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Synthesis and pharmacological characterization of the selective GluK1 radioligand (*S*)-2-amino-3-(6-[^{3}H]-2,4-dioxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-1(2*H*)-yl)propanoic acid ([^{3}H]-NF608)†‡

Anna Alcaide,[a] Laura Marconi,[ad] Ales Marek,[b] Isabell Haym,[a] Birgitte Nielsen,[a] Stine Møllerud,[a] Mikael Jensen,[c] Paola Conti,[d] Darryl S. Pickering[a] and Lennart Bunch*[a]

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The kainic acid receptors belong to the class of ionotropic glutamate receptors and comprise five subunits named GluK1–5. Radioligands are essential tools for use in binding assays aimed at ligand–receptor structure–activity-relationship studies. Previous work has led to the synthesis of GluK1 radioligands [^{3}H]-SYM2081, [^{3}H]-UBP310 and [^{3}H]-ATPA, however all strategies were work-intensive and thus not attractive. Herein, we report the synthesis of [^{3}H]-NF608 and subsequent pharmacological evaluation at homomeric recombinant rat GluK1 receptors. Binding affinities of a series of standard GluK1 ligands were shown to be in line with previously reported affinities obtained by use of already reported radioligands.

Introduction

The kainic acid (KA, Fig. 1A) receptors belong to the class of ionotropic glutamate receptors and are believed to mediate a modulatory excitatory response in the central nervous system. The KA receptors comprise five subunits, termed GluK1–5 and are tetrameric in structure (dimer of dimers). While the subunits GluK1–3 may form functional homomeric as well as heteromeric receptors, subunits GluK4,5 only form functional receptors with GluK1–3. To study the role and function of each of the KA receptor subtypes, it is essential that subtype selective ligands (pharmacological tools) are available. While selective GluK1 agonists and antagonists have been reported (Fig. 1B), selective ligands for the GluK2–5 subtypes remain to be discovered.[1,2] In order to achieve this, extensive structure–activity-studies are continuously performed, which includes binding affinity studies of synthesized compounds at homomeric, recombinant KA receptor subtypes. Readily accessible radioligands are essential to accomplish this, and previous work-intensive strategies have led to the synthesis of [^{3}H]-UBP310[3] and [^{3}H]-ATPA (ref. 4) (Fig. 1C) as GluK1-selective radioligands. The high affinity KA receptor ligand SYM2081 (Fig. 1C) has also been employed as the radioligand in binding assays of homomeric GluK1–3 receptors,[5] however its difficult purification makes it less attractive to produce and it is no longer commercially available. While commercially available [^{3}H]-KA can be successfully used in radioligand binding studies for GluK2 (K_d = 6.5 nM) (Fig. 2), GluK3 (K_d = 8.0 nM) (Fig. 2), GluK4 LBD (K_d = 1.9 nM) (Kristensen *et al.*, 2016)[17] and GluK5 (K_d = 6.9 nM) (Møllerud *et al.*, 2016)[16] it has 10-fold lower affinity at GluK1 (K_d = 67 nM) (Fig. 2) making it a less than ideal radiolabel for binding studies at GluK1. Here we report the synthesis and pharmacological characterization of [^{3}H]-NF608 – a new high affinity, GluK1-selective radioligand.

Results and discussion

Synthesis

Firstly, we pursued a direct bromination of commercially available UBP310 to obtain the dibromothiophene precursor previously reported for the synthesis of [^{3}H]-UBP310 (Scheme S1, ESI‡).[3] However, bromination took place at the uracil ring only, and all attempts failed to following incorporate deuterium (see ESI‡ for details).

We next turned to investigate the high affinity, selective GluK1 ligand NF608 (Fig. 1) which also comprises a thiophene ring. Its synthesis is described in only two steps from commercially available thieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-

[a] *Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. E-mail: lebu@sund.ku.dk; Fax: +45 35336041; Tel: +45 35336244*
[b] *Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo nám. 2, Prague 6, 16610, Czech Republic*
[c] *Nutech Hevesy Laboratory, The Technical University of Denmark, Denmark*
[d] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, Via Mangiagalli 25, 20133 Milano, Italy*

† The authors declare no competing interests.

‡ Electronic supplementary information (ESI) available: Detailed description and experimental data of failed strategy towards the synthesis of [^{3}H]-UBP310 from UBP310. HRMS spectrum (negative mode) of ^{3}H-NF608. See DOI: 10.1039/c6md00339g

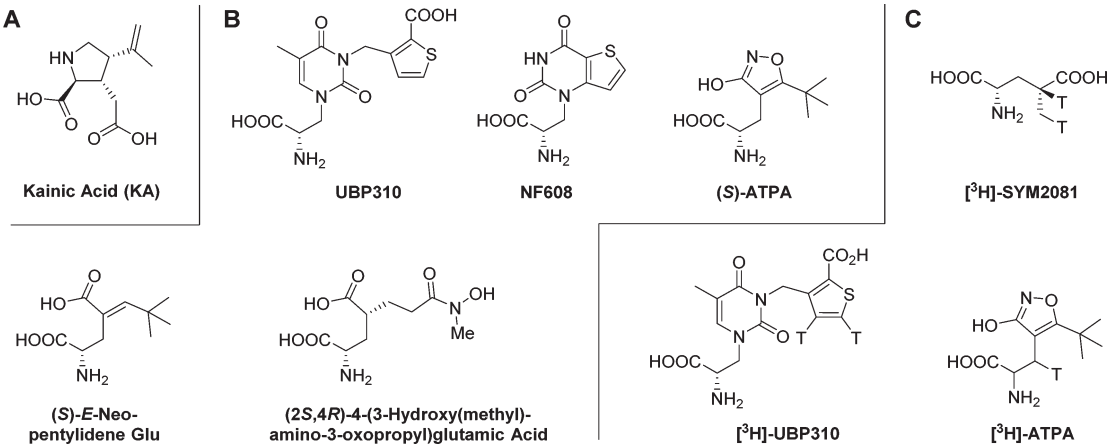


Fig. 1 A) Chemical structure of kainic acid (KA). B) Reported high affinity subtype selective GluK1 ligands UBP310,[6] NF608,[7] (*S*)-ATPA,[8] (*S*)-*E*-neopentylidene Glu,[9] and (2*S*,4*R*)-4-(3-hydroxy(methyl)amino-3-oxopropyl)glutamic acid.[10] C) Reported tritium labeled high affinity GluK1 ligands, [^{3}H]-UBP310,[3] [^{3}H]-ATPA,[4] and [^{3}H]-SYM2081.[11]

dione (**1**) (Scheme 1).[7] Firstly, we attempted bromination of the thiophene ring of **3** under standard conditions (Br_2 in AcOH or TFA, rt to 90 °C) (Scheme 1). However, none of the corresponding bromo or dibromo derivatives comprised by general structure **4** were observed but only the expected cleavage of the BOC group.

