Thieno[2,3-*d*]pyrimidines and -[1,3]oxazines as Glutamate Antagonists and Investigations on the Inhibitory Potency toward Human Leukocyte Elastase

Detlef Briel,[a] Anastasiya Rybak,[a] Christiane Kronbach,[b] Klaus Unverferth,[b] Camino M. González Tanarro,[c] and Michael Gütschow[c]*

[a]Pharmaceutical Chemistry, Institute of Pharmacy, University of Leipzig, Brüderstraße 34, D-04103 Leipzig, Germany
[b]Biotie Therapies GmbH, Meißner Straße 35, D-01445 Radebeul, Germany
[c]Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany
*E-mail: guetschow@uni-bonn.de
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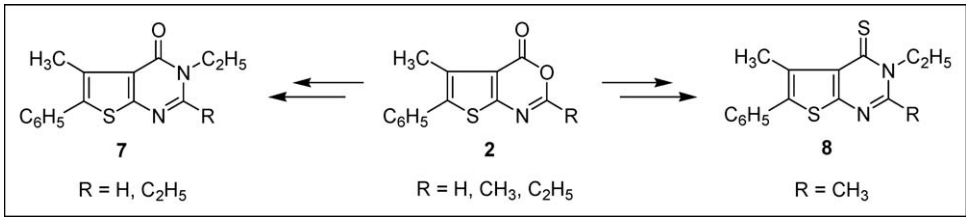

A series of fused thiophene derivatives, that is, representatives of thieno[2,3-*d*]pyrimidines, thieno[2,3-*d*][1,3]oxazines and thieno[2,3-*d*][1,3]thiazines, with the common 5-methyl-6-phenyl substitution pattern was synthesized. The target compounds, *e.g.*, **7** or **8**, were designed as cyclic analogs of ethyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate, an antagonist at the GluR6 kainate receptor. Thieno[2,3-*d*][1,3]oxazin-4-one **2** (R = C_2H_5) was identified as new a potent inhibitor (IC_{50} = 17 μ*M*) of this receptor subtype. The inhibitory potency of **2** (R = C_2H_5) against human leukocyte elastase was also examined. The compound was characterized as a noncovalent inhibitor with an IC_{50} value of 8.8 μ*M*.

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INTRODUCTION

Even well-established anticonvulsants, such as carbamazepine, valproic acid, phenytoin, or benzodiazepines can cause undesired side effects. Moreover, certain forms of epilepsy, that is, focal seizures, cannot be treated sufficiently with such drugs. This provided the impetus behind the development of new drugs to improve the prospects for mono- and combined therapy. In the course of the continuing search for new anticonvulsants, substituted quinazolines and the bioisosteric thieno[2,3-*d*]pyrimidines have been reported to be active [1,2]. Kainate glutamate receptors may represent an interesting new target for the development of innovative anticonvulsants [3,4]. The kainate receptor subtype GluR6, expressed in the excitatory pyramid cells of the hippocampus, appears to be particularly significant. The GluR6 and GlyR5 subtypes might play opposing roles during the hippocampal excitation [3,5].

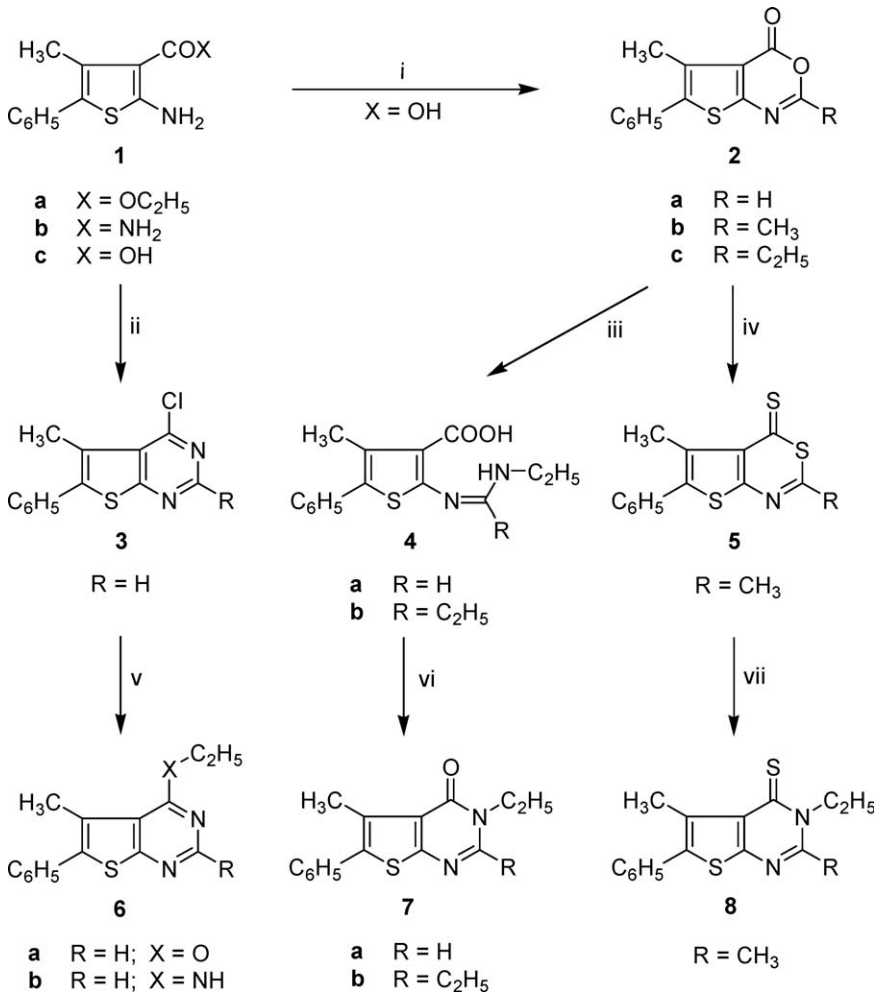
The know antagonists of the GluR6 kainate receptor comprise two groups, compounds which contain glutamate or its isosterically modified fragments on the one hand, and compounds with a structure not related to glutamate on the other hand. Diarylureas and quinoxalinediones are predominant examples of the latter group [6–10].

Substituted alkyl thiophene-3-carboxylates have been identified as a new class of selective GluR6 antagonists [11]. As a result of a screening of several thiophene esters, 2-amino-4-methyl-5-phenylthiophene derivatives proved to be particularly active. The ethyl ester **1a** (IC_{50} = 0.75 μ*M*) exceeded other esters, e.g., methyl, propyl, with respect to selectivity for the GluR6 kainate receptor [11]. On the basis of these findings, we envisaged cyclic analogs of **1a** and focused on the synthesis of thienopyrimidines (Scheme 1). Selected compounds were also evaluated as inhibitors of human leukocyte elastase (HLE), a serine protease of the chymotrypsin family. Under normal conditions, the activity of HLE is regulated by endogenous inhibitors, but uncontrolled activity of HLE may result in several pathological states, including emphysema, chronic obstructive pulmonary disease, cystic fibrosis and rheumatoid arthritis. HLE inhibitors are therefore of relevance for the therapy of such afflictions [12–15].

RESULTS AND DISCUSSION

The synthetic routes to bicyclic thiophenes with the common 5-methyl-6-phenyl substitution pattern are outlined in Scheme 1. Using known methods, the fused pyrimidine ring was formed in the reaction of the thiophene-3-carboxamide

Scheme 1. Conditions: i) $HC(OC_2H_5)_3$, 12 h, reflux, or $(RCO)_2O$, 1.5 h, reflux; ii) 1. $HC(OC_2H_5)_3$, 12 h, reflux, 2. $POCl_3$, 5 h, reflux; iii) $NH_2CH_2CH_3$ (70% aqueous solution); iv) P_4S_{10} / toluene, 3 h, reflux; v) $NaOCH_2CH_3$ / CH_2CH_3OH, 12 h, or $NH_2CH_2CH_3$ (70% aqueous solution) / CH_2Cl_2; vi) $SOCl_2$, $CHCl_3$, 30 min, reflux; vii) $NH_2CH_2CH_3$ (70% aqueous solution).



1b [16] with ethyl orthoformate. Subsequent treatment with phosphorous oxychloride afforded the 4-chlorothienopyrimidine **3**. The conversion of **3** with sodium ethoxide gave **6a**, in which the carboxylic ester moiety of **1a** is replaced by a semicyclic ethyl imidate substructure. The corresponding replacement by an ethyl amidine substructure in **6b** was accomplished when **3** was treated with ethylamine. The reactions of the thiophene-3-carboxylic acid **1c** [17] with carboxylic anhydrides or carboxylic acid ortho esters provided an access to thieno[2,3-*d*][1,3]oxazin-4-ones **2**. Oxazinones **2** bear two electrophilic sites, C-2 and C-4. Ethylamine exclusively attacked **2** at the C-2 carbon, leading to the formation of amidino carboxylic acids **4**. Treatment of **4** with thionyl chloride furnished compounds **7a**, **b** with N-ethyl lactam structure. The thieno[2,3-*d*][1,3]thiazine-4-thione **5**, accessible by thionation of the corresponding oxazinone **2b** with diphosphorous pentasulfide [17], was reacted with ethylamine to give the thienopyrimidine **8** with N-ethyl thiolactam structure. It should be noted that **8** was directly produced and a corresponding ring-open thiophene derivative could not be isolated. Such a compound with amidine and dithiocarboxylate moieties, formed through an attack of ethylamine at C-2 of **5**, might remain in solution or undergo different transformations, thus accounting for the low yield of **8**. In the mass spectra of the thienopyrimidines **7** and **8**, the molecular peaks appear with highest intensities. Elimination of C_2H_4 was the main fragmentation reaction leading to the detection of fragment ions with the anticipated structure **9**. Similarly, the loss the ethyl chain was the predominant fragmentation of **6**.

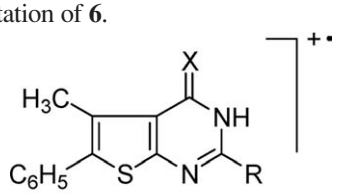


Table 1

Inhibition (% of Control) of fractional luminescence or IC_{50} value.

Compd.	GluR5	GluR6
2c	11	$IC_{50} = 17$ μ*M*
6a	9	14
6b	−7	3
7a	−6	9
7b	7	5
8	−11	0

The thienopyrimidines **6a**,**b**, **7a**,**b**, **8**, and the oxazinone **2c** were evaluated as antagonists of the kainate receptor subtypes GluR5 and GluR6 (Table 1). The experiments were performed with human embryonic kidney (HEK) cells stably expressing the GluR5 and GluR6 receptor. The antagonistic activity was determined by measuring the glutamate-mediated luminescence. The photoprotein aequorin was used as a bioluminescent reporter. The complete luminescence of the cells was determined with the detergent triton X-100 [11]. While the thienopyrimidines **6a**,**b**, **7a**,**b**, and **8**, at a concentration of 10 μ*M*, did barely influence the luminescence signal in the GluR5 and GluR6 assays, 2-ethylthieno[2,3-*d*][1,3]oxazin-4-one (**2c**) inhibited the glutamate response in the GluR6 aequorin assay with an IC_{50} value of 17 μ*M*. Thus, this compound will serve as a lead for further chemical modification to develop new anticonvulsants.

Representatives of 3,1-benzoxazin-4-ones have been reported as alternate substrate inhibitors of HLE [18–20]. It has been shown that the introduction of small alkyl groups connected *via* an O, S, or N atom to the position 2 of the heterocyclic system resulted in potent inhibition [18]. Bioisosteric thieno[1,3]oxazin-4-ones react in an analogous manner as alternate substrate inhibitors with serine proteases and esterases [21–24]. The interaction involves the nucleophilic attack of the active site serine (SerOH), formation of an acyl-enzyme and hydrolytic cleavage to release the modified ring-opened inhibitor and the free enzyme (Fig. 1).

We have selected the thieno[2,3-*d*][1,3]oxazin-4-ones **2a**–**c** and determined their inhibitory activity against HLE. Compound **2c** was identified as an HLE inhibitor ($IC_{50} = 8.8$ μ*M*, Fig. 2), whereas **2a** was not sufficiently soluble in the assay medium, and **2b** failed to inhibit HLE ($IC_{50} > 40$ μ*M*). It can be suspected, that the ethyl group in **2c** interacts with the S1 pocket of HLE, thus reflecting the primary substrate specificity for small aliphatic amino acids at P1 position of a substrate.

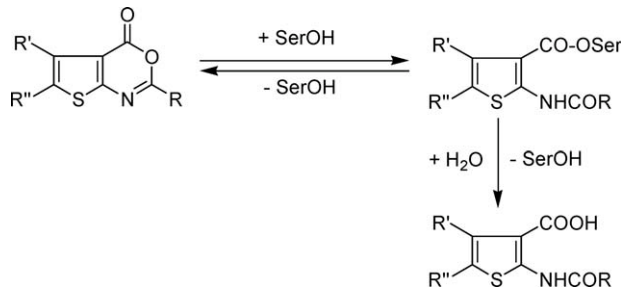


Figure 1. Enzyme-catalyzed conversion of thieno[2,3-*d*][1,3]oxazin-4-ones.

To elucidate the mechanism of elastase inhibition by **2c**, we examined a possible enzyme-catalyzed degradation of the inhibitor by means of HPLC. Compound **2c** was incubated at 25°C, pH 7.8, with HLE in a 200-fold higher concentration compared to that used in the inhibition assays. Despite the inhibition of HLE by **2c**, an accelerated hydrolysis in the presence of such a high amount of enzyme could be expected [24]. However, the decrease in concentration of **2c** was weak (<20% within 4 h) and similarly observed in the control experiment, where **2c** was incubated in the absence of HLE. It can therefore be concluded that **2c** does not act as an alternate substrate inhibitor of HLE, but most probably as a competitive noncovalent inhibitor. This behavior differs from that of thieno[1,3]oxazin-4-ones with alkoxy, alkylthio, or (di)alkylamino substituents at 2-position. Such compounds have been characterized [22] to interact with HLE in the way outlined in Figure 1.

EXPERIMENTAL

Melting points were obtained on a Büchi melting point apparatus 535 and are uncorrected. Mass spectra (EI, 70 eV) were measured on a VG Analytics VG ZAB-HSQ spectrometer. ESI-HRMS spectra were recorded on a Bruker Daltonics

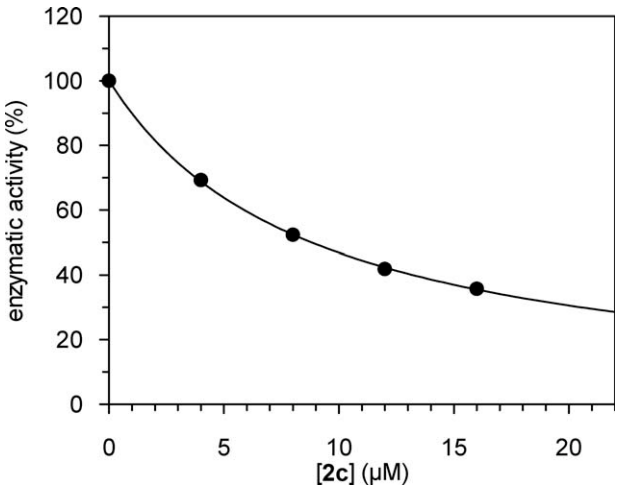


Figure 2. Inhibition of HLE by **2c** in the presence of 100 μ*M* of the chromogenic substrate MeO-Suc-Ala-Ala-Pro-Val-pNA. The data are mean values of duplicate measurements. The reactions were followed over 10 min, and the rates, *v*, were determined by linear regression. The rates in absence of inhibitor, v_0, were set to 100%. Nonlinear regression according to the equation $v = v_0/([I]/(IC_{50} + 1)$ gave a value $IC_{50} = 8.8 \pm 0.1$ μ*M*.

7T Apex II FT-ICR mass spectrometer. ^{1}H NMR spectra were recorded on a Varian Gemini-300 spectrometer at 300.08 MHz. ^{13}C NMR spectra were recorded on a Varian Gemini-300 spectrometer at 75.45 MHz. IR spectra were recorded on a Perkin–Elmer FT-IR PC 16 instrument. 5-Methyl-6-phenylthieno[2,3-*d*][1,3]oxazin-4-one (**2a**) [17], 2,5-dimethyl-6-phenylthieno[2,3-*d*][1,3]oxazin-4-one (**2b**) [17], and 4-chloro-5-methyl-6-phenylthieno[2,3-*d*]pyrimidine (**3**) [16] were prepared as reported.

2-Ethyl-5-methyl-6-phenylthieno[2,3-*d*][1,3]oxazin-4-one (2c). A mixture of compound **1c** [11] (2 g, 8.58 mmol) and propionic anhydride (10 mL, 78 mmol) was refluxed for 90 min. The precipitate, formed after cooling, was filtered off, dried, and recrystallized from ethanol. Yield 1.2 g (34%). Colorless crystals, mp 99–100°C; ms: *m/z* (%) 271 ($M^{+\cdot}$, 100), 242 (40); ^{1}H NMR (deuteriochloroform): δ 1.33 (t, 3H, CH_3, J = 7.5 Hz), 2.56 (s, 3H, CH_3), 2.72 (q, 2H, CH_2, J = 7.5 Hz), 7.39–7.46 (m, 5H, phenyl-H); ir (potassium bromide): ν 1744 (C=O), 1596, 1158–1075 cm^{-1}. Anal. Calcd. for $C_{15}H_{13}NO_2S \times 0.5\ H_2O$: C, 64.26; H, 5.03; N, 5.00; S, 11.44. Found C, 64.03; H, 5.16; N, 5.37; S, 11.48.

2-(Ethylaminomethylenamino)-4-methyl-5-phenylthiophene-3-carboxylic acid (4a). Ethylamine (10 mL of a 70% aqueous solution, 0.126 mol) was added dropwise to compound **2a** [17] (1.4 g, 5.7 mmol). The mixture was kept at 0°C until the crystallization was finished. The precipitate was separated and dried to obtain a crude product which was not further purified. Yield 1.1 g (70%). Beige solid, mp 180–183°C; ms: *m/z* (%) 288 ($M^{+\cdot}$, 97), 215 (100); ESI-HRMS: *m/z* 577.19433 ($[2M+H]^+$)$^\cdot$ ($C_{30}H_{33}N_4O_4S_2^+$ requires 577.19377); 289.10042 ($[M+H]^+$)$^\cdot$ ($C_{15}H_{17}N_2O_2S^+$ requires 289.10052); 599.17642 ($[2M+Na]^+$)$^\cdot$ ($C_{30}H_{32}N_4NaO_4S_2^+$ requires 599.17572); 311.08269 ($[M+Na]^+$)$^\cdot$ ($C_{15}H_{16}N_2NaO_2S^+$ requires 311.08247); ^{1}H NMR (DMSO-d_6): δ 1.16 (t, 3H, CH_3, J = 7.2 Hz), 2.36 (s, 3H, CH_3), 3.29 (q, 2H, CH_2, J = 7.0 Hz), 7.38–7.50 (m, 5H, phenyl-H), 8.14 (s, 1H, CH), 8.34 (s, br, 0.5H, NH, exchangeable with D_2O), 14.30 (s, br, 0.5H, OH, exchangeable with D_2O); ir (potassium bromide): ν 3221 (N—H), 3069–2873, 1694 (C=O), 1632, 1577 cm^{-1}.

2-(1-Ethylaminopropane-1-ylidenamino)-4-methyl-5-phenylthiophene-3-carboxylic acid (4b). Compound **4b** was prepared from **2c** (1.2 g, 4.4 mmol) following the aforementioned procedure. Yield 1.2 g (89%). Beige solid, mp 152–155°C (crude product); ms: *m/z* (%) 316 ($M^{+\cdot}$, 18), 242 (100); ^{1}H NMR (DMSO-d_6): δ 1.20 (t, 6H, 2 CH_3, J = 7.5 Hz), 2.41 (s, 3H, CH_3), 2.7 (q, 2H, CH_2, J = 7.8 Hz), 3.27 (q, 2H, CH_2, J = 7.5 Hz), 7.40–7.54 (m, 5H, phenyl-H), 8.42 (s, br, 1H, NH, exchangeable with D_2O); ir (potassium bromide): ν 3237 (N—H), 2978–2937, 1684 (C=O), 1592, 1490, 1238, 1073 cm^{-1}. Anal. Calcd. for $C_{17}H_{20}N_2O_2S$: C, 64.53; H, 6.37; N, 8.85; S, 10.13. Found C, 64.22; H, 6.34; N, 8.79; S, 10.07.

2,5-Dimethyl-6-phenylthieno[2,3-*d*][1,3]thiazin-4-thione (5) [17]. Compound **2b** [17] (2.57 g, 10 mmol) was dissolved in 80 mL of dry toluene. After addition of diphosphorous pentasulfide (22.2 g, 100 mmol), the mixture was refluxed for 2 h. The formed inorganic precipitate was filtered off and washed several times with hot toluene. The combined toluene solutions were evaporated and the precipitated crude product was separated and recrystallized from ethanol. Yield 1.16 g (40%). Red crystals, mp 135°C (C_2H_5OH); ms: *m/z* (%) 291 ([$M^{+\cdot}$ + 2], 26), 289 ($M^{+\cdot}$, 100), 274 (10), 256 (12), 247 (15), 230 (11), 224 (12), 215 (41), 203 (13), 184 (9), 171 (31), 127 (10), 121 (17), 115 (27), 89 (9), 77 (14); ^{1}H NMR (deuteriochloroform): δ 2.61 (s, 3H, CH_3), 2.78 (s, 3H, CH_3), 7.48–7.51 (m 5H, phenyl-H); ^{13}C NMR (deuteriochloroform): δ 19.0 (5-CH_3), 25.5 (2-CH_3), 127.1 (C-4a), 128.7 (C-4′), 128.9 (C-2′, C-6′), 130.1 (C-3′, C-5′), 130.8 (C-6), 132.1 (C-1′), 132.8 (C-5), 136.8 (C-7a), 170.1 (C-2), 201.6 (C=S); ir (potassium bromide): ν 1545, 1262, 1052 cm^{-1}.

4-Ethoxy-5-methyl-6-phenylthieno[2,3-*d*]pyrimidine (6a). Compound **3** [16] (0.5 g, 1.9 mmol) was refluxed in 10 mL of a solution of sodium ethoxide in ethanol (1 mol/L) for 12 h. The solvent was evaporated, the precipitate was isolated, washed with water and recrystallized from ethanol. Yield 0.4 g (78%). Colorless crystals, mp 103°C (C_2H_5OH); ms: *m/z* (%) 270 ($M^{+\cdot}$, 100), 255 (36), 242 (78); ^{1}H NMR (DMSO-d_6): δ 1.40 (t, 3H, CH_3, J = 7.2 Hz), 2.50 (s, 3H, CH_3), 4.53 (q, 2H, CH_2, J = 7.2 Hz), 7.44–7.52 (m, 5H, phenyl-H), 8.60 (s, 1H, CH); ^{13}C NMR (deuteriochloroform): δ 14.9 (CH_3), 15.4 (CH_3), 63.3 (OCH_2), 120.2 (C_q), 126.3 (C_q), 129.3 (CH, Ph), 129.7 (2 × CH, Ph), 130.2 (2 × CH, Ph), 133.4 (C_q), 135.4 (C_q), 153.7 (CH), 164.7 (C_q), 167.1 (C_q); ESI-HRMS: *m/z* 271.09014 ($[M+H]^+$)$^\cdot$ ($C_{15}H_{15}N_2OS^+$ requires 271.08996); 563.15462 ($[2M+Na]^+$)$^\cdot$ ($C_{30}H_{28}N_4NaO_2S_2^+$ requires 563.15459); 293.07211 ($[M+Na]^+$)$^\cdot$ ($C_{15}H_{14}N_4NaOS^{+\cdot}$ requires 293.07190); ir (potassium bromide): ν 3446 (br, traces from water), 3064–2978, 1555–1450, 1332, 1063, 1044 cm^{-1}. Anal. Calcd. for $C_{15}H_{14}N_2OS \times H_2O$: C, 62.47; H, 5.59; N, 9.71; Found C, 62.69; H, 4.96; N, 9.67.

4-Ethylamino-5-methyl-6-phenylthieno[2,3-*d*]pyrimidine (6b). Compound **3** [16] (0.3 g, 1.15 mmol) was dissolved in dichloromