	>100	>100	>100
2a	>100	>100	19 [18;21]
2b	>100	>100	21 [19;24]
2c	>100	>100	14 [13;15]
2d	>100	>100	41 [37;46]
2e	>100	>100	86 [74;99]
2f	>100	>100	80 [75;86]
2g	>100	>100	>100

[a] Values are expressed as the antilog to the log mean of three individual experiments. The numbers in brackets [min;max] indicate ± S.E.M. according to a logarithmic distribution of K_i. [b] Ref. 26. [c] Ref. 27. [d] Ref. 8.

Table 4 Pharmacological activities at cloned mGluRs expressed in CHO cells[a]

	EC_{50}/μM [pEC_{50} ± S.E.M.]		
	mGluR1	mGluR2	mGluR4
(*S*)-Glu	13[b]	4.4[b]	13[b]
Ibo	43 [4.37 ± 0.01][b]	110 [3.97 ± 0.04][b]	>1000[b]
Thio-Ibo	12 [4.96 ± 0.11][c]	52 [4.30 ± 0.06][c]	2.6 [5.63 ± 0.14][c]
1	>1000	>1000	>1000
2a	>1000	150 [3.89 ± 0.16]	295 [3.53 ± 0.18][d]
2b	>1000	89 [4.05 ± 0.02]	>1000
2c	>1000	140 [3.94 ± 0.20]	>1000
2d	>1000	140 [3.92 ± 0.13]	>1000
2e	>1000	>1000	>1000
2f	>1000	>1000	>1000
2g	>1000	>1000	>1000

[a] Functional data were obtained from CHO cell lines stably expressing the mGluR1a, 2 or 4a receptor subtype. Results on mGluR1a receptors were obtained by measurement of intracellular Ca^{2+} levels and results for mGluR2 and 4a were determined by measurement of intracellular cAMP. Data are given as means of at least three independent experiments. [b] Ref. 9. [c] Ref. 8. [d] Partial agonism (relative efficacy = 54%).

receptor subunits NR1 and NR2A-D (Fig. 3 and Table 3). Ibo and Thio-Ibo were found to be partial agonists at all four subunit combinations, with maximal activities ranging from 57–79% relative to Glu. The chloro-substituted compound **2a** was a partial agonist at the NR1/NR2A, NR1/NR2B and NR1/NR2D subtypes, as illustrated by the concentration–response curves in Fig. 3, whereas **2a** was an antagonist at the NR1/NR2C subtype combination as illustrated by the electrophysiological traces in Fig. 4. Compounds **2b** and **2d** were antagonists at all four subunit combinations (data not shown), and compound **2c** was a partial agonist at NR2B and NR2D containing receptors and an antagonist at NR2A and NR2C containing receptors (Fig. 3 and 4).

The compounds were also tested for activity at mGluRs using mGluR1, mGluR2 and mGluR4 as representatives of metabotropic Group I, II and III receptors, respectively. In contrast to Ibo and Thio-Ibo, none of the compounds **1** and **2a–2g** showed any activity at mGluR1 at 1 mM. Only compound **2a** showed activity as a partial agonist at mGluR4 (Table 4). At mGluR2, activity was lost for compounds **1** and **2e–g**, whereas compounds containing smaller substituents (**2a–2d**) retained mGluR2 agonist activity comparable to that of Ibo and Thio-Ibo. None of the compounds had antagonist activity at any of the mGluRs (at 1 mM).

Molecular modeling

Ligand binding domains from all three classes of iGluRs have been crystallized and homology models have been constructed.[1,28] The X-ray crystal structures of soluble AMPA-selective GluR2 constructs of the extracellular Glu binding domain (S1S2) cut off from the membrane spanning part have revealed a clamshell-like binding domain which closes around the ligand.[29] This activation of the GluR2 receptor leads to a positive correlation between the agonist efficacy of the ligand and the degree of domain closure it can induce. We have recently performed a mutation study using a homology model of the Glu binding domain of NR2B based on the crystal structure of the glycine binding domain of NR1.[30] The published crystal structure of the Glu binding domain of NR2A[31] prompted us to rebuild the model of NR2B–S1S2 using Prime 3.5[32] now including two water molecules in the binding site. Although there are small changes from the previous structure, the models of NR2B produce similar results in the ligand–protein docking experiments. Automated flexible docking of compounds **2a–2d** in the new NR2B model using Glide 3.5[32] resulted in binding modes for all four compounds that were the same as for **2c** shown in Fig. 5A. The amino acid moiety binds like Glu in the NR2A

Table 3 Electrophysiological activities at recombinant NMDA receptors expressed in *Xenopus* oocytes[a]

	NR1/NR2A		NR1/NR2B		NR1/NR2C		NR1/NR2D	
	EC_{50}/μM	Relative I_{max}	EC_{50}/μM	Relative I_{max}	EC_{50}/μM	Relative I_{max}	EC_{50}/μM	Relative I_{max}
Glu	2.9 [2.5;3.2]	1	1.8 [1.5;2.1]	1	1.5 [1.3;1.7]	1	0.51 [0.47;0.56]	1
Ibo	40 [36;44]	0.68 ± 0.07	27 [25;28]	0.76 ± 0.04	39 [35;43]	0.66 ± 0.02	17 [16;18]	0.78 ± 0.01
Thio-Ibo	124 [112;139]	0.62 ± 0.06	109 [105;113]	0.79 ± 0.02	115 [110;120]	0.57 ± 0.01	49 [43;55]	0.73 ± 0.01
2a	190 [177;205]	0.17 ± 0.04	322 [272;382]	0.39 ± 0.07	—[b]		234 [220;248]	0.42 ± 0.04
2b	—[b]		—[b]		—[b]		—[b]	
2c	—[b]		245 [233;257]	0.24 ± 0.02	—[b]		327 [295;363]	0.29 ± 0.03
2d	—[b]		—[b]		—[b]		—[b]	

[a] EC_{50}-values are expressed as the antilog of the mean of the log of at least three individual experiments. The numbers in brackets [min;max] indicate ± S.E.M. according to a logarithmic distribution. The I_{max}-values ± S.E.M. are relative to maximal steady-state currents evoked by glutamate. [b] No response to 1 mM.

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Fig. 3 A–D: Mean concentration–response curves obtained by two-electrode voltage-clamp recordings at NR1/NR2A-D receptors expressed in *Xenopus* oocytes.

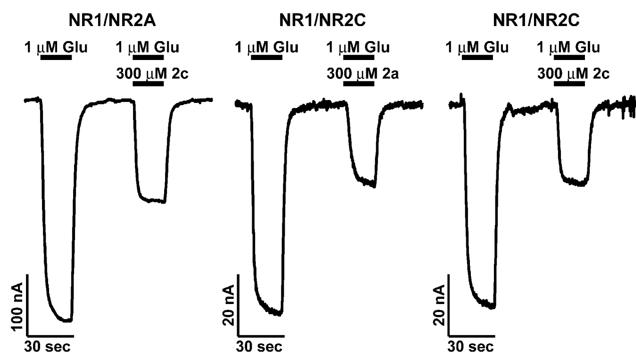

Fig. 4 Two-electrode voltage-clamp recordings showing antagonist activities of compound **2c** at NR1/NR2A and of compounds **2a** and **2c** at NR1/NR2C.

structure and the heterocycle is positioned by hydrogen bonds to the hydroxyl group and amide N-H of T691. In this binding mode the substituents in the 4-position of the heterocycle points like an interdomain wedge between H486 and V686 that are part of the upper and lower domain in the clamshell structure (Fig. 5B). Thus, it seems likely that the decreasing efficacy with increasing size of the substituent arises from steric clash with these two residues, resulting in steric hindrance of clamshell closure. This finding is in agreement with our mutation study on NR2B, where His486 and Val686 are mutated to residues of increased size resulting in decreased efficacy of ligands containing substituents protruding towards this region.[30]

Discussion

The striking difference in the pharmacological profiles of Ibo and Thio-Ibo[8] prompted the synthesis of a series of α- and 4-substituted analogues of Thio-Ibo. Direct functionalization of a protected Thio-Ibo derivative was not feasible and instead the analogues were prepared *via* 4-substituted 3-benzyloxyisothiazole derivatives. Incorporation of substituents into the molecule of Thio-Ibo significantly narrowed the pharmacological profile compared to Thio-Ibo. Most of the activity at mGluRs was lost although four compounds were equipotent with Thio-Ibo as weak agonists at mGluR2. However, the NMDA receptor binding

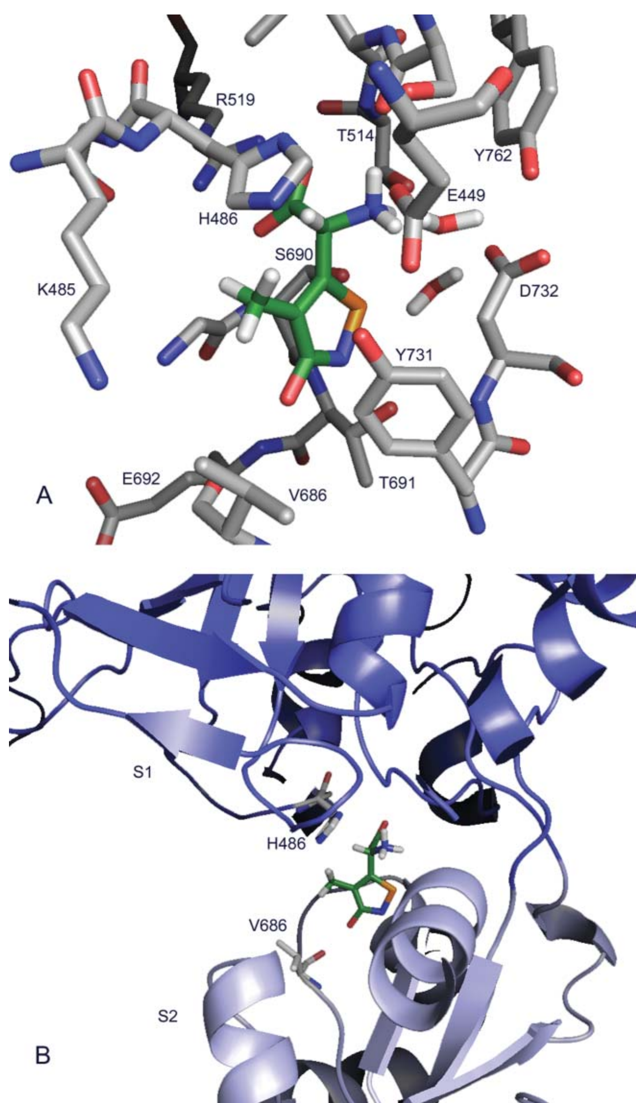

Fig. 5 A: (S)-**2c** (green carbon atoms) docked in a homology model of NR2B binding domain showing residues within 5 Å (grey carbon atoms). B: (S)-**2c** docked in a homology model of NR2B showing the protein backbone as well as residue H486 (upper residue) and V686 (lower residue). H486 from S1 (dark blue) and V686 from S2 (light blue) may be important determinants for the agonist efficacy of the Thio-Ibo analogues.

site seemed to accommodate most of the substituents in the 4-position. In particular, analogues containing smaller substituents retained affinity similar to that of Thio-Ibo. Therefore, the 4-substituted chloro, bromo, methyl and ethyl Thio-Ibo analogues, **2a–2d**, were characterized at recombinant NMDA receptors. Interestingly, this series of compounds displayed quite heterogeneous pharmacological profiles on these subtypes. Thio-Ibo analogues containing the more bulky bromo and ethyl substituents, **2b** and **2d**, did not activate any of the NMDA receptor subtypes. In contrast, the smaller chloro and methyl substituents in **2a** and **2c** mediate agonist activity at several NMDA receptor subtypes, and Thio-Ibo activates all subtypes. It is notable that the change in efficacy, when substituent bulk is increased, is different between the individual subtypes. Thus, the relative agonist efficacy of Thio-Ibo at NR1/NR2A is approximately 4-fold higher than that of the chloro derivative (**2a**), whereas methyl substitution (**2c**) leads to antagonism at this subtype. In contrast, only a minor decrease in relative agonist efficacy is observed for **2a** and **2c** at NR1/NR2B and NR1/NR2D. At NR1/NR2C agonist activity is lost for all four Thio-Ibo analogues (**2a–2d**).

The determination of the pK_a values has substantiated that the compounds may function as Glu bioisosteres. The pK_a values of the 3-isothiazolol, representing the distal carboxylic acid in Glu, are in the range of 4.5–6.3 as compared with Ibo which has a pK_a value of 5.04. Thus, at physiological pH the compounds are expected to be virtually fully ionized. No correlation between pharmacological activity and the range of pK_a values observed can be concluded.

In this series of Thio-Ibo derivatives it seems plausible, that the increased bulk of the substituents could limit domain closure due to steric clashes. The observed differences in efficacy across subtypes may reflect that the Thio-Ibo analogues are recognized by the individual subtypes in a dissimilar manner or that activation of the receptor subtypes are differently coupled to binding of the Thio-Ibo derivatives. A difference in relative efficacy at various subtypes based on variations in the recognition of the ligands does not seem likely, given that the residues in the ligand binding domain of the NMDA receptor are fully conserved throughout the different NR2 subtypes. Consequently, it may be hypothesized that the ligands induce similar conformational changes in the ligand binding domain in all subtypes, but that the energy barrier to activate the ion channel part of the receptor varies among the subtypes.

In conclusion, the substituted Thio-Ibo analogues **2a** and **2c** are the first examples of NMDA receptor agonists that differentiate among the individual receptor subtypes.

Experimental

Chemistry

Detailed experimental procedures and compound characterization data of compounds **1**, **2b–g**, **3**, **5**, **6**, **7b–c**, **f–g**, **9c**,**f–g**, **10**, **11**, **12**, **13b–g**, **14b–g** are described in supplementary information.†

(*RS*)-2-Amino-(4-chloro-3-hydroxy-5-isothiazolyl)acetic acid (2a). (*RS*)-(3-Benzyloxy-4-chloro-5-isothiazolyl)-(*N*-*tert*-butoxycarbonylamino)acetic acid (**14a**) (236 mg, 0.6 mmol) was dissolved in AcOH (3 mL). 10% HBr in AcOH (3 mL) was added. After stirring for 8 h the reaction was concentrated *in vacuo*. The solid was triturated with Et_2O, redissolved in 70% EtOH (aq), treated with propylene oxide whereupon the zwitterion of **2a** was isolated as colorless crystals, which turned light brown overnight (104 mg, 84%). Mp. >140 °C (Found: C, 28.6; H, 2.5; N, 13.1. Calc. for $C_5H_5ClN_2O_3S$: C, 28.8; H, 2.4; N, 13.4%); TLC (*n*BuOH–H_2O–AcOH–EtOAc 1 : 1 : 1 : 1) R_f 0.4; δ_H(DMSO-d_6) 4.60 (s, 1H).

3-Benzyloxy-4-chloroisothiazole (7a). 3-Benzyloxyisothiazole (**4**) (3.0 g, 16 mmol) was dissolved in MeCN (50 mL) and NCS (2.3 g, 17 mmol) was added. The mixture was left stirring for 3 d at rt. The mixture was concentrated to approximately 2 mL and EtOAc was added. The organic solution was washed with H_2O (2×), followed by brine and dried using $MgSO_4$. Concentration *in vacuo* followed by FC (petroleum ether–toluene 1 : 0–1 : 1) gave pure **7a** (2.0 g, 58%) as a clear oil and 0.2 g of 3-benzyloxy-4-chloro-5-chloroisothiazole as a yellow oil (Found: 226.0082 [M + H$^+$].

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Calc. for $C_{10}H_9ClNOS$: 226.0093 [M + H$^+$]); TLC (petroleum ether–toluene 1 : 1) R_f 0.6; δ_H($CDCl_3$) 5.41 (s, 2H), 7.30–7.44 (m, 5H), 8.15 (s, 1H); δ_C($CDCl_3$) 71.0 (CH_2), 111.1 (C), 127.9 (CH), 128.1 (CH), 128.4 (CH), 135.9 (C), 143.2 (CH), 163.6 (C); *m/z* (EI) 225 (9%, M$^+$), 190, 106, 91.

3-Benzyloxy-4-ethylisothiazole (7d). 1-(3-Benzyloxy-4-isothiazolyl)ethanol (**9d**) (1.00 g, 4 mmol) was dissolved in dry CH_2Cl_2 (15 mL) under N_2 and cooled to 0 °C. Triethylsilane (1.15 mL, 7 mmol) was added followed by the addition of trifluoroacetic acid (10 mL). The mixture was left at ~5 °C overnight with no stirring. H_2O (20 mL) was added to the cooled reaction mixture followed by extraction with Et_2O (3×). The combined organic phases were washed with satd. $NaHCO_3$ (aq) until a pH of 7 was reached. The organic phase was washed with brine and dried with $MgSO_4$. A silane impurity was removed under high vacuum overnight. FC (petroleum ether–toluene 2 : 1–1 : 1) afforded **7d** as a clear oil (0.66 g, 70%) (Found: 220.0789 [M + H$^+$]. Calc. for $C_{12}H_{14}NOS$: 220.0796 [M + H$^+$]); TLC (petroleum ether–toluene 1 : 1) R_f 0.3; δ_H($CDCl_3$) 1.22 (t, *J* 7, 3H), 2.52 (dq, *J* 1 and 7, 2H), 5.42 (s, 2H), 7.23–7.45 (m, 5H), 8.03 (t, *J* 1, 1H); δ_C($CDCl_3$) 13.6 (CH_3), 19.8 (CH_2), 70.3 (CH_2), 127.8 (CH), 128.0 (CH), 128.3 (C), 128.5 (CH), 137.0 (C), 142.5 (CH), 167.1 (C); *m/z* (EI) 219 (6%, M$^+$), 202, 186, 106, 91.

3-Benzyloxy-4-phenylisothiazole (7e). 3-Benzyloxy-4-iodoisothiazole (**8**) (700 mg, 2.2 mmol) was dissolved in dry DMF (14 mL) under N_2. $PhB(OH)_2$ (540 mg, 4.4 mmol) and $PdCl_2(PPh_3)_2$ (77 mg, 0.1 mmol) was added under N_2. 3M K_2CO_3 (aq) (1.5 mL) was added and the mixture was left refluxing at 75 °C overnight under N_2. The reaction mixture was cooled to rt and H_2O (20 mL) was added. The reaction mixture was extracted with Et_2O (3×). The combined organic phases were washed with 2 M NaOH (2 × 20 mL). The organic phase was further washed with H_2O and brine and dried using $MgSO_4$. The mixture was concentrated *in vacuo* and purified using FC (petroleum ether–toluene 1 : 1) giving **7e** as a clear oil (552 mg, 94%) (Found: 268.0791 [M + H$^+$]. Calc. for $C_{16}H_{14}NOS$: 268.0796 [M + H$^+$]); TLC (petroleum ether–toluene 1 : 1) R_f 0.27; δ_H($CDCl_3$) 5.54 (s, 2H), 7.31–7.42 (m, 6H), 7.45–7.49 (m, 2H), 7.67–7.70 (m, 2H), 8.53 (s, 1H); δ_C($CDCl_3$) 70.8 (CH_2), 126.1 (C), 127.6 (CH), 127.7 (CH), 127.7 (CH), 128.0 (CH), 128.5 (CH), 128.6 (CH), 132.2 (C), 136.7 (C), 144.9 (CH), 165.8 (C); *m/z* (EI) 267 (11%, M$^+$), 162, 134, 106, 91.

3-Benzyloxy-4-iodoisothiazole (8). 3-Benzyloxyisothiazole (**4**) (3.0 g, 16 mmol) was dissolved in $CHCl_3$ (20 mL). Dry K_2CO_3 (3.2 g, 22 mmol) was added followed by ICl (3.8 g, 24 mmol) in $CHCl_3$ (10 mL). The reaction mixture was refluxed for 2 d under N_2. The reaction was quenched with 10 mL of 1 M Na_2SO_3 (aq), followed by extraction with CH_2Cl_2. The combined organic phases were dried with $MgSO_4$, concentrated *in vacuo* and purified by FC (petroleum ether–toluene 1 : 1) giving **8** as a clear oil (4.1 g, 82%) (Found: 317.9455 [M + H$^+$]. Calc. for $C_{10}H_9INOS$: 317.9450 [M + H$^+$]); TLC (petroleum ether–toluene 1 : 1) R_f 0.4; δ_H($CDCl_3$) 5.44 (s, 2H), 7.30–7.47 (m, 5H), 8.46 (s, 1H); δ_C($CDCl_3$) 65.0 (C), 71.3 (CH_2), 127.8 (CH), 128.2 (CH), 128.6 (CH), 136.3 (C), 151.6 (CH), 167.9 (C); *m/z* (EI) 317 (3%, M$^+$), 190, 106, 91.

1-(3-Benzyloxy-4-isothiazolyl)ethanol (9d). **8** (3.00 g, 9.5 mmol) was dissolved in dry THF (30 mL) under N_2. The mixture was cooled to −30 °C and *i*PrMgCl in Et_2O (5.7 mL, 2 M, 11 mmol) was added to the mixture. The mixture was slowly warmed to 0 °C and left at 0 °C for 20 min before acetaldehyde (0.53 mL, 9.5 mmol) in THF (2 mL) was added. The mixture was left at rt overnight. The reaction was quenched with satd. NH_4Cl (aq) (10 mL) and additional H_2O (40 mL) was added. The pH was adjusted to ~7 using 1 M HCl (aq). The aqueous mixture was extracted with EtOAc (3×) and the combined organic phases were washed with brine and dried with $MgSO_4$. FC (toluene–EtOAc 9 : 1) gave **9d** as a brown oil (1.88 g, 84%) (Found: 236.0759 [M + H$^+$]. Calc. for $C_{12}H_{14}NO_2S$: 236.0745 [M + H$^+$]); TLC (toluene–EtOAc 9 : 1) R_f 0.22; δ_H($CDCl_3$) 1.48 (d, *J* 7, 3H), 2.63 (br s, 1H), 4.87 (dq, *J* 7, 1H), 5.43 (s, 2H), 7.32–7.44 (m, 5H), 8.27 (s, 1H); δ_C($CDCl_3$) 22.9 (CH_3), 63.8 (CH), 70.6 (CH_2), 127.9 (CH), 128.2 (CH), 128.5 (CH), 130.2 (C), 136.4 (C), 143.6 (CH), 165.6 (C).

2-(3-Benzyloxy-4-chloro-5-isothiazolyl)-2-(*N*-*tert*-butoxycarbonylamino)malonic acid diethyl ester (13a). **7a** (1.00 g, 4.4 mmol) in Et_2O (10 mL) was added over 5 min to freshly prepared LDA (5.8 mmol) in Et_2O (40 mL) at −78 °C under N_2. The mixture was left stirring at −78 °C for 2 h. 2-(*N*-*tert*-Butoxycarbonylimino)malonic acid diethyl ester (1.30 g, 4.8 mmol) in Et_2O (5 mL) was added slowly. The mixture was warmed over 1 h to −30 °C. Satd. NH_4Cl (aq) was added and the mixture was warmed to rt. The mixture was extracted with EtOAc (3×), washed with brine, dried with $MgSO_4$. and concentrated *in vacuo*. FC (petroleum ether–toluene–EtOAc 4 : 4 : 1) yielded **13a** as a yellow oil (0.93 g, 42%). Starting material **7a** was recovered (0.25 g, 25%). TLC (petroleum ether–toluene–EtOAc 4 : 4 : 1) R_f 0.3; δ_H($CDCl_3$) 1.24 (t, *J* 7, 2 × CH_3, 6H), 1.39 (s, *t*Bu, 9H), 4.24–4.34 (m, 2 × CH_2, 4H), 5.42 (s, CH_2, 2H), 6.64 (br s, NH, 1H), 7.30–7.44 (m, 5H); δ_C($CDCl_3$) 13.9 (CH_3), 28.1 (CH_3), 63.9 (CH_2), 65.8 (C), 70.5 (CH_2), 81.2 (C) 109.4 (C), 128.0 (CH), 128.2 (CH), 128.5 (CH), 136.2 (C), 153.3 (C), 154.5 (C), 162.3 (C), 165.2 (C).

(*RS*)-(3-Benzyloxy-4-chloro-5-isothiazolyl)-(*N*-*tert*-butoxycarbonylamino)acetic acid (14a). The title compound was prepared according to the procedure described for **6** starting with **13a** (900 mg, 1.80 mmol) and a reaction time of $3\frac{1}{2}$ h. Purification using FC (CH_2Cl_2–MeOH–AcOH 100 : 5 : 2) followed by recrystallization (EtOAc–petroleum ether) yielded **14a** as colorless crystals (337 mg, 47%). Mp. 128.9–129.2 °C (Found, C, 51.13; H, 4.93; N, 6.84. Calc. for $C_{17}H_{19}ClN_2O_5S$: C, 51.19; H, 4.80; N, 7.03%); TLC (CH_2Cl_2–MeOH–AcOH 100 : 5 : 2) R_f 0.19; δ_H($CDCl_3$) 1.30 and 1.45 (2 × br s, *t*Bu, 9H), 5.43–5.66 (m, CH_2, α-H, NH (0.4H), 3.4H), 7.33–7.47 (m, phenyl, 5H), 8.10 (br s, COOH, 1H); δ_C($CDCl_3$) 28.2 (CH_3), 52.8 (CH), 70.8 (CH_2), 83.4 (C), 110.9 (C), 128.1 (CH), 128.4 (CH), 128.7 (CH), 136.1 (C), 156.8 (C), 158.5 (C), 163.4 (C), 170.3 (C).

Pharmacology

iGluR receptor binding. Rat brain membrane preparations used in the receptor binding experiments for iGluRs were prepared according to the method described by Ransom and Stec.[33] Affinities for native AMPA, KA and NMDA receptors were determined using 5 nM [^{3}H]AMPA,[34] 5 nM [^{3}H]KA[35] and 2 nM [^{3}H]CGP39653,[36] respectively, with some modifications (see Hermit *et al.*[8] for further details).

Two-electrode voltage-clamp electrophysiology. For expression in *Xenopus* oocytes, rat NR1-1a (GenBank U11418) cDNA was subcloned into a pCI-IRES-neo vector and rat NR2A (GenBank D13211), NR2B (GenBank M91562), NR2C (GenBank D13212), and NR2D (GenBank D13214) cDNAs were subcloned into a pCI-IRES-bla vector containing a T7 site upstream from the 5′ untranslated region. Constructs used for expression in *Xenopus* oocytes were linearized by restriction enzymes in order to produce cRNAs, using mMessage mMachine kits.[37] Oocytes were surgically removed from mature female *Xenopus laevis* as previously described[27] and co-injected with cRNA encoding NR1-1a and NR2B at a 1 : 1 ratio and maintained at 18 °C in Barth's solution (in mM: 88 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 0.3 $Ca(NO_3)_2$, and 15 HEPES pH 7.6) supplemented with 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Invitrogen, Carlsbad, CA). The electrophysiological recordings and data analysis were performed as previously described.[30]

mGluR activity. Chinese hamster ovary (CHO) cell lines stably expressing rat mGluR1a, mGluR2 and mGluR4a were prepared as previously described[38] (see also Clausen *et al.*[27] for further details).

Measurement of intracellular Ca^{2+} levels and cyclic AMP formation: pharmacological activity at mGluR1a was assessed by measurement of intracellular Ca^{2+} levels as previously described.[13] Pharmacological activity at mGluR2 and mGluR4a was assessed by measuring intracellular cAMP levels as previously described[39] (see also Clausen *et al.*[27] for further details).

Molecular modeling

A homology model of the agonized ligand binding domain of NR2B was constructed using the crystal structure of the soluble NR2A–S1S2 construct in complex with Glu (Protein Data Bank code 2A5S) as a template. The sequence of NR2B was aligned with NR2A and truncated so that a sequence consisting of residues D404–R540 from S1, followed by the GT linker and residues Q662–H826 from S2 forms a virtual NR2B–S1S2 construct. Residues are numbered according to the sequence of total NR2B protein. From this sequence, a model structure of NR2B was generated using Prime 3.5.[32] Glu and water molecules 6 and 48 from 2A5S were added to the resulting structure and submitted to the standard preparation and refinement procedure in Glide 3.5[32] to assign charges, add hydrogens, and perform a series of constrained minimizations (OPLS-AA force field). On this final model, Van der Waals and electrostatic grids within a 20 $Å^3$ box around the ligand were calculated using Glide 3.5, and these grids were then used for ligand docking. The ligands **2a–2d** were submitted to a conformational search (Monte Carlo) in tri-ionized forms using the MMFFs force field and water as solvent in Macromodel 9.0[32] The lowest energy conformations were docked with Glide 3.5 to the agonist binding site of the NR2B–S1S2 model using the automated flexible procedure. Default parameters were used. All figures of the models were prepared using PyMol software.[32]

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