We therefore revised the strategy to introduce the bromine functionality earlier in the synthesis (Scheme 1), and indeed we were able to brominate the thiophene ring of **1** in the 7 position to give **5**. Disappointingly, alkylation at N^1 with lactone **2** (ref. 12) as the electrophile was unproductive (NaH, DMF, −65 to 80 °C) presumably due to the bulkiness of the bromine atom (Scheme 1). For that reason, we turned to pursue bromination of the 6 position of the thienopyrimidine ring in order to allow for following alkylation at N^1.

The synthesis of key bromo intermediate **13** (Scheme 2) commenced with *N*-protection of commercially available methyl 3-aminothiophene-2-carboxylate (**7**) with trichloroacetyl isocyanate in THF to afford compound **8** in 93% yields.[13] Subsequent bromination with Br_2 in acetic acid at 80 °C successfully provided exclusively 5-bromo-thiophene **9**, which was then treated with 2 M ammonia in methanol to afford urea **10**.[13] Cyclization with potassium *tert*-butoxide in DMF, furnished the desired heterocyclic product **11**.[13] Alkylation at N^1 with lactone **2**[12] was performed using sodium hydride as base in DMF, as described in a similar procedure.[7] The alkylation product **12** was obtained in only 18% – a yield which could likely be optimized. Cleavage of the BOC group in the presence of 2 M aq HCl provided key bromo amino acid intermediate **13**, in overall 6 steps. Deuteration over Pd/C in a basic aqueous media gave 80% deuterium incorporation, furnishing deuterated product [D6]-NF608 together with NF608, in overall 57% yield.

Tritiation

Having established the method for deuterium incorporation, tritiation was carried out by use of T_2 (gas) with Pd/C as catalyst in water to give [^{3}H]-NF608 in high yield (Scheme 3) at a specific activity of 16.3 Ci mmol^{-1}.

Radiochemical stability

Formulation of [^{3}H]-NF608 was chosen as a 1 mCi mL^{-1} solution in H_2O–EtOH (1 : 1) and the radiochemical stability was monitored over time. When stored at −21 °C, the stability was determined to be 98% after 42 days and 97.5% after 90 days. When stored at −196 °C for 90 days, no decomposition could be detected.

GluK1 binding pharmacology

Assay validation

Based upon our previous publication we anticipated [^{3}H]-NF608 to be a high affinity radioligand at GluK1 since unlabeled NF608 had shown a K_i of 5.3 nM.[7] Initial tests indicated high level of specific binding of the radiolabel to homomeric rat GluK1 expressed in *sf9* cell membranes and subsequently an assay validation was conducted using 1 mM (*S*)-glutamate to define nonspecific binding. No specific binding was seen using uninfected *sf9* cell membranes. A series of buffers, temperatures, pH conditions and washing protocols were evaluated (Table 1) and 50 mM Tris-HCl pH 7.1 at 4 °C with 2 × 4 mL filter washes was found to be optimal for specific binding to GluK1. There was no pH dependency of the binding between pH 7.1 and 7.8. Binding of [^{3}H]-NF608 to native GluK1 in rat brain P2 membranes was tested using the high affinity GluK1-selective ligand 10 μM UBP310 to evaluate nonspecific binding and, although a small amount of specific binding was detected, it was insufficient to perform pharmacological analyses at native GluK1. This is likely due to the low specific activity of the radioligand (16.3 Ci mmol^{-1}).

Kinetics

The association and dissociation kinetics of [^{3}H]-NF608 binding were examined at GluK1 at 4 °C (Fig. 3). The association

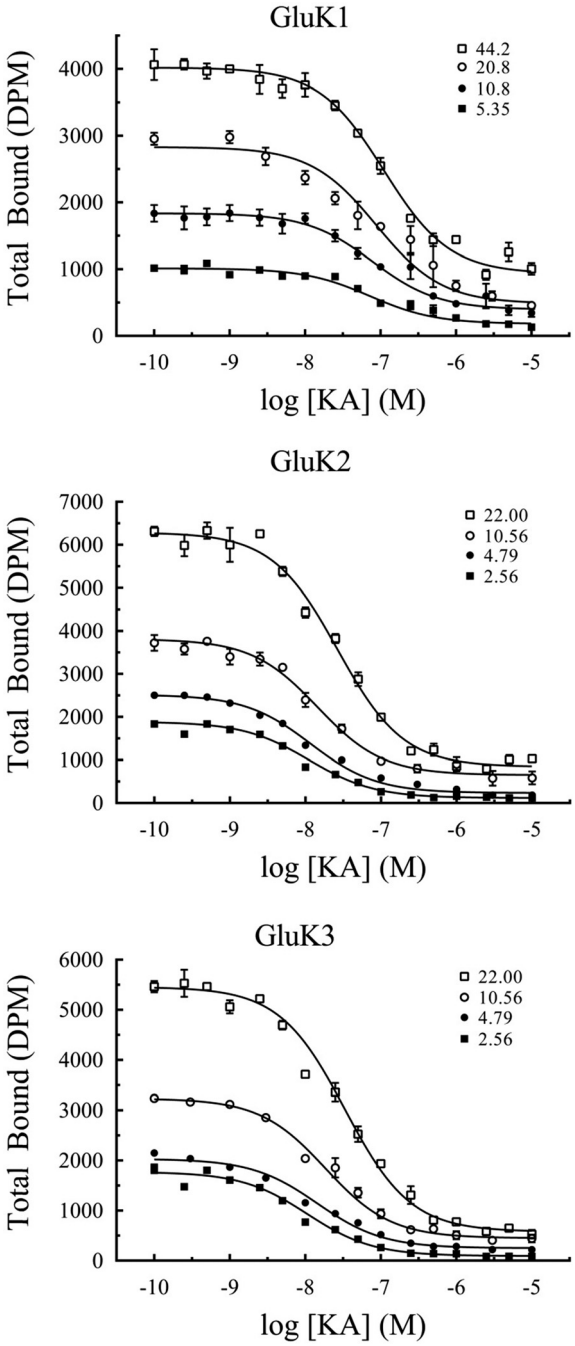


Fig. 2 Homologous competition binding of [^{3}H]KA at recombinant, homomeric rat GluK1–3 expressed in *sf9* cell membranes. [^{3}H]KA (44.0 Ci mmol^{-1}) concentrations (nM) are indicated in the figure insets. GluK1 pK_d = 7.173; GluK2 pK_d = 8.185; GluK3 pK_d = 8.097. Hill values are close to unity for all. Shown are pooled data (mean ± SEM) from single experiments conducted in triplicate.

rate was measured as (mean ± SEM) k_{on} = 10.05 ± 1.30 × 10^6 min^{-1} nM^{-1} (n = 3) and the dissociation rate determined as (mean ± SEM) k_{off} = 0.0274 ± 0.0017 min^{-1} (n = 4). The time course of binding at 4 °C indicated that equilibrium is attained by 1 h. Calculation of the affinity from the kinetic rate constants gave a kinetic K_d (mean ± SEM) = 2.72 ± 0.39 nM, which agrees well with the previously determined affinity of NF608 at GluK1 (K_i = 5.29 ± 0.66 nM).[7]

Saturation

The equilibrium binding affinity of [^{3}H]-NF608 at GluK1 was measured in saturation binding (Fig. 4) where (mean ± SEM, n = 3) K_d = 6.68 ± 0.88 nM, B_{max} = 5829 ± 248 fmol mg^{-1} protein. This affinity is not statistically significantly different (p = 0.251, t-test) from our previously determined affinity of NF608 at GluK1 using [^{3}H]SYM2081 as the radioligand.

Competition

The pharmacological profile of a selected series of KA receptor ligands was examined using [^{3}H]-NF608 (Fig. 5). A comparison of the profile to that previously observed using [^{3}H] SYM2081 as the radioligand[7] shows reasonable agreement and Hill values are near unity for all ligands tested (Table 2). Analysis of homologous competition binding with NF608 yielded a K_d = 6.93 nM (pK_d = 8.160 ± 0.047) (Fig. 6), which is identical to the K_d determined from saturation analysis.

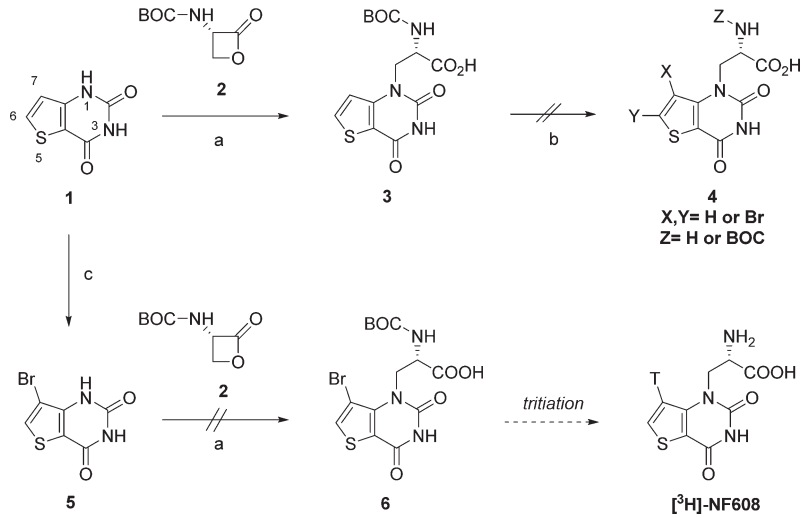
Conclusion

In conclusion we have reported a short and efficient method for the radiosynthesis of the selective GluK1 radioligand [^{3}H]-NF608. The radioligand was characterized in *in vitro* binding assays at cloned homomeric GluK1 receptors and binding affinities (K_i) of a series of standard GluK1 ligands were shown to be in line with previously reported affinities obtained by use of already reported radioligands.

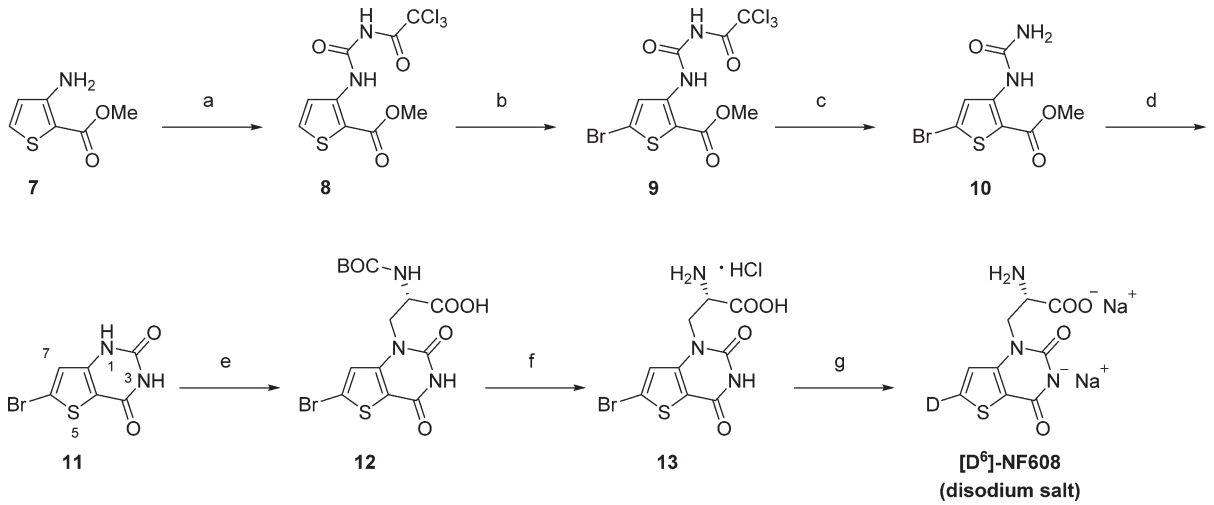
Experimental section

Chemistry

All reactions involving dry solvents or sensitive agents were performed under a nitrogen or argon atmosphere, and glassware was dried prior to use. Commercially available chemicals were used without further purification. Solvents were dried prior to use with an SG water solvent purification system or dried by standard methods. Reactions were monitored by analytical thin-layer chromatography (TLC, Merck silica gel 60 F254 aluminum sheets), analytical HPLC or UPLC. Flash chromatography was carried out using the Merck silica gel 60 (15–40 μm) or Merck silica gel 60 (40–63 μm). ^{1}H NMR spectra were recorded on a 400 MHz Bruker Avance III or 600 MHz Bruker Avance III HD, and ^{13}C NMR spectra on a 101 MHz Bruker Avance III or 151 MHz Bruker Avance III HD. Chemical shifts are reported in δ (ppm) relative to the singlet at δ = 7.26 ppm of $CDCl_3$, the quintet at 2.50 ppm of DMSO-d_6, and the singlet at 4.79 ppm of D_2O for ^{1}H NMR, and to the centre line of the triplet at δ = 77.16 ppm of $CDCl_3$, the heptuplet at 39.52 ppm of DMSO-d_6 for ^{13}C-NMR. Analytical HPLC was performed using a Dionex UltiMate 3000 pump and Dionex Ultimate 3000 Diode Array Detector (200, 210, 225 and 254 nm) installed with a Phenomenex Gemini-NX 3μ C18 110A, 250 × 4.60 mm column.

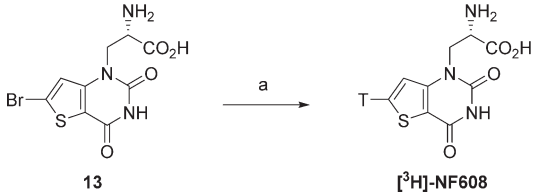


Scheme 1 Attempted bromination of **3** to give bromo-analogs comprised by general structure **4**. Reagents and conditions. a) NaH, DMF, −65 to 80 °C; b) Br_2, AcOH or TFA, rt to 90 °C (only the HCl or TFA salt of NF608 was observed) c) Br_2, AcOH, 90 °C (97%).



Scheme 2 Synthesis of key 6-bromo precursor **13** and incorporation of deuterium to give [D6]-NF608. Reagents and conditions. (a) Trichloroacetyl isocyanate, THF, 0 °C to rt, 2 h, 93%; (b) Br_2, acetic acid, 0–80 °C, overnight, 69%; (c) 2 N NH_3 in methanol, 0 °C to rt, 30 min, 89%; (d) *tert*-BuOK, DMF, rt, overnight, 95%; (e) NaH, DMF, 0 °C to rt, then lactone **2** in DMF, 0 °C to rt, 18% (f) 2 M aq HCl, 50 °C, 1 h, 94% (g) D_2 (g), Pd/C, NaOH, H_2O, rt, overnight, 57%.

Solvent A: H_2O + 0.1% TFA; solvent B: MeCN–H_2O 9 : 1 + 0.1% TFA. For HPLC control, data collection and data handling, Chromeleon software v. 6.80 was used. Preparative HPLC was carried out on an Ultimate 3000 Thermo SCIENTIFIC system with a Dionex Ultimate 3000 series pump, a Dionex Ultimate 3000 Diode Array Detector (200, 210, 225 and 254 nm), and a Phenomenex Gemini-NX 5μ C18 110A, 250 × 21.20 mm column for preparative purifications or a Phenomenex Gemini-NX 5μ C18 110A, 250 × 10.00 mm column for semi-preparative purifications. Solvent A: H_2O + 0.1% TFA; solvent B: MeCN–H_2O 9 : 1 + 0.1% TFA. For HPLC control, data collection and data handling, Chromeleon software v. 6.80 was used. UPLC-MS spectra were recorded using an Acquity UPLC H-Class Waters series solvent delivery system equipped with an autoinjector coupled to an Acquity QDa and TUV detectors installed with an Acquity UPLC®BECH C18 1.7 μm column. Solvent A: 5% aq MeCN + 0.1% HCO_2H: solvent B: MeCN + 0.1% HCO_2H. Usually, gradients from A : B 1 : 0 to 0 : 1 (5 min) or A : B 1 : 0 to 0 : 50 (5



Scheme 3 Tritiation of **13**. Reagents and conditions. a) T_2 (515 mbar), Pd/C (30%), NaOH (1 M), H_2O, 3 h, r.t. [23.5 mCi (>99.9% R.C.P.), 23% R.C.Y.]

Table 1 Binding assay conditions evaluated

Buffers (50 mM)
Tris-HCl
Tris-HCl + 100 mM NaCl
Bis-Tris-HCl
Na^+HEPES
Na^+MOPS
Na^+TES
NaH_2PO_4
Na^+PIPES
pH
7.1
7.4
7.6
7.8
Temperature (°C)
4
22
37
Filter washing (mL)
2 × 3
3 × 3
2 × 4
3 × 4
2 × 5
3 × 5

Overview of the various assay conditions tested. The pH, temperature and washing conditions were only evaluated using 50 mM Tris-HCl buffer.

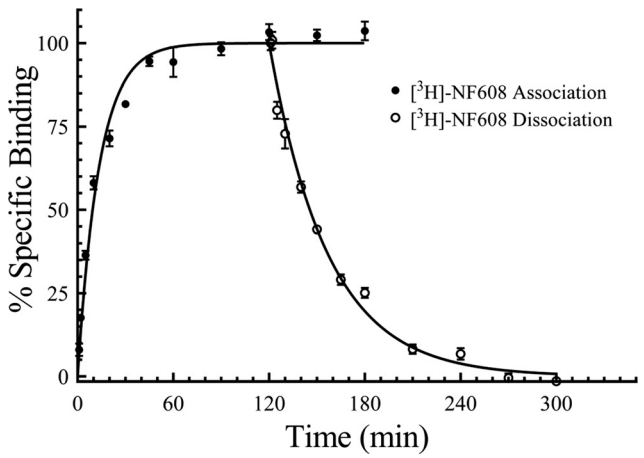


Fig. 3 Association and dissociation kinetic experiments of [^{3}H]-NF608 binding to recombinant, homomeric rat GluK1 expressed in *sf9* cell membranes. Dissociation was initiated after a 2 h equilibration with the radioligand. Shown are pooled, normalized data from 3–4 experiments conducted in triplicate. Nonspecific binding was determined in the presence of 1 mM (*S*)-glutamate.

min), were performed depending on the polarity of the compounds. For data collection and data handling, MassLynx software was used. Compounds were dried under high vacuum or freeze dried using a ScanVac Cool Safe Freeze Drier. The purity of compounds submitted for pharmacological characterization was determined by ^{1}H-NMR and HPLC to be >95%.

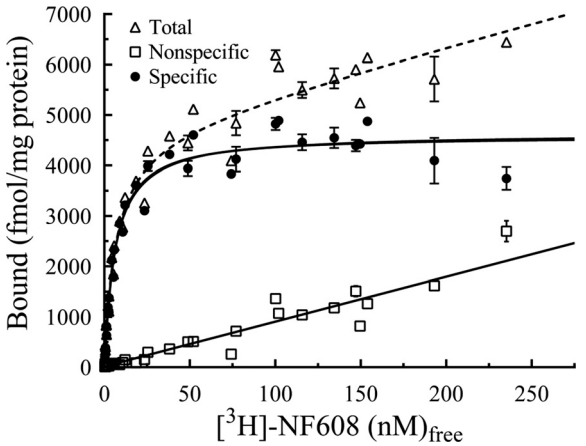


Fig. 4 Saturation binding of [^{3}H]-NF608 (16.3 Ci mmol^{-1}) to recombinant, homomeric rat GluK1 expressed in *sf9* cell membranes. Shown are pooled, normalized data from 3 experiments conducted in triplicate. Nonspecific binding was determined in the presence of 1 mM (*S*)-glutamate. From the pooled analysis: K_d = 6.61 ± 0.50 nM, B_{max} = 4675 ± 76 fmol mg^{-1} protein, n_H = 1.07 ± 0.07.

For the tritiation experiments

^{1}H-, ^{3}H- and ^{13}C NMR spectra were recorded at 300/320 MHz and 75 MHz, respectively, with a Bruker Avance II 300 MHz instrument at 25 °C. The residual solvent signals in the ^{1}H and ^{13}C NMR spectra were used as an internal reference ($CDCl_3$: δ = 7.26 for ^{1}H and δ = 77.23 for ^{13}C). The mass spectra were obtained by the Bruker Daltonics Esquire 4000 system with a direct input (ESI, stream ACN–H_2O, a mass range of 50–1200 Da, Esquire Control Software). The HR-mass spectra were obtained in the ESI mode either on a Waters-

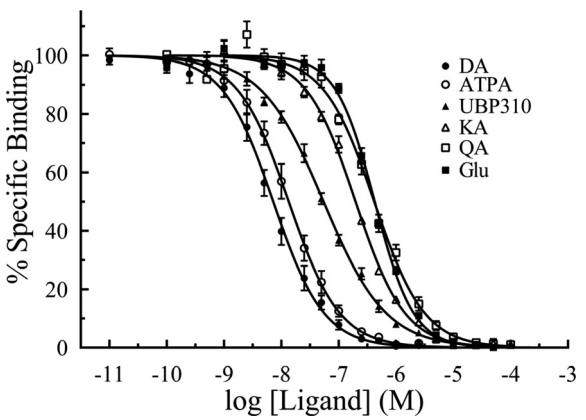


Fig. 5 Competition binding at recombinant, homomeric rat GluK1 expressed in *sf9* cell membranes. Shown are pooled, normalized data from 3 experiments conducted in triplicate. DA, domoic acid; ATPA, (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid; KA, kainic acid; QA, quisqualic acid; Glu, (*S*)-glutamic acid; UBP310, (*S*)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-thiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione.

Table 2 Pharmacological profile of GluK1 ligands at recombinant, homomeric rat GluK1 expressed in *sf*9 cell membranes

Radioligand →	[^{3}H]-SYM2081		[^{3}H]-NF608	
Competitor ↓	K_i (nM)	n_H	K_i (nM)	n_H
(*S*)-NF608	5.29 ± 0.66[a]	0.99 ± 0.03	9.9 ± 2.6	0.96 ± 0.04
UBP310	22.1 ± 4.6[b]	0.78 ± 0.03	44.6 ± 6.6	0.81 ± 0.03
(*RS*)-ATPA	2.13 ± 0.21	0.96 ± 0.03	11.9 ± 4.6	1.04 ± 0.06
Kainic acid	76 ± 12[a]	0.99 ± 0.04	135 ± 5	1.01 ± 0.02
Quisqualic acid	171 ± 93[c]	0.93 ± 0.03	344 ± 72	1.00 ± 0.03
Domoic acid	1.11 ± 0.20[c]	0.99 ± 0.04	6.41 ± 2.48	1.04 ± 0.01
(*S*)-Glutamic acid	140 ± 3[a]	0.98 ± 0.03	292 ± 24	1.30 ± 0.08

Shown are means ± SEM of at least 3 experiments conducted in triplicate at 12–16 ligand concentrations.[a] Ref. 7. [b] Ref. 15. [c] Ref. 5.

Micromass Q-TOF Micro mass spectrometer or on a Thermo Fisher Scientific LTQ Orbitrap XLc. The tritiation reaction was performed on a custom-designed tritium manifold system manufactured by RC Tritec AG, Switzerland. Activities were measured on a Perkin-Elmer TriCarb 2900TR liquid scintillation counter (LSC) in a Zinsser Quicksafe A cocktail. The HPLC was performed on a system consisting of a WATERS Delta 600 Pump and Controller, a WATERS 2487 UV detector and a RAMONA radio chromatographic detector from Raytest (Germany) with interchangeable fluid cells. For the preparative runs, the cell with a single small crystal of solid scintillator was used; for analytical runs, the column effluent was mixed with a Zinsser Quickszint Flow 302 cocktail at the ratio of 1 : 3.

***tert*-Butyl (*S*)-(2-oxooxetan-3-yl)carbamate (2).** To an ice-cooled solution of triphenylphosphine (95%, 7.3 g, 26.53 mmol) in anhydrous THF (50 mL), was added diethyl azidodicarboxylate (DEAD, 97%, 4.6 mL, 28.95 mmol). The resulting solution was stirred at 0 °C for 15 minutes and a solution of *N*-(*tert*-butoxycarbonyl)-L-serine (99%, 5 g, 24.12 mmol) in anhydrous THF (36 mL) was added dropwise. The reaction mixture was stirred and allowed to reach room temperature overnight. Next day, the mixture was quenched with water (80 mL) and then it was diluted with EtOAc (40 mL). The aqueous layer was extracted with EtOAc (3 × 80 mL) and the collected organic layers were washed with brine (80 mL), dried over $MgSO_4$ and filtered. After that, the solvent of the filtrate was removed under reduced pressure to give a residue that was purified by flash chromatography (silica gel, heptane–EtOAc 95 : 5 to 7 : 3, gradient) to afford the lactone 2 as a white solid (1.7 g, 37%). ^{1}H NMR (600 MHz, CDCl3) δ 5.26 (br s, 1H), 5.20–5.05 (m, 1H), 4.47 (br t, J = 5.1 Hz, 1H), 4.43 (br t, J = 4.7 Hz, 1H), 1.48 (s, 9H). Product described in ref. 12.

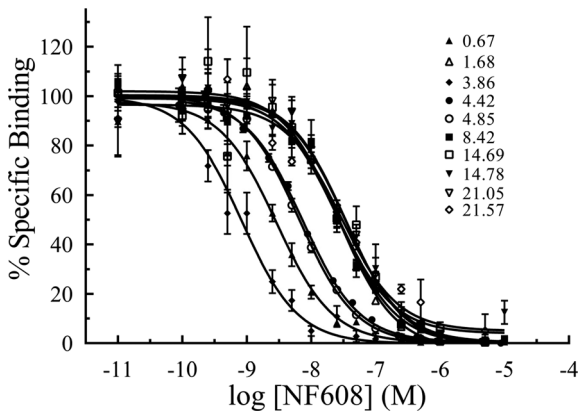


Fig. 6 Homologous competition binding of [^{3}H]-NF608 *vs.* NF608 at recombinant, homomeric rat GluK1 expressed in *sf*9 cell membranes. Shown are means ± SEM of triplicate values normalized for the maximum specific binding in each individual experiment. Nonspecific binding is determined using 1 mM (*S*)-glutamate. Inset: [^{3}H]-NF608 concentrations (nM). Analysis of the pooled data to solve for a common ligand affinity when the nonspecific binding is fixed at zero% and the B_{max} is floated gave (mean ± SEM) K_d = 6.93 nM (pK_d = 8.160 ± 0.047). Solving for a common hill value gave (mean ± SEM) n_H = 0.9899 ± 0.0455.

(*S*)-1-((2′-*tert*-Butoxycarbonyl)amino-2′carboxyethyl)thieno-[3,2-*d*]pyrimidin-2,4-dione (3). A solution of thieno[3,2-*d*]pyrimidin-4(3*H*)-one (98%, 1 g, 5.88 mmol) in anhydrous DMF (40 mL) was cooled to 0 °C and then sodium hydride (60% w/w in mineral oil, 214 mg, 5.34 mmol) was added. The resulting suspension was stirred 50 minutes at room temperature. After that time, the reaction mixture was cooled again to 0 °C and a solution of *tert*-butyl (*S*)-(2-oxooxetan-3-yl)carbamate (2) (1 g, 5.34 mmol) in anhydrous DMF (20 mL) was added. The resulting mixture was stirred at room temperature overnight. Then, the reaction mixture was quenched by adding water dropwise until no reaction was observed. The solvent of the reaction mixture was co-evaporated with toluene (3 × 20 mL) to get a white solid that was divided in 6 portions and each one was dissolved in DMSO–MeCN (15 : 1, 1 mL) and purified by 6 consecutive preparative HPLC purifications (Rt: 19.53 min; flow: 10 mL min^{-1}; A–B 1 : 0 to 25 : 75 (25 min); A–B 25 : 75 (5 min); preferably λ = 254 nm, since DMSO does not absorb) to afford the desired compound 3 as a white solid (206 mg, 11%), recovering also the starting material thieno[3,2-*d*]pyrimidin-4(3*H*)-one (Rt: 14.29 min; 109 mg, 11%). ^{1}H NMR (600 MHz, DMSO-d$_6$) δ 12.99 (s, 1H), 11.49 (s, 1H), 8.10 (d, J = 5.4 Hz, 1H), 7.29 (d, J = 5.4 Hz, 1H), 7.01 (d, J = 8.6 Hz, 1H), 4.45–4.36 (m, 2H), 4.03 (dd, J = 15.3, 11.0 Hz, 1H), 1.20 (s, 9H). ^{13}C NMR (151 MHz, DMSO-d$_6$) δ 171.2, 158.2, 155.1, 151.3, 147.9, 135.1, 118.1, 112.2, 78.3, 51.1, 46.5, 27.8. UPLC-MS (*m*/*z*) calcd for $C14H_{17}N_3O_6S$ [M − H]$^-$, 354.1; found, 354.0.

Methyl 3-(3-(2,2,2-trichloroacetyl)ureido)thiophene-2-carboxylate (8); methyl 5-bromo-3-(3-(2,2,2-trichloroacetyl)-ureido)thiophene-2-carboxylate (9). Methyl 3-(3-(2,2,2-trichloroacetyl)ureido)thiophene-2-carboxylate (**8**); methyl 5-bromo-3-(3-(2,2,2-trichloroacetyl)ureido)thiophene-2-carboxylate (**9**) were synthesized according to ref. 13.

Methyl 5-bromo-3-ureidothiophene-2-carboxylate (10). To an ice-cooled suspension of **9** (7.84 g, 17.55 mmol) in anhydrous MeOH (70 mL), was added 2 M NH_3 solution (in MeOH, 17.5 mL, 35.09 mmol) and this mixture was stirred at room temperature for 30 minutes, during which time a white solid precipitated. After this period, the white solid was filtered and washed with cold MeOH (3 × 10 mL) to give the compound **10** (4.37 g, 89%) as a white solid with a purity of 98% (determined by ^{1}H-NMR). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23 (s, 1H), 8.06 (s, 1H), 6.86 (br s, 2H), 3.80 (s, 3H). Product described in ref. 13.

6-Bromothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (11). 6-Bromothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (**11**) was synthesized according to literature procedure.[13]

(*S*)-3-(6-Bromo-2,4-dioxo-3,4-dihydrothieno[3,2-*d*]-pyrimidin-1(2*H*)-yl)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (12). A solution of 6-bromothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (**11**) (581 mg, 2.35 mmol) in anhydrous DMF (11.4 mL) was cooled to 0 °C and then sodium hydride (60% w/w in mineral oil, 85 mg, 2.14 mmol) was added. The resulting suspension was stirred 40 minutes at room temperature. After that time, the reaction mixture was cooled again to 0 °C and a solution of *tert*-butyl (*S*)-(2-oxooxetan-3-yl)carbamate (**2**) (400 mg, 2.14 mmol) in anhydrous DMF (10 mL) was added. The resulting mixture was stirred at room temperature overnight. Then, the reaction mixture was quenched by adding water dropwise until no reaction was observed. The solvent of the reaction mixture was co-evaporated with toluene (3 × 10 mL) to get a white solid that was divided into 7 portions and each one was dissolved in DMSO–MeCN (15 : 1, 1.5 mL) and purified by 7 consecutive preparative HPLC purifications (Rt: 28.60 min; flow: 10 mL min^{-1}; A (10 min), A–B 75 : 25 to 25 : 75 (25 min), preferably λ = 254 nm because DMSO do not absorbs) to afford the desired compound as a white solid (187 mg, 18%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.06 (br s, 1H), 11.64 (s, 1H), 7.57 (s, 1H), 7.04 (d, J = 8.9 Hz, 1H), 4.44–4.37 (m, 2H), 3.95 (dd, J = 15.2, 11.0 Hz, 1H), 1.21 (s, 9H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 170.9, 157.2, 155.1, 150.9, 147.5, 122.4, 122.1, 112.9, 78.2, 51.1, 46.8, 27.8. UPLC-MS (*m*/*z*) calcd for $C_9H_9BrN_3O_4S$ [M − Boc]$^+$, 333.9; found, 333.9.

(*S*)-2-(6-Bromo-2,4-dioxo-3,4-dihydrothieno[3,2-*d*]-pyrimidin-1(2*H*)-yl)-1-carboxyethan-1-ammonium chloride (13). A suspension of (*S*)-3-(6-Bromo-2,4-dioxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-1(2*H*)-yl)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (**12**) (122 mg, 0.28 mmol) in 2 M aq HCl (6 mL), was heated to 50 °C during 1 hour. After that time, the solvent and volatiles were removed under reduced pressure to afford the title compound as a white solid (98 mg, 94%), which was used in next reaction step without further purification. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.73 (s, 1H), 8.40 (s, 3H), 7.68 (s, 1H), 4.44 (dd, J = 15.0, 6.3 Hz, 1H), 4.27 (dd, J = 15.0, 7.9 Hz, 1H), 4.10 (br t, J = 6.3 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.3, 157.2, 151.4, 146.6, 123.0, 121.6, 113.6, 50.5, 44.7. UPLC-MS (*m*/*z*) calcd for $C_9H_9BrN_3O_4S$ [M + H]$^+$, 333.9; found, 333.9.

Sodium (*S*)-1-(2-amino-2-carboxylatoethyl)-2,4-dioxo-1,4-dihydro-2*H*-thieno[3,2-*d*]pyrimidin-3-ide-6-*d* ([D6]-NF608 disodium salt) and sodium (*S*)-1-(2-amino-2-carboxylatoethyl)-2,4-dioxo-1,4-dihydro-2*H*-thieno[3,2-*d*]pyrimidin-3-ide (NF608 disodium salt). Pd/C (1.8 mg, 10% Pd on activated C, water-wet) was weighed in a small flask that was repeatedly filled and evacuated with N_2 (3 times), and then D_2 (3 times). In parallel, (*S*)-2-(6-bromo-2,4-dioxo-3,4-dihydrothieno[3,2-*d*]-pyrimidin-1(2*H*)-yl)-1-carboxyethan-1-aminium chloride (**13**) (9 mg, 0.024 mmol) was weighed in a separate flask that was repeatedly filled and evacuated with N_2 (3 times). Then, water (240 μL) and 1 M aq NaOH (69 μL, 0.069 mmol) were added to afford a suspension that was heated to 60 °C for 5 minutes until getting a clear solution that was transferred to the previous flask with Pd/C. The resulting mixture was repeatedly filled and evacuated with D_2 (3 times), and stirred vigorously at room temperature overnight under D_2 (1 atm). After this period, the reaction mixture was filtered through a plug of Celite®, and the filtrand was washed with water (3 × 3 mL). Then, the filtrate was concentrated *in vacuo* to give the deuterated : hydrogenated disodium salts of products [D6]-NF608 : NF608 in a 4 : 1 ratio, as a white solid (4 mg, 57%, 80% D-incorporation).

Sodium (*S*)-1-(2-amino-2-carboxylatoethyl)-2,4-dioxo-1,4-dihydro-2*H*-thieno[3,2-*d*]pyrimidin-3-ide-6-d ([D6]-NF608). ^{1}H NMR (600 MHz, D_2O) δ 7.29 (br s, 1H), 4.29 (d, J = 7.1 Hz, 2H), 3.75 (t, J = 7.1 Hz, 1H). ^{13}C NMR (151 MHz, D_2O) δ 178.6, 163.6, 155.3, 148.5, 136.3, 117.1, 113.3, 54.8, 49.0. UPLC-MS (*m*/*z*) calcd for $C_9H_9DN_3O_4S$ [M + H]$^+$, 257.0; found, 257.0.

Sodium (*S*)-1-(2-amino-2-carboxylatoethyl)-2,4-dioxo-1,4-dihydro-2*H*-thieno[3,2-*d*]pyrimidin-3-ide (NF608). ^{1}H NMR (600 MHz, D_2O) δ 8.02 (d, J = 5.5 Hz, 1H), 7.29 (d, J = 5.5 Hz, 1H), 4.29 (d, J = 7.1 Hz, 2H), 3.75 (t, J = 7.1 Hz, 1H). ^{13}C NMR (151 MHz, D_2O) δ 178.6, 163.6, 155.3, 148.5, 136.3, 117.2, 113.3, 54.8, 49.0.

[^{3}H]-(*S*)-2-Amino-3-(2,4-dioxo-3,4-dihydrothieno[3,2-d]-pyrimidin-1(2*H*)-yl)propanoic acid ([^{3}H]-NF608). A solution of **13** (1.3 mg, 3.5 μmol) with Pd/C [30%] (2.6 mg, 2 w/w eq.) in H_2O (1 mL) and NaOH (20 μL, 1 M) in round reaction flask (2 mL) equipped with a Teflon-coated stir bar was mounted onto tritium manifold system. The reaction mixture was degassed three times in a repeated freeze–thaw cycle (liquid nitrogen), and pressure of released tritium gas was adjusted to 515 mbar (6.9 Ci). The reaction mixture was vigorously stirred for 3 hours. The reaction mixture was frozen with liquid nitrogen and unreacted tritium gas was reabsorbed in the uranium bad. The reaction mixture was heated up to room temperature and the catalyst was filtered off with a PTFE syringe filter (0.45 μm). The tritiation flask and filter

were rinsed with H_2O (3 × 0.6 mL). To remove labile activity, the solvent with addition of EtOH (2 mL) was repeatedly lyophilized (3 × 3 mL H_2O/EtOH 50 : 50). The activity of crude product (63% R.C.P.) was determined at 65 mCi. The crude product was dissolved in H_2O (1 mL) and purified by radio-HPLC on a Synergi 4μ POLAR RP80A 250 × 4.6 mm semi-preparative column using gradient elution with A = water, B = acetonitrile (flow of 4.7 mL min^{-1}, 25 °C, 100% of A for 25 min, 100% of B in 35 min). The retention time of [^{3}H]-NF608 was detected at 19.3 min. Pure [^{3}H]-NF608 was isolated in total activity 23.5 mCi (16.3 Ci $mmol^{-1}$), 23% R.C.Y. The specific activity was determined by HRMS at 16.3 Ci $mmol^{-1}$. ^{3}H-NMR (320 MHz, D_2O): δ 8.08 (1 T, s). HRMS (ESI): calc. for $C_9H_7{}^3H_1N_3O_4S$ [M − 1] 256.0318; found 256.0319.

Pharmacology

Recombinant homomeric rat GluK1$(Q)_{1b}$, GluK2$(V,C,R)_a$ and GluK3$_a$ were expressed in *sf9* insect cells as previously described.[5] Rat whole brain (mixed sex) crude synaptosomal (P2) membranes were prepared by standard methods.[14] For use in binding assays, an aliquot of membranes was thawed and washed four times by centrifugation 48 000 × *g*, 15 min at 4 °C with Ultra-Turrax resuspension in 25 mL ice-cold hypotonic buffer (5 mM Tris-HCl, pH 7.4, 4 °C). Membranes were then resuspended in assay buffer (50 mM Tris-HCl, pH 7.1, 4 °C). Binding assays were conducted in a volume of 250 μL at 4 °C and kinetic and saturation assays terminated by rapid filtration through 0.3% (w/v) polyethyleneimine-treated GF/C-type glass fiber filters (VWR filter #696; VWR, Denmark) on 12-well Millipore filtration manifolds (Millipore, Darmstadt, Germany), washing twice with 4 mL ice-cold assay buffer. Filters were equilibrated in 3 mL EcoScint scintillation fluid (National Diagnostics, Atlanta, GA) for 12 h and radioactivity detected as DPM using a TriCarb 2900 scintillation counter (PerkinElmer, Waltham, MA). Non-specific binding was determined in the presence of 1 mM (*S*)-glutamate. In competition experiments, bound and free radioligand were separated by cold filtration through GF/B glass fiber filters in UniFilter-96 microtitre plates (PerkinElmer) on a FilterMate manifold (PerkinElmer) using two washes with ice-cold assay buffer. Filters were dried 1 hour at 70 °C and 50 μL Microscint 20 (PerkinElmer) was added. Radioactivity was detected as DPM using a TopCounter (PerkinElmer).

Binding data were analyzed with GraphPad Prism v6 (GraphPad Software, San Diego, CA) using: one-site K_i and four-parameter logistic equations for competition data, one site-homologous equation for [^{3}H]kainate K_d determination, mono-exponential rate equations for association and dissociation data and one-site total and nonspecific binding for [^{3}H]-NF608 saturation binding.

Abbreviations

KA	Kainic acid
Glu	(*S*)-Glutamic acid
DA	Domoic acid
QA	Quisqualic acid
NF608	(*S*)-2-Amino-3-(2,4-dioxo-3,4-dihydrothieno[3,2-*d*] pyrimidin-1(2*H*)-yl)propanoic acid
ATPA	(*RS*)-2-Amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid
UBP310	(*S*)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxy-thiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione

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