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Original article

Substituted 2-Aminothiopen-derivatives: A potential new class of GluR6-Antagonists

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ABSTRACT

In the course of search for new therapeutic agents against epilepsy new inhibitors for the kainate receptor subtypes GluR5 and GluR6 were synthesized.

We were able to synthesize new substituted thieno[2,3-*d*]pyrimidines **3a,b**, **4a,b**, **5a,b** as well as thiophene-3-carboxamides **2a-d** and a multitude of substituted 4-methyl-5-phenylthiophene-3-carboxylic acids.

All compounds described herein were tested for their antagonistic effect towards the kainate receptor subtypes GluR5 and GluR6. The highest activity was observed for ethyl 2-amino-4-methyl-5-phenyl-thiophene-3-carboxylate **1c** with an $IC_{50} = 0.75$ μM at the GluR6 receptor.

1. Introduction

Epilepsy is a chronic disease and needs continuous or lifelong therapy [1]. In pathogenesis of epileptic diseases the disbalance between GABA- and glutamate-based neurotransmission in the central nervous system plays an essential role. As the most important excitatory neurotransmitter glutamate is involved in many different processes in CNS of mammals [2]. Kainate receptors [3], a subtype of ionotropic glutamate receptors, are of special interest. Kainate, as analog of glutamate, is able to induce epileptic seizures in rodents [4]. Thus the inhibition of glutamate receptors is an approach for the therapy of epileptic diseases. Especially subtypes GluR5 and GluR6 are discussed in genesis of different kinds of epilepsy [5–10]. Due to this kainate receptors are interesting potential targets for the development of new antiepileptics [10–12]. Among these receptors a special significance to the kainate receptor subtype GluR6 is described. This subtype is primarily expressed in the excitatoric pyramidal cells of the hippocampus. There are hints that the GluR6 and the GluR5 subtype play an opposing role in the hippocampal activation [11,13]. In search for new antiepileptics some quinalines and thieno[2,3-*d*]pyrimidines proved to be successful anticonvulsants [14,15]. All known inhibitors of GluR5 and GluR6 have distinct structural similarities to endogenous agonist glutamate, including pyrrolidine-, isoxazole-, oxa(thia)diazole-, pyrimidine- and hexahydrofuropyrane derivatives. EC_{50} values of glutamate (631 μM GluR5, 270–762 μM GluR6) and kainate (33.6 μM GluR5, 299 μM GluR6) on different glutamate receptors are summarized in literature [7]. Our researches gave indications that thieno[2,3-*d*]pyrimidines are specific kainate receptor inhibitors [16,17,18]. Another new chemical class of GluR6 kainate receptor antagonists are 2-aminothiophene-3-carboxylic acid derivatives. In high throughput screenings ethyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate **1c** was identified as a non-competitive GluR6-subtype specific Inhibitor. This compound has an IC_{50} of 0.75 μM *in vitro* and therefore is an interesting target for further structure optimization and modification.

2. Results and discussion

2.1. Chemistry

Based on thiophenes **1a-d** different thieno[2,3-*d*]pyrimidines **3a,b**, **4a,b**, **5a,b** were synthesized (Scheme 1). Conversion of 2-aminothiophen-3-carboxamides **1a,b** using thiophosgene afforded the 2-thioxo-2,3-dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-ones **3a,b**, which could be alkylated by iodoethane to give the 2-(Ethylthio)-thieno[2,3-*d*]pyrimidin-4(3*H*)-ones **4a,b**. Ethyl 2-aminothiophene-

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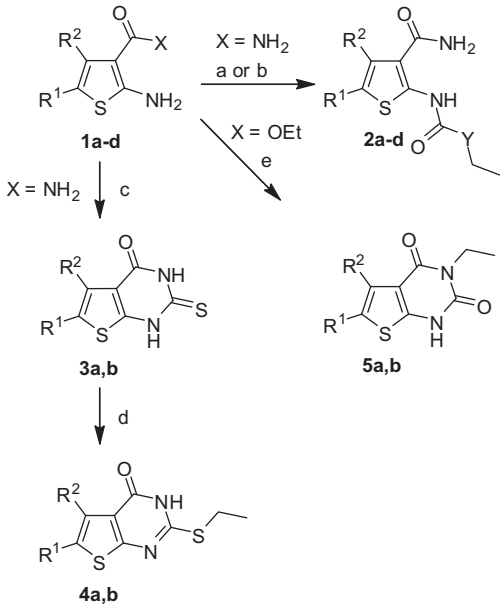


Scheme 1. (a) for Y = O (**2a,b**): ClCOOEt, K_2CO_3, toluene, r.t., 3 h; (b) for Y = NH (**2c,d**): EtNCO, CH_2Cl_2, reflux, 5 d; (c) $SCCl_2$, K_2CO_3, CH_2Cl_2, r.t., 1.5 h; (d) $NaOCH_3$, EtI, EtOH, r.t., 30 min; (e) (i) EtNCO, CH_2Cl_2, reflux, 5 d; (ii) NaOH, EtOH, reflux, 1.5 h.

3-carboxylates **1c,d** were used for the synthesis of 3-ethyl-thieno[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-diones **5a,b** via conversion with *N*-ethylisocyanate followed by the cyclization using sodium hydroxide. 2-*N*-substituted thiophene-3-carboxamides **2a-d** were synthesized based on 2-aminothiophen-3-carboxamides **1a,b** by conversion with ethyl chloroformate or *N*-ethylisocyanate. Under various conditions no cyclization of thiophene-3-carboxamides **2a-d** to the corresponding thienopyrimidines was observed.

Based on the good results of compound **1c** has an IC_{50} of 0.75 μM the substituents R^1 and R^2 were modified stepwise. For R^1 phenyl-groups and for R^2 H or phenyl-groups should be established. Furthermore R^1—R^2 was replaced by phenyl tetra methylen-residue.

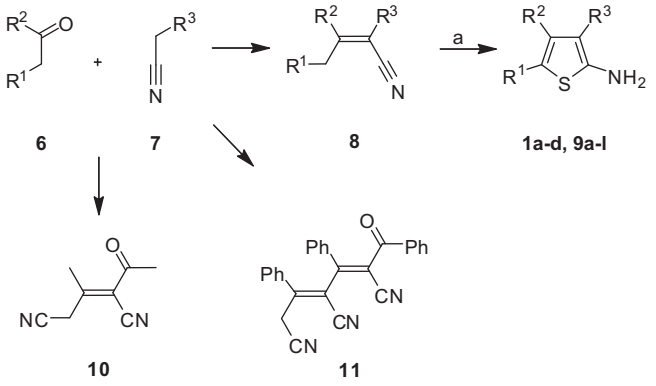
The thiophene derivatives **1a-d** and **9a-l** were obtained via Gewald reaction (Scheme 2). [19,20] The first step was a Knoevenagel Cope [21] condensation between ketone **6** and a acceptor-substituted acetonitrile **7** to produce the crotonic acid nitrile **8**. Subsequent reaction of **8** with sulfur in the presence of diethylamine or morpholine gave the cyclization to the thiophenes **1a-d** and **9a-l**. Sometimes it was possible to perform the reaction as an one-pot-reaction without isolation of **8**. But in most cases it was convenient to isolate **8** first.

By the method of Gewald **9a-f** were synthesized and tested. For these compounds a decreased GluR6-antagonism was observed in comparison to **1c** (see 2.2). For that reason phenylacetone **6** was used for further synthesis to give **9g-l** with R^1 = phenyl and R^2 = methyl.

Reaction of phenylacetone **6** with benzoylacetonitrile **7** formed the crotonic acid nitrile **8l** in 25% yield. The main product **11** which was formed via condensation of three molecules of benzoylacetonitrile **7**. Thereby benzoylacetonitrile **7** acts not only as the carbonyl-compound but also as methyl-compound (Scheme 2).

If acetylacetonitrile was used instead of benzoylacetonitrile only **10** could be isolated in 33% yield, because the carbonyl reactivity is much stronger (Scheme 2). These unwanted reactions are well known in literature [22,23,24] for β-cyanketones with CH-acidic group in position 2.

Using **1c** as starting material a multitude of thiophene compounds could be synthesized bearing different modifications especially in 2-position (Scheme 3).



Scheme 2. (a) S_8, morpholine or $NH(Et)_2$, EtOH, r.t., 1–3 h.

Alkaline hydrolysis of **1c** with sodium hydroxide solution gave the carboxylic acid **12**. Various experiments to get decarboxylation of **12**, e.g. reflux in different solvents, quinoline, addition of $Ba(OH)_2$ or Cu, afforded only complex mixtures. Probably the formed 2-amino-thiophene undergoes consecutive reactions. These instability would be equivalent to the properties of unsubstituted 2-aminothiophene, which already polymerizes at room temperature [25–28]. In contrast the heterocycle of 2-aminothiophenes bearing a acceptor in 3- or 5-position is stabilized by a "push-pull-effect" [19,29,30].

Due to instability of 2-aminothiophene decarboxylation of **12** succeeded only with ethyl chloroformate. Simultaneous acylation of the amino group obviously stabilizes the intermediate. Anyway the decarboxylation product **13** is only a side product. The main product is carboxylic acid **14**.

Conversion of **1c** using carboxylic acid-anhydrides or –chlorides gave 2-acylaminothiophenes **15a-d**. *N*-ethylisocyanate formed derivatives **15e,h** via acylation. The use of bromoacetyl bromide as acylating reagent led to derivative **15f**.

Substitution of bromine with morpholine, benzylamine and butylamine delivered **16a-c** (Scheme 4).

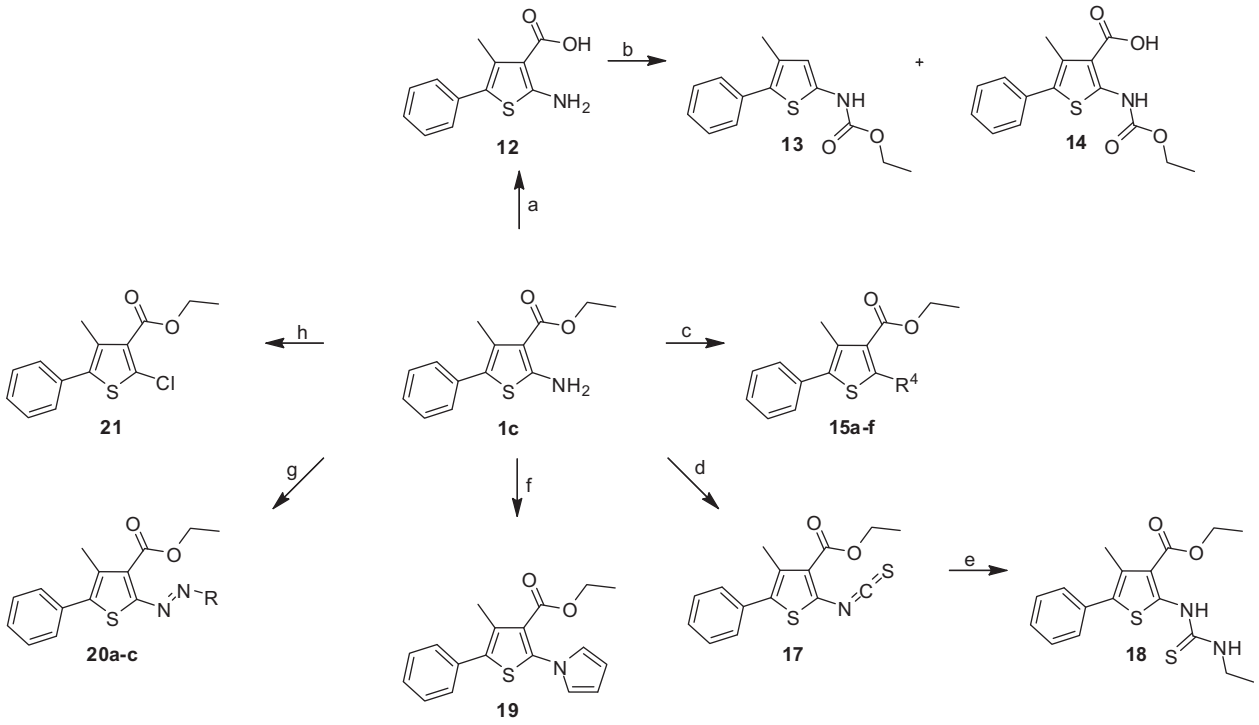
To prepare a thiourea derivative **1c** was converted with thiophosgene to the isothiocyanate **17** followed by reaction with ethylamine to give **18**.

Methylation of the weak basic amine group was carried out with methyl iodide under pressure to afford **15g**.

Reaction of **1c** with 2,5-dimethoxy-tetrahydrofurane formed pyrrol derivative **19**, where the amino-group of thiopene is involved in a pyrrol-system and lost the ability to build hydrogen bonds. Additional variations of the basic substituent in 2-position were obtained by conversion with sodium nitrite/HCl to the intermediate diazoniumsalt followed by the reaction with dimethylamine, morpholine, cupper chloride or cyanide. Thereby the diazo-derivatives **20a-c** and chloride **21** were synthesized.

2.2. Biology

The synthesized thiophene derivatives were studied for their antagonistic activity towards the kainate-receptor subtypes GluR5 and GluR6. Therefore we established a test system. Human embryonic kidney cells (HEK-cells) characterized by a stable recombinant expression of aequorin together with the GluR5 or GluR6, respectively, kainate receptor were used as test system. The antagonistic effect of substances on kainate receptors was determined using a luminescence reporter assay. The application of kainate receptor agonist glutamate (at its respective EC_{50} concentration, 275 μM) caused an influx of Ca^{2+} ions through the opened ion channel which lead to a luminescent signal. The signal was



Scheme 3. (a) NaOH, EtOH, reflux, 2.5 h; (b) ClCOOEt, K_2CO_3, CH_3CN, r.t., 8 h; (c) acid chloride, K_2CO_3, r.t., 3 h (**15c,d,f**), acid anhydride, reflux, 2.5 h (**15a,b,g**) or EtNCO, CH_2Cl_2, reflux, 5 d (**15e,h**); (d) $CSCl_2$, K_2CO_3, CH_2Cl_2, r.t., 1 h; (e) NH_2Et, CH_2Cl_2, r.t., 1 h; (f) 2,5-dimethoxytetrahydrofuran, AcOH, reflux, 20 min; (g) (i) $NaNO_2$/HCl, 0 °C, 20 min; (ii) $NH(CH_3)_2$ (**20a**), morpholine (**20b**) or NaCN (**20c**), 0 °C, 30 min; (h) (i) $NaNO_2$/HCl, 0 °C, 20 min; (ii) CuCl/HCl, 0 °C, 30 min.

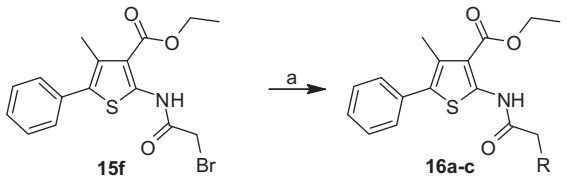
caused by the reaction of Ca^{2+} with coelenterazine, the cofactor of the Ca^{2+} affinic photoprotein aequorine. Antagonistic activity of compounds was determined by inhibition of the glutamate specific luminescence signal. The overall luminescence of the cells was detected after lysis by triton-X100.

It appeared that the thienopyrimidine derivatives **3a,b**, **4a,b** and **5a,b** and the thiophene derivatives **2a-d** have only less activity (Table 1). In contrast compound **1c** showed a surprising result. The IC_{50} value in GluR6 kainate-receptor-assay of derivative **1c** was 0.75 μM. The known glutamate receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) has a IC_{50} of 23 μM using the same test conditions. NBQX, SYM 2081 and miconazole were also tested in this assay. The IC_{50}-values are 74 μM, 4 μM and 6.5 μM respectively.

To gain more information about the structure-activitiy-relationship more thiophene derivatives were synthesized.

The results of GluR5- and GluR6-aequorin-assay of all thiophene compounds synthesized are summarized in Table 2.

Change of substituents in 4- or 5 position leads to a decreased antagonistic activity against GluR6-receptor. This applies for the introduction of substituents at phenyl residues in 5-position (**9a-c**) as well as the replacement of methyl-group in 4-position against H or phenyl (**9d,e**) or anellation of a phenyltetramethylene-residue (**9f**). For that reason these positions remained unchanged in a majority of tested thiophenes ain analogy to lead structure **1c**.



Scheme 4. (a) morpholine (**16a**), benzylamine (**16b**) or butylamine (**16c**) was used, CH_3CN, r.t., 1 h.

The most active members of 4-methyl-5-phenylthiophenes proved to be the 2-aminothiophenes having a alkylester-group in 3-position (**9g-i**). For these compounds IC_{50} of 2.4–4.1 μM were obtained in *in vitro* kainate-receptor-assay. The inhibition-values of methylester **9g** and propylester **9h** show reduced activity in GluR-assay in comparison to ethylester **1c**.

Exchange of ethylester-group in 3-position against amide (**9j**), nitrile (**9k**), benzoyl (**9l**) or carboxylic acid (**12**) leads to decreased antagonistic activity.

Also by acylation of 2-aminofunction it was not possible to enhance the antagonistic activity. The acylated compounds **13**, **14**, **15a-e** and **16 a-c** showed only weak inhibition of fractional luminescence. In some cases the inhibition was lower than 25% and thus within the margin of error of the bioluminescence methode.

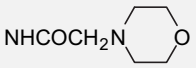
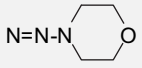
Equally weak inhibition was observed for the 2-thioureido-thiophene **18** and 2-methylaminothiophene **15g**.

Table 1
Substituted thiophenes and thienopyrimidines.

	R^1	R^2	X	Y	c (μM)	GluR5	GluR6
1a	C_6H_5	CH_3	NH_2		100	2[a]	3[a]
1b	$(CH_2)_4$		NH_2		10	4[a]	13[a]
1c	C_6H_5	CH_3	$COOC_2H_5$		10	12[a]	0.75[b]
1d	$(CH_2)_4$		$COOC_2H_5$		10	7[a]	11[a]
2a	C_6H_5	CH_3		O	10	19[a]	12[a]
2b	$(CH_2)_4$			O	10	14[a]	17[a]
2c	C_6H_5	CH_3		NH	10	5.8[a]	17[a]
2d	$(CH_2)_4$			NH	10	0.1[a]	12[a]
3a	C_6H_5	CH_3			10	10[a]	−2[a]
3b	$(CH_2)_4$				10	17[a]	0[a]
4a	C_6H_5	CH_3			10	1[a]	0[a]
4b	$(CH_2)_4$				10	8[a]	4[a]
5a	C_6H_5	CH_3			10	11.8[a]	9.5[a]
5b	$(CH_2)_4$				10	6[a]	7.1[a]

[a] Inhibition of luminescence (% of control).
[b] IC_{50} (μM).

Table 2
Substituted thiophenes.

	R^1	R^2	R^3	R^4	c (μM)	GluR5	GluR6
1c	C_6H_5	CH_3	$COOC_2H_5$	NH_2	10	12[a]	0.75[b]
9a	4-CH_3–O–C_6H_4	CH_3	$COOC_2H_5$	NH_2	10	1[a]	8.6[b]
9b	4-F–C_6H_4	CH_3	$COOC_2H_5$	NH_2	10	0[a]	12[b]
9c	2-Cl–C_6H_4	CH_3	$COOC_2H_5$	NH_2	10	10[a]	5.7[b]
9d	C_6H_5	H	$COOC_2H_5$	NH_2	100	25[a]	-1[a]
9e	C_6H_5	C_6H_5	$COOC_2H_5$	NH_2	10	13[a]	4.5[b]
9f	$CH_2CH(C_6H_5)CH_2CH_2$		$COOC_2H_5$	NH_2	10	1[a]	4[a]
9g	C_6H_5	CH_3	$COOCH_3$	NH_2	10	30[b]	4.1[b]
9h	C_6H_5	CH_3	$COOC_3H_7$	NH_2	10	5[a]	2.4[b]
9i	C_6H_5	CH_3	$COOC(CH_3)_3$	NH_2	10	4[a]	3.6[b]
9j	C_6H_5	CH_3	$CONHC_2H_5$	NH_2	10	9[a]	4[a]
9k	C_6H_5	CH_3	CN	NH_2	10	17[a]	71[b]
9l	C_6H_5	CH_3	COC_6H_5	NH_2	10	17[a]	10.6[b]
12	C_6H_5	CH_3	COOH	NH_2	100	20[a]	44[a]
13	C_6H_5	CH_3	H	$NHCOOC_2H_5$	10	1[a]	18[a]
14	C_6H_5	CH_3	COOH	$NHCOOC_2H_5$	10	12[a]	7[a]
15a	C_6H_5	CH_3	$COOC_2H_5$	$NHCOCH_3$	10	3[a]	4[a]
15b	C_6H_5	CH_3	$COOC_2H_5$	$NHCOCF_3$	10	18[a]	20[a]
15c	C_6H_5	CH_3	$COOC_2H_5$	$NHCOC_6H_5$	10	−3[a]	5[a]
15d	C_6H_5	CH_3	$COOC_2H_5$	$NHCOCOOC_2H_5$	10	4[a]	10[a]
15e	C_6H_5	CH_3	$COOC_2H_5$	$NHCONHC_2H_5$	10	0.5[a]	9[a]
15g	C_6H_5	CH_3	$COOC_2H_5$	$NHCH_3$	10	4.5[a]	19[a]
16a	C_6H_5	CH_3	$COOC_2H_5$		10	14[a]	9[a]
16b	C_6H_5	CH_3	$COOC_2H_5$	$NHCOCH_2NHCH_2C_6H_5$	10	25[a]	4[a]
16c	C_6H_5	CH_3	$COOC_2H_5$	$NHCOCH_2NHCH_2C_4H_9$	10	−3[a]	10[a]
17	C_6H_5	CH_3	$COOC_2H_5$	NCS	10	0[a]	3.5[b]
18	C_6H_5	CH_3	$COOC_2H_5$	$NHCSNHC_2H_5$	10	7[a]	−6.5[a]
19	C_6H_5	CH_3	$COOC_2H_5$		10	−1[a]	2[a]
20a	C_6H_5	CH_3	$COOC_2H_5$	N=N–$N(CH_3)_2$	10	10[a]	38[b]
20b	C_6H_5	CH_3	$COOC_2H_5$		10	4[a]	54[b]
20c	C_6H_5	CH_3	$COOC_2H_5$	N=N–CN	10	7[a]	21[b]
21	C_6H_5	CH_3	$COOC_2H_5$	Cl	10	0[a]	10[b]

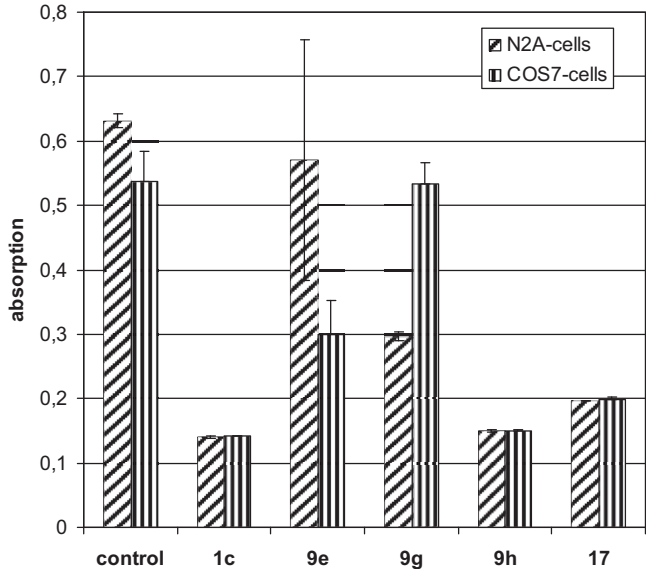
[a] Inhibition of luminescence (% of control).
[b] IC_{50} (μM).

We found potent compounds in the group of thiophene derivatives having a 2-amino-group, which can act as H-donor of a hydrogen bond. This applies for the 2-diazenylthiophenes **20a-c**, 2-chlorothiophene **21** and isothiocyanate **17**. The most effective compound of this group proved to be **17** having an IC_{50} of 3.5 μM in GluR6-assay.

The effect of thiophenes **1c**, **9e,g,k** and **17** on metabolic activity of cells was determined using a MTT-assay [31]. Toxic effects of thiophene derivatives against N2A- and COS7-cells were detectable at a concentration of 100 μM only and after an incubation time of 24 h (Scheme 5).

Compounds **1c**, **9h** and **17** inhibited the activity of mitochondria in both cell lines in this concentration in comparison to control.

However **9e** showed only slightly effect towards N2A-cells. In the presence of **9g** no significant differences of formazan formation in COS7-cell line was observed.



Scheme 5. Influence of thiophenes **1c**, **9e**, **9g**, **9h** and **17** (100 μM) on activity of mitochondrial dehydrogenases in N2A- and COS7-cells.

3. Conclusion

We were able to identify 2-aminothiophenes with hydrophobic substituents in 4- and 5-position as a new class of selective GluR6-Antagonists. The aim of this work was to increase the selectivity of GluR6-specific inhibition. The lead compound of previous studies of

5-phenylthiopene-3-carboxylic acid methylesters was modified. By this way the influence of decreasing the log *P*-value was analysed. Without changing the chemical structure a variety of substitution patterns were realized keeping a hydrophobic region (4- and 5-position) and a hydrophilic region (2- and 3- position). To gather information about the influence of the amino group for receptor binding the electronic properties as well as the hydrogen donor properties in this position were changed.

In MTT-assay toxic effects were observed only in high concentrations (100 μM) and long incubation times (24 h). Having these preliminary results the tested compounds can be considered as less toxic.

4. Experimental

4.1. Chemistry

Unless otherwise noted all reagents and solvents were used as supplied commercially (Merck, Sigma–Aldrich, Fluka, Lancaster). All reactions were performed in oven-dried glassware. Analytical thin layer chromatography (TLC) was performed on Merck Silica gel 60 F_{254} plates. Flash column chromatography was performed on Merck Silica gel 60 (particle size 63 μM–200 μM) and Lancaster Silica gel 60 (particle size 40 μM). Nuclear magnetic resonance spectra were recorded on a Varian Gemini-300, Bruker DRX-400 or Bruker DRX-600. The chemical shifts are reported in ppm and the residual solvent signal is used as an internal standard. Infrared spectra were collected on a Perkin-Elmer FT-IR PC 16 spectrometer. For mass spectra a VG Analytics VG ZAB-HSQ (EI, 70 eV), Bruker Daltonics 7 T Apex™ II FT-ICR-MS (ESI-HRMS, EI, 70 eV), Perkin-Elmer Micro-HPLC 200/PE SCIEX API 3000 (LC/MS) or Hewlett Packard HP 1050/MS Engine HP 5989 A (LC/MS) (negative and positive chemical ionisation, EI, 70 eV) were used. Melting points were determined on a BÜCHI Melting Point 535 or on a Boetius. The declared values are not corrected.

4.1.1. Synthesis of 2-aminothiophenederivatives ***1a-d*** and ***9a-l*** by Gewald

4.1.1.1. General method A. To a stirring mixture of ketone (0.1 mol), nitrile (0.1 mol) and powdered sulfur (0.1–0.11 mol) in ethanol (30 ml) diethylamine or morpholine (10 ml) was added dropwise, keeping the temperature lower than 50 °C. After 1–3 hours the reactions were complete and the reaction mixtures were cooled in a fridge for crystallization. If no crystallization takes place the mixtures were poured in to 2–3 fold volume of water. The precipitates were filtered and recrystalized from ethanol.

4.1.1.2. 2-Amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide ***1b***. Yield 61%; mp 190 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.68 (m, 4 H), 2.51 (m, 4 H), 6.52 (bs, 2 H), 6.89 (s, 2 H); LRMS-EI: *m/z* calcd 196.1, found 196.0; IR (KBr): 1420, 1481, 1589, 1645, 2854, 3157, 3388, 3405, 3482, 3937 cm^{-1}.

4.1.1.3. Ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate ***1d***. Yield 73%; mp 111–115 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.33 (t, J = 7.2 Hz, 3 H), 1.76 (m, 4 H), 2.50 (t, J = 5.7 Hz, 2 H), 2.69 (t, J = 5.7 Hz, 2 H), 4.25 (q, J = 7.2 Hz, 2 H), 5.93 (s, 2 H); LRMS-EI: *m/z* calcd 225.1, found 225.1; IR (KBr): 1028, 1154, 1179, 1276, 1295, 1412, 1491, 1499, 1576, 1596, 1647, 2841-2986, 3299, 3404 cm^{-1}.

4.1.1.4. Ethyl 2-amino-5-phenylthiophene-3-carboxylate ***9d***. Yield 75%; mp 123-124 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.37 (t, J = 7.2 Hz, 3 H), 4.30 (q, J = 7.2 Hz, 2 H), 6.00 (s, 2 H), 7.33 (m, 6 H); LRMS-EI: *m/z* calcd 247.2, found 247.2; IR (KBr): 1094, 1204, 1264, 1413, 1480, 1496, 1548, 1585, 1667, 2932, 2978, 3322, 3455 cm^{-1}.

4.1.1.5. Ethyl 2-amino-6-phenyl-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate ***9f***. Yield 86%; mp 101–103 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.35 (t, J = 7.2 Hz, 3 H), 1.91 (m, 1 H), 2.10 (m, 1 H), 2.75 (m, 3 H), 2.99 (m, 2 H), 4.29 (q, J = 7.2 Hz, 2 H), 5.96 (s, 2 H), 7.28 (m, 5 H); ^{13}C-NMR ($CDCl_3$): δ (ppm) 14.6, 24.4, 30.2, 32.5, 40.9, 59.6, 107.0, 118.4, 126.4, 127.0, 128.6, 132.5, 146.1, 160.2, 166.1; LRMS-EI: *m/z* calcd 301.1, found 301.0; IR (KBr): 1244, 1258, 1480, 1572, 1663, 2834, 2988, 3355, 3471 cm^{-1}.

4.1.1.6. General method B. Crotonic acid nitrile (0.1 mol), sulfur (0.1 mol) and diethylamin or morpholin (5–10 ml) in ethanol (30 ml) were converted and isolated like described in general method A.

4.1.1.7. 2-Amino-4-methyl-5-phenylthiophene-3-carboxamide ***1a***. Yield 60%; mp 185 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 2.26 (s, 3 H), 6.86 (s, 2 H), 6.94 (s, 2 H), 7.34 (m, 5 H); LRMS-EI: *m/z* calcd 232.1, found 232.1; IR (KBr): 1279, 1413, 1468, 1552, 1563, 1592, 1651, 3170, 3312, 3440, 3501 cm^{-1}.

4.1.1.8. Ethyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate ***1c***. Yield 77%; mp 95–97 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.37 (t, J = 7.2 Hz, 3 H), 2.33 (s, 3 H); 4.32 (q, J = 7.2 Hz, 2 H), 6.10 (s, 2 H), 7.34 (m, 5 H); LRMS-EI: *m/z* calcd 261.1, found 261.1; IR (KBr): 1070, 1103, 1232, 1256, 1326, 1405, 1475, 1557, 1568, 1596, 1655, 2926, 2988, 3356, 3477 cm^{-1}.

4.1.1.9. Ethyl 2-amino-5-(4-methoxyphenyl)-4-methylthiophene-3-carboxylate ***9a***. Yield 53%; mp 102–105 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.37 (t, J = 7.2 Hz, 3 H), 2.28 (s, 3 H), 3.83 (s, 3 H), 4.31 (q, J = 7.2 Hz, 2 H), 6.06 (s, 2 H), 6.92 (m, 2 H), 7.26 (m, 2 H); LRMS-EI: *m/z* calcd 291.3, found 291.3; IR (KBr): 1180, 1270, 1467, 1570, 1654, 2925, 2984, 3322, 3447 cm^{-1}.

4.1.1.10. Ethyl 2-amino-5-(4-fluorophenyl)-4-methylthiophene-3-carboxylate ***9b***. Yield 49%; mp 79–82 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.37 (t, J = 7.2 Hz, 3 H), 2.28 (s, 3 H), 4.31 (q, J = 7.2 Hz, 2 H), 6.09 (s, 2 H), 7.07 (m, 2 H), 7.30 (m, 2 H); ^{19}F-NMR ($CDCl_3$): δ (ppm) −37.1; LRMS-EI: *m/z* calcd 279.2, found 279.2; IR (KBr): 1134, 1225, 1269, 1466, 1560, 1654, 2929, 2985, 3320, 3447 cm^{-1}.

4.1.1.11. Ethyl 2-amino-5-(2-chlorophenyl)-4-methylthiophene-3-carboxylate ***9c***. The crude product was purified by column chromatography (toluene); yield 28%; mp 96-98 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.11 (t, J = 7.2 Hz, 3 H), 2.11 (s, 3 H), 4.14 (q, J = 7.2 Hz, 2 H), 6.12 (s, 2 H), 7.24 (m, 4 H); ^{13}C-NMR ($CDCl_3$): δ (ppm) 14.7, 16.6, 36.0, 59.9, 105.2, 127.0, 127.6, 129.4, 130.0, 130.4, 133.8, 138.9, 164.9, 166.6; HRMS-ESI: *m/z* calcd 318.03260 $[M + Na]^+$, found 318.03265; IR (KBr): 1064, 1134, 1277, 1490, 1593, 1649, 2976, 3309, 3424 cm^{-1}.

4.1.1.12. Ethyl 2-amino-4,5-diphenylthiophene-3-carboxylate ***9e***. Yield 59%; mp 146 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 0.73 (t, J = 7.2 Hz, 3 H), 3.82 (q, J = 7.2 Hz, 2 H), 7.11 (m, 10 H), 7.55 (s, 2 H); HRMS-ESI: *m/z* calcd 346.08722 $[M + Na]^+$, found 346.08738; IR (KBr): 1235, 1275, 1465, 1484, 1547, 1576, 1645, 2978, 3059, 3312, 3425 cm^{-1}.

4.1.1.13. Methyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate ***9g***. Yield 30%; mp 118 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 2.24 (s, 3 H), 3.73 (s, 3 H), 7.37 (m, 12 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 16.6, 51.2, 105.5, 117.9, 127.4, 129.4, 129.8, 131.3, 134.7, 164.5, 166.2; LRMS-EI: *m/z* calcd 247.1, found 247.1; IR (KBr): 1239, 1271, 1437, 1480, 1550, 1581, 1670, 2948, 3304, 3469 cm^{-1}.

4.1.1.14. Propyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate ***9h***. The crude product was purified by column chromatography

(toluene); yield 20%; mp 66 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.03 (t, $J = 7.5$ Hz, 3 H), 1.78 (m, 2 H), 2.34 (s, 3 H), 4.23 (t, $J = 6.6$ Hz, 2 H), 6.12 (s, 2 H), 7.33 (m, 5 H); HRMS-ESI: *m/z* calcd 298.08722 $[M + Na]^+$, found 298.08736; IR (KBr): 1235, 1272, 1479, 1551, 1576, 1666, 2964, 3317, 3427 cm^{-1}.

4.1.1.15. tert-Butyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate ***9i***. The crude product was purified by column chromatography (CH_2Cl_2); yield 20%; mp 101-103 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.59 (s, 9 H), 2.31 (s, 3 H), 6.06 (bs, 2 H), 7.37 (m, 5 H); HRMS-ESI: *m/z* calcd 312.10287 $[M + Na]^+$, found 312.10308; IR (KBr): 1169, 1251, 1453, 1577, 1664, 2928, 2975, 3328, 3441 cm^{-1}.

4.1.1.16. 2-Amino-N-ethyl-4-methyl-5-phenylthiophene-3-carboxamide ***9j***. The product was purified by column chromatography (1,4-dioxane/toluene 1:2); yield 20%; mp 65 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.24 (t, $J = 7.2$ Hz, 3 H), 2.34 (s, 3 H), 3.47 (m, 2 H), 5.77 (bs, 1 H), 5.97 (s, 2 H), 7.35 (m, 5 H); ^{13}C-NMR ($CDCl_3$): δ (ppm) 15.2, 16.3, 34.4, 111.8, 127.1, 127.5, 128.6, 129.8, 130.4, 134.3, 159.4, 166.7; HRMS-ESI: *m/z* calcd 283.08755 $[M + Na]^+$, found 283.08795; IR (KBr): 1452, 1675, 2972, 3289 cm^{-1}.

4.1.1.17. 2-Amino-4-methyl-5-phenylthiophene-3-carbonitrile ***9k***. The crude product was purified by column chromatography (EtOAc/*n*-hexane, 1:9); yield 10%; mp 137 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 2.27 (s, 3 H), 4.75 (s, 2 H), 7.35 (m, 5 H); HRMS-ESI: *m/z* calcd 237.04569 $[M + Na]^+$, found 237.04591; IR (KBr): 1517, 1654, 2198, 2922, 3336, 3426 cm^{-1}.

4.1.1.18. (2-Amino-4-methyl-5-phenylthiophen-3-yl)(phenyl)methanone ***9l***. The crude product was purified by column chromatography (CH_2Cl_2); yield 22%; mp 80 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.72 (s, 3 H), 6.53 (s, 2 H), 7.45 (m, 10 H); LRMS-EI: *m/z* calcd 293.1, found 292.9; IR (KBr): 1178, 1268, 1447, 1567, 1654, 2923, 3282, 3421 cm^{-1}.

4.1.2. Synthesis of 2-acylamino-thiophene-3-carboxylic adic derivatives

4.1.2.1. Method A Acylation via carboxylic acid chlorides. To a stirred mixture of the corresponding 2-aminothiophene-3-carboxylic acid derivative (0.50 g) and K_2CO_3 (10 eq.) in toluene (10 ml) the corresponding carboxylic acid chloride (1.2–10 eq) was added at 0 °C. After 3 h at r.t. the inorganic solid were filtered off, solvent was removed by filtration and the residue was recrystallized from 1,4-dioxane.

4.1.2.2. Ethyl 3-carbamoyl-4-methyl-5-phenylthiophen-2-ylcarbamate ***2a***. Originated from **1a** 24 and ethyl chloroformate (0.25 ml, 2.63 mmol); yield 23%; mp 194–195 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.33 (t, $J = 7.2$ Hz, 3 H), 2.42 (s, 3 H), 4.28 (q, $J = 7.2$ Hz, 2 H), 5.82 (bs, 2 H), 7.37 (m, 5 H), 11.20 (s, 1 H); LRMS-EI: *m/z* calcd 304.1, found 304.1; IR (KBr): 1045, 1078, 1117, 1205, 1331, 1546, 1584, 1520, 1635, 1727, 2990, 3329 cm^{-1}.

4.1.2.3. Ethyl 3-carbamoyl-4,5,6,7-tetrahydrobenzo[b]thiophen-2-ylcarbamate ***2b***. Originated from **1b** 25 and ethyl chloroformate (0.25 ml, 2.63 mmol); yield 31%; mp 209–210 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.31 (t, $J = 7.2$ Hz, 3 H), 1.84 (m, 2 H), 2.68 (m, 2 H), 4.25 (q, $J = 7.2$ Hz, 2 H), 5.64 (bs, 2 H), 11.19 (s, 1 H); LRMS-EI: *m/z* calcd 268.1, found 268.1; IR (KBr): 1209, 1534, 1587, 1629, 1725, 2990, 3180, 3380 cm^{-1}.

4.1.2.4. Ethyl 4-methyl-5-phenylthiophen-2-ylcarbamate ***13***. Originated from **12** and ethyl chloroformate, acetonitrile was used instead of toluene, reaction time was 8 h, the solvent was removed under reduced pressure, the solid (mixture of **13** and **14**) was solved in $CHCl_3$ and washed with NaOH (0.5 M). The phases were separated, solvent was removed and the residue was solved in DMF. Water was added to precipitate the decarboxylated compound **13** (8%), which was recrystallized from ethanol; mp 132–134 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.32 (t, $J = 7.2$ Hz, 3 H), 2.25 (s, 3 H), 4.26 (q, $J = 7.2$ Hz, 2 H), 6.45 (s, 1 H), 6.96 (s, 1 H), 7.35 (m, 5 H); LRMS-EI: *m/z* calcd 261.0, found 261.0; IR (KBr): 1264, 1311, 1586, 1690, 3432 cm^{-1}.

4.1.2.5. 2-(Ethoxycarbonylamino)-4-methyl-5-phenylthiophene-3-carboxylic acid ***14***. Originated from **12** and ethyl chloroformate, acetonitrile was used instead of toluene, reaction time was 8 h; yield 40% containing 5% decarboxylated compound **13**; mp 174–178 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.27 (t, $J = 7.2$ Hz, 3 H), 2.31 (s, 3 H), 4.23 (q, $J = 7.2$ Hz, 2 H), 7.43 (m, 5 H), 10.73 (s, 1 H); HRMS-ESI: *m/z* calcd 328.06140 $[M + Na]^+$, found 328.06143; IR (KBr): 1206, 1253, 1410, 1460, 1530, 1690, 1734, 3200, 3400, 3298 cm^{-1}.

4.1.2.6. Ethyl 2-benzamido-4-methyl-5-phenylthiophene-3-carboxylate ***15c***. Originated from **1c** and benzoyl chloride (0.5 ml, 4.3 mmol); yield 70%; mp 143 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.45 (t, $J = 7.2$ Hz, 3 H), 2.42 (s, 3 H), 4.44 (q, $J = 7.2$ Hz, 2 H), 7.47 (m, 8 H), 8.04 (m 2 H), 12.46 (s 1 H); LRMS-EI: *m/z* calcd 365.1, found 365.0; IR (KBr): 1222, 1268, 1298, 1530, 1559, 1599, 1654, 1670, 2928, 2977, 3440 cm^{-1}.

4.1.2.7. Ethyl 2-(2-ethoxy-2-oxoacetamido)-4-methyl-5-phenylthiophene-3-carboxylate ***15d***. Originated from **1c** and ethyl 2-chloro-2-oxoacetate, benzene was used instead of toluene; yield 70%; mp 123–124 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.45 (m, 6 H), 2.41 (s, 3 H), 4.45 (m, 4 H), 7.41 (m, 5 H), 12.55 (s, 1 H); LRMS-EI: *m/z* calcd 361.1, found 361.1; IR (KBr): 1237, 1282, 1381, 1444, 1526, 1556, 1673, 1702, 1734, 2936, 2978, 3440 cm^{-1}.

4.1.2.8. ethyl 2-(2-bromoacetamido)-4-methyl-5-phenylthiophene-3-carboxylate ***15f***. originated from **1c** and 2-bromoacetyl bromide (2.00 ml, 23 mmol), benzene was used instead of toluene; yield 71%; mp 53–54 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.34 (t, $J = 6.9$ Hz, 3 H), 2.32 (s, 3 H), 4.35 (q, $J = 6.9$ Hz, 2 H), 4.42 (s, 2 H), 7.42 (m, 5 H), 11.57 (s, 1 H); LRMS-EI: *m/z* calcd 381.0, found 383.0; IR (KBr): 1161, 1198, 1252, 1375, 1441, 1531, 1552, 1626, 1670, 1747, 2986, 3212, 3444 cm^{-1}.

4.1.2.9. Method B Acylation via carboxylic acid anhydrides. The corresponding carboxylic acid anhydride (50 mmol) and the corresponding 2-amino-3-carboxylic acid derivative (2 mmol) were heated under reflux for 2.5 h. The solvent was removed under reduced pressure and the residues were dried and recrystallized from ethanol.

4.1.2.10. Ethyl 2-acetamido-4-methyl-5-phenylthiophene-3-carboxylate ***15a***. Originated from **1c** and acetic anhydride; yield 71%; mp 121–126 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.42 (t, $J = 7.2$ Hz, 3 H), 2.29 (s, 3 H), 2.38 (s, 3 H), 4.38 (q, $J = 7.2$ Hz, 2 H), 7.41 (m, 5 H), 11.41 (s, 1 H); ^{13}C-NMR ($CDCl_3$): δ (ppm) 14.6, 15.9, 24.0, 61.0, 113.2, 127.7, 128.8, 129.3, 129.7, 130.3, 134.1, 149.3, 167.1, 167.5; LRMS-EI: *m/z* calcd 303.1, found 303.1; IR (KBr): 1206, 1248, 1306, 1404, 1439, 1523, 1554, 1598, 1657, 1688, 2939, 2988, 3240 cm^{-1}.

4.1.2.11. Ethyl 4-methyl-5-phenyl-2-(2,2,2-trifluoroacetamido)thiophene-3-carboxylate ***15b***. Originated from **1c** and trifluoroacetic anhydride; yield 97%; mp 88–92 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.44 (t, $J = 7.2$ Hz, 3 H), 2.40 (s, 3 H), 4.44 (q, $J = 7.2$ Hz, 2 H), 7.41 (m, 5 H), 12.44 (s, 1 H); ^{19}F-NMR ($CDCl_3$): δ (ppm) 2.36; LRMS-EI: *m/z* calcd 357.2, found 357.2; IR (KBr): 1240, 1322, 1525, 1565, 1668, 1719, 2934, 2996, 3426 cm^{-1}.

4.1.3. Synthesis of ethyl 4-methyl-2-(methylamino)-5-phenylthiophene-3-carboxylate ***15g***

A mixture of **1c** (0.15 g, 0.60 mmol), iodomethane (0.36 ml, 6 mmol) and triethylamine (0.84 ml, 6 mmol) in acetonitrile (3 ml)

were converted in a high pressure reactor (20 bar) at 105 °C for 30 h. The solvent was removed under reduced pressure and the crude product was recrystallized from ethanol to give **15g** 139 (0.10 g, 0.36 mmol, 61%). mp 140 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.38 (t, $J = 7.2$ Hz, 3 H), 2.34 (s, 3 H), 3.01 (d, $J = 4.7$ Hz, 3 H), 4.29 (q, $J = 7.2$ Hz, 2 H), 7.32 (m, 5 H), 7.79 (s, 1 H); LRMS-EI: *m/z* calcd 275.1, found 275.0; IR (KBr): 1082, 1225, 1528, 1596, 1647, 2978, 3307 cm^{-1}.

4.1.4. Synthesis of 2-(3-ethylureido)-thiophene-carboxylic acid derivatives

4.1.4.1. General procedure. To a solution of the corresponding 2-aminothiophene (0.80 mmol) in dry CH_2Cl_2 (5 ml) *N*-ethyl-isocyanate (1.00 ml, 12.8 mmol) was added and the mixture was refluxed for 5 days. After cooling down to r.t. 50% aqueous ethanol was added and the precipitate was filtered off and recrystallized from ethanol.

*4.1.4.2. 2-(3-Ethylureido)-4-methyl-5-phenylthiophene-3-carboxamide **2c**.* Originated from **1a**; yield 83%; mp 223–225 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.05 (t, $J = 7.2$ Hz, 3 H), 2.29 (s, 3 H), 3.11 (m, 2 H), 7.38 (m, 7 H), 7.59 (s, 1 H), 10.17 (s, 1 H); LRMS-EI: *m/z* calcd 303.1, found 303.0; IR (KBr): 1154, 1247, 1294, 1523, 1557, 1594, 1645, 1664, 2931, 2974, 3100, 3399 cm^{-1}.

*4.1.4.3. 2-(3-Ethylureido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide **2d**.* Originated from **1b**; yield 92%; mp 194–198 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.03 (t, $J = 7.2$ Hz, 3 H), 1.70 (m, 4 H), 2.52 (m, 4 H), 3.08 (m, 2 H), 6.97 (bs, 2 H), 7.53 (s, 1 H), 10.48 (s, 1 H); LRMS-EI: *m/z* calcd 267.1, found 267.0; IR (KBr): 1228, 1278, 1298, 1524, 1559, 1592, 1637, 1677, 2856, 2974, 3088, 3473 cm^{-1}.

*4.1.4.4. Ethyl 2-(3-ethylureido)-4-methyl-5-phenylthiophene-3-carboxylate **15e**.* Originated from **1c**; yield 75%; mp 138–140 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.07 (t, $J = 7.2$ Hz, 3 H), 1.33 (t, $J = 7.2$ Hz, 3 H), 2.29 (s, 3 H), 3.13 (m, 2 H), 4.31 (q, $J = 7.2$ Hz, 2 H), 7.39 (m, 5 H), 7.91 (s, 1 H), 10.36 (s, 1 H); LRMS-EI: *m/z* calcd 332.1, found 332.0; IR (KBr): 1034, 1337, 1534, 1557, 1648, 1664, 2931, 2974, 3286 cm^{-1}.

*4.1.4.5. Ethyl 2-(3-ethylureido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate **15h**.* Originated from **1d**; yield 84%; mp 165–169 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.06 (t, $J = 6.9$ Hz, 3 H), 1.29 (t, $J = 6.9$ Hz, 3 H), 1.70 (m, 4 H), 2.51 (m, 2 H), 2.67 (m, 2 H), 3.11 (m, 2 H), 4.25 (q, $J = 6.9$ Hz, 2 H), 7.80 (s, 1 H), 10.24 (s, 1 H); LRMS-EI: *m/z* calcd 296.1, found 296.0; IR (KBr): 1040, 1229, 1544, 1565, 1654, 1684, 2931, 2975, 3230, 3283 cm^{-1}.

*4.1.5. Synthesis of 2-amino-4-methyl-5-phenylthiophene-3-carboxylic acid **12***

A solution of **1c** (67.7 g, 0.26 mol) in ethanol (650 ml) and aqueous NaOH (10.0 M, 260 ml) was refluxed for 2.5 h. The solvent was removed under reduced pressure, the precipitate of sodium salt was filtered off and solved in water. The free acid was precipitated by the addition of HCl (3 M), filtered off, washed with cold water and dried to give **12** (50.4 g, 0.22 mol, 83%); mp 153 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 2.24 (s, 3 H), 7.34 (m, 5 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 16.0, 105.6, 116.8, 126.6, 128.7, 128.0, 131.0, 134.2, 163.9, 167.0; HRMS-ESI: *m/z* calcd 234.05833 $[M + H]^+$, found 234.05866; IR (KBr): 1260, 1375, 1451, 1470, 1579, 1647, 2924, 3380, 3470 cm^{-1}.

*4.1.6. Synthesis of ethyl 2-isothiocyanato-4-methyl-5-phenylthiophene-3-carboxylate **17***

To a mixture of **1c** (1.00 g, 3.83 mmol) and K_2CO_3 (0.97 g, 7.00 mmol) in CH_2Cl_2 (5 ml) thiophosgene in CH_2Cl_2 (0.80 ml, 10.4 mmol) was added at 0 °C. After 1 h at r.t. the inorganic solid was removed by filtration, solvent was removed under reduced pressure and the residue was washed with *n*-hexane to give **17** (0.52 g, 1.71 mmol, 45%); mp 58-60 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.44 (t, $J = 7.2$ Hz, 3 H), 2.37 (s, 3 H), 4.40 (q, $J = 7.2$ Hz, 2 H), 7.40 (m, 5 H); ^{13}C-NMR ($CDCl_3$): δ (ppm) 14.6, 15.2, 61.2, 128.4, 128.5, 128.9, 129.9, 132.7, 133.2, 133.8, 162.5; LRMS-EI: *m/z* calcd 303.0, found 303.0; IR (KBr): 1196, 1302, 1456, 1541, 1707, 2102, 2979 cm^{-1}.

*4.1.7. Synthesis of ethyl 2-(3-ethylthioureido)-4-methyl-5-phenylthiophene-3-carboxylate **18***

To a solution of **17** (0.50 g, 1.65 mmol) in CH_2Cl_2 (4.5 ml) ethylamine (0.2 ml, 3.06 mmol) was added dropwise. After 1 h at r.t. the reaction mixture was acidified using HCl (1 M in ethanol). The precipitate was filtered off and recrystallized from ethanol to give **18** (0.40 g, 1.15 mmol, 70%); mp 178–180 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.15 (t, $J = 6.6$ Hz, 3 H), 1.34 (t, $J = 7.5$ Hz, 3 H), 2.31 (s, 3 H), 3.44 (m, 2 H), 4.35 (q, $J = 7.5$ Hz, 2 H), 7.41 (m, 5 H), 9.52 (s, 1 H), 11.58 (s, 1 H); LRMS-EI: *m/z* calcd 348.1, found 348.0; IR (KBr): 1067, 1227, 1442, 1550, 1578, 1654, 2926, 2973, 3228 cm^{-1}.

*4.1.8. Synthesis of ethyl 4-methyl-5-phenyl-2-(1H-pyrrol-1-yl)thiophene-3-carboxylate **19***

A solution of **1c** (0.50 g, 1.92 mmol) and 2,5-dimethoxytetrahydrofuran (0.32 ml, 2.5 mmol) in acetic acid (5 ml) was refluxed for 20 min. The reaction mixture was poured into crushed ice, made basic and extracted with CH_2Cl_2. The organic phase was washed several times with water, died (Na_2SO_4) and evaporated. Column chromatography (CH_2Cl_2/acetone 9.5:0.5) of the residue gave **19** (0.51 g, 1.64 mmol, 85%) as an oil; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.16 (t, $J = 7.2$ Hz, 3 H), 2.37 (s, 3 H), 4.20 (q, $J = 7.2$ Hz, 2 H), 6.30 (m, 2 H), 6.78 (m, 2 H), 7.42 (m, 5 H); LRMS-EI: *m/z* calcd 311.1, found 311.0; IR ($CHCl_3$): 1078, 1218, 1328, 1379, 1405, 1464, 1514, 1560, 1716, 2927, 2980 cm^{-1}.

*4.1.9. Conversion of **15f** with amines*

4.1.9.1. General procedure. To a solution of **15f** in acetonitrile (3.5 ml) the corresponding amine (10 eq.) was added dropwise at 0 °C. The precipitate was filtered off after 1 h, dried and recrystallized from ethanol.

*4.1.9.2. Ethyl 4-methyl-2-(2-morpholinoacetamido)-5-phenylthiophene-3-carboxylate **16a**.* Morpholine was used; yield 31%; mp 142 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.35 (t, $J = 7.2$ Hz, 3 H), 2.33 (s, 3 H), 2.55 (s, 4 H), 3.24 (s, 2 H), 3.65 (s, 4 H), 4.37 (q, $J = 7.2$ Hz, 2 H), 7.43 (m, 5 H), 12.21 (s, 1 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 14.9, 16.1, 54.0, 61.3, 61.3, 66.9, 114.0, 128.4, 128.6, 129.5, 130.0, 130.2, 134.0, 147.7, 165.7, 169.1; LRMS-EI: *m/z* calcd 388.1, found 388.2; IR (KBr): 1112, 1244, 1273, 1523, 1552, 1676, 2822, 2964, 3440 cm^{-1}.

*4.1.9.3. Ethyl 2-(2-(benzylamino)acetamido)-4-methyl-5-phenylthiophene-3-carboxylate **16b**.* Benzylamine was used; yield 50%; mp 122–123 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.42 (t, $J = 7.2$ Hz, 3 H), 2.14 (bs, 1 H), 2.41 (s, 3 H), 3.56 (s, 2 H), 3.88 (s, 2 H), 4.42 (q, $J = 7.2$ Hz, 2 H), 7.36 (m, 10 H), 12.43 (s, 1 H); LRMS-EI: *m/z* calcd 408.2, found 408.0; IR (KBr): 1174, 1242, 1305, 1517, 1551, 1668, 2927, 2985, 3435 cm^{-1}.

*4.1.9.4. Ethyl 2-(2-(butylamino)acetamido)-4-methyl-5-phenylthiophene-3-carboxylate **16c**.* Butylamine was used; yield 38%; mp 97-99 °C; ^{1}H-NMR ($CDCl_3$): δ (ppm) 0.93 (t, $J = 7.2$ Hz, 3 H), 1.48 (m, 7 H), 2.40 (s, 3 H), 2.69 (t, $J = 7.5$ Hz, 2 H), 3.53 (s, 2 H), 4.41 (q, $J = 7.2$ Hz, 2 H), 7.37 (m, 5 H), 12.33 (s, 1 H); LRMS-EI: *m/z* calcd 374.0, found 374.0; IR (KBr): 1185, 1239, 1307, 1423, 1441, 1512, 1548, 1670, 2860, 2926, 2956, 3446 cm^{-1}.

4.1.10. Diazotation of ***1c***

4.1.10.1. General procedure. To a mixture of **1c** (0.5 g, 1.92 mmol), water (7 ml) and conc. HCl (3 ml) $NaNO_2$-solution (0.20 g in 1 ml water, 2.68 mmol) was added at 0 °C during 20 min. After 30 more min in an ice bath the mixture was filtered and the diazonium-salt-solution was added dropwise to a 0 °C cold solution of the corresponding nucleophile. The reaction mixture was stirred for 30 min, extracted with Et_2O (3 × 10 ml), dried (Na_2SO_4) and evaporated. Crude products were purified by column chromatography (Et_2O).

4.1.10.2. Ethyl 2-(3,3-dimethyltriaz-1-enyl)-4-methyl-5-phenylthiophene-3-carboxylate ***20a***. Dimethylamin (40% aqueous solution, 4 ml) was used as nucleophile; yield 52%; mp 88-92 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.29 (t, J = 7.2 Hz, 3 H), 2.23 (s, 3 H), 3.19 (s, 3 H), 3.49 (s, 3 H), 4.26 (q, J = 7.2 Hz, 2 H), 7.40 (m, 5 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 14.9, 15.0, 37.4, 43.8, 60.8, 123.9, 128.3, 129.5, 129.7, 130.4, 131.9, 134.5, 158.1, 165.0; LRMS-ESI: *m/z* calcd 340.1 $[M + Na]^+$, found 340.1; IR (KBr): 1057, 1100, 1227, 1372, 1443, 1474, 1596, 1697, 2850, 2919 cm^{-1}.

4.1.10.3. Ethyl 4-methyl-2-(morpholinodiazenyl)-5-phenylthiophene-3-carboxylate ***20b***. Morpholine (10 ml) was used as nucleophile; yield 83%; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.38 (t, J = 7.2 Hz, 3 H), 2.33 (s, 3 H), 3.84 (m, 8 H), 4.38 (q, J = 7.2 Hz, 2 H), 7.42 (m, 5 H); HRMS-ESI: *m/z* calcd 382.11958 $[M + Na]^+$, found 382.11997; IR (KBr): 1109, 1171, 1221, 1306, 1349, 1377, 1422, 1598, 1706, 2859, 2976 cm^{-1}.

4.1.10.4. Ethyl 2-(cyanodiazenyl)-4-methyl-5-phenylthiophene-3-carboxylate ***20c***. Sodium cyanide (0.94 g, 19.2 mmol) in water (5 ml) was used as nucleophile; yield 87%, mp 60-62 °C; %; ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.42 (t, J = 7.2 Hz, 3 H), 2.41 (s, 3 H), 4.48 (q, J = 7.2 Hz, 2 H), 7.51 (m, 5 H); ^{13}C-NMR ($CDCl_3$): δ (ppm) 14.1, 14.4, 62.8, 116.4, 129.3, 129.4, 130.6, 132.2, 137.1, 145.9, 153.3, 155.0, 163.3; HRMS-ESI: *m/z* calcd 322.06207 $[M + Na]^+$, found 322.06207; IR (KBr): 1069, 1221, 1278, 1328, 1380, 1447, 1654, 1718, 2176, 2988 cm^{-1}.

4.1.10.5. Ethyl 2-chloro-4-methyl-5-phenylthiophene-3-carboxylate ***21***. CuCl-solution (0.80 g, 8.08 mmol) in conc. HCl (20 ml) was used as nucleophile; yield 92%; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.30 (t, J = 7.2 Hz, 3 H), 2.39 (s, 3 H), 4.27 (q, J = 7.2 Hz, 2 H), 7.46 (m, 5 H); LRMS-EI: *m/z* calcd 280.0, found 282.1; IR ($CHCl_3$): 1065, 1222, 1297, 1306, 1378, 1450, 1600, 1716, 2871, 2958 cm^{-1}.

4.1.11. Synthesis of 2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-ones ***3a,b***

4.1.11.1. General procedure. To a mixture of 2-aminothiophene-3-carboxamide **1a,b** (3.80 mmol), water (0.50 ml) and K_2CO_3 (0.97 g, 7 mmol) in CH_2Cl_2 (5 ml) was added thiophosgene (0.80 ml, 10.4 mmol) in CH_2Cl_2 (5 ml) at 0 °C. After 1.5 h the precipitate was filtered off, washed with cold CH_2Cl_2 and dried.

4.1.11.2. 5-Methyl-6-phenyl-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one ***3a***. Originated from **1a**; yield 93%; mp 296-300 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 2.44 (s, 3 H), 7.39 (m, 5 H), 12.38 (s, 1 H), 13.47 (s, 1 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 14.7, 118.6, 128.9, 129.7, 129.8, 130.1, 132.8, 151.7, 158.2, 173.8; LRMS-EI: *m/z* calcd 274.0, found 274.0.

4.1.11.3. 5,6,7,8-Tetrahydrobenzo[b]-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one ***3b***. Originated from **1b**; yield 82%; mp 287-290 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.73 (m, 4 H), 2.64 (m, 2 H), 2.74 (m, 2 H), 12.27 (s, 1 H), 13.30 (s, 1 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 21.5, 22.5, 23.9, 24.9, 116.4, 128.2, 130.8, 150.0, 157.0, 173.8; LRMS-EI: *m/z* calcd 238.0, found 238.0.

4.1.12. Synthesis of 2-(Ethylthio)-thieno[2,3-d]pyrimidin-4(3H)-ones ***4a,b***

4.1.12.1. General procedure. 2-Thioxo-2,3-dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-one **3a,b** (1.10 mmol) was solved in a mixture of ethanol (5 ml) and sodium methanolate solution (1 M, 10 ml). Iodoethane (3.30 mmol) was added and the mixture was stirred for 30 min. The solvent was evaporated, the precipitate was filtered off, washed with water and recrystallized from ethanol.

4.1.12.2. 2-(Ethylthio)-5-methyl-6-phenylthieno[2,3-d]pyrimidin-4(3H)-one ***4a***. Originated from **3a**; yield 82%; mp > 312 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.26 (t, J = 7.5 Hz, 3 H), 2.57 (s, 3 H), 2.97 (q, J = 7.5 Hz, 2 H), 7.36 (m, 5 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 15.2, 15.3, 23.8, 120.0, 124.0, 126.6, 128.6, 128.9, 129.4, 134.9, 165.0, 169.0; LRMS-EI: *m/z* calcd 302.1, found 301.9; IR (KBr): 1028, 1055, 1362, 1550, 1600, 1654, 2868, 2968, 3406 cm^{-1}.

4.1.12.3. 2-(Ethylthio)- 5,6,7,8-tetrahydrobenzo[b]-thieno[2,3-d]pyrimidin-4(3H)-one ***4b***. Originated from **3b**; yield 82%; mp 297-298 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.26 (t, J = 6.9 Hz, 3 H), 1.74 (m, 4 H), 2.65 (m, 2 H), 2.81 (m, 2 H), 3.03 (q, J = 6.9 Hz, 2 H), 12.45 (bs, 1 H); ^{13}C-NMR (DMSO-d_6): δ (ppm) 15.0, 22.2, 22.9, 23.9, 24.5, 25.8, 118.5, 125.3, 130.4, 161.3, 163.9, 164.9; LRMS-EI: *m/z* calcd 266.1, found 265.9; IR (KBr): 1028, 1233, 1387, 1489, 1540, 1654, 2856, 2930, 3432 cm^{-1}.

4.1.13. Synthesis of 3-Ethylthieno[2,3-d]pyrimidine-2,4(1H,3H)-diones ***5a,b***

4.1.13.1. General procedure. Ethyl 2-(3-ethylureido)-thiophene-3-carboxylates **15e,h** (0.30 mmol) and NaOH (4 ml, 10 M in ethanol) were refluxed for 1.5 h and acidified with HCl (1 M). The precipitate was filtered off and recrystallized from ethanol.

4.1.13.2. 3-Ethyl-5-methyl-6-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione ***5a***. Originated from **15e**; yield 74%; mp 280-283 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.23 (t, J = 6.9 Hz, 3 H), 2.44 (s, 3 H), 3.89 (q, J = 6.9 Hz, 2 H), 7.43 (m, 5 H), 12.22 (s, 1 H); LRMS-EI: *m/z* calcd 286.1, found 286.0; IR (KBr): 1041, 1204, 1525, 1567, 1601, 1644, 1706, 2868, 2976 cm^{-1}.

4.1.13.3. 3-Ethyl-5,6,7,8-tetrahydrobenzo[b]-thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione ***5b***. Originated from **15h**; yield 72%; mp 260-364 °C; ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.09 (t, J = 7.2 Hz, 3 H), 1.73 (m, 4 H), 2.61 (m, 2 H), 2.76 (m, 2 H), 3.84 (q, J = 7.2 Hz, 2 H), 12.02 (s, 1 H); LRMS-EI: *m/z* calcd 250.1, found 250.0; IR (KBr): 1028, 1060, 1207, 1532, 1579, 1634, 1701, 2856, 2981 cm^{-1}.

4.2. Biology

The kainate-receptor assay was performed by C. Kronbach, Biotie Therapies GmbH, Radebeul [10].

Human embryonic kidney cells (HEK 293-cells) which stably express the GluR5 or GluR6 receptor, respectively, together with the luminescent protein aequorin were used as screening assay for kainate receptor antagonists. GluR5- (or GluR6-) and aequorin-expressing HEK 293-cells were cultivated in a MEM growing medium together with Earle's salts and Glutamax-I (Life Technologies), containing 10% FKS, 1% non-essential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 600 μg/ml G 418 (Calbiochem) and 500 nM ouabaine.

One day before the measurement 60.000 cells per cavity were seeded into white, non-transparent 96-well microtiterplates (Costar). On the test day cells were incubated with 5 μM coelenterazine for 1 h at 37 °C. Then the medium was poured off and

replaced by 80 μl assay buffer and 10 μl test compound was added. The assay-buffer contained 150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 10 mM glucose, 0.3 mg/ml concanavaline A (ConA) and 100 mM (GluR6) $CaCl_2$ (pH = 7.3). Afterwards the cells were incubated for 10 min at room temperature.

The luminescence measurement was performed in a luminometer (LUMIstar, BMG) equipped with two computer-controlled injectors, over a period of 26 s per cavity (13 intervals of 2 s). After the first second 10 μl of a 2.75 mM glutamate-solution in assay-buffer was injected (in order to have glutamate concentration of 275 μM per cavity, according to the EC_{50} value of glutamate for GluR6) or 10 μl of 0.8 mM glutamate (due to 80 μM = EC_{50} for GluR5). A second injection of 100 μl Triton in assay-buffer (without Ca^{2+}) was carried out after 20 seconds into the same cavity.

The calculation of specific channel activity induced by an agonist was determined as fractional luminescence, which was calculated from respective sum of signals of agonist- and triton-induced luminescence.

For determination of IC_{50} values Hill-plot (4-parameter model) was used.

The MTT-assay was performed analog to an already described method [31]. This test was used to determine the viability of cell. As a substrate the pale yellow tetrazoniumsalt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which can be converted to dark blue formazan by the mitochondrional dehydrogenases of living cells. After desintegration of cell membranes formazan is released using special lysis buffer. The intensity of solution is measured spectrophotometrical and correlates with the number of living cells.

The absorption of samples was measured at 590 nm. Two different cell lines were used, neuroblastoma cells (N2A) and monkey kidney cells (COS7). The investigated compounds were tested in concentrations of 0.1 μM, 1 μM, 10 μM and 100 μM.

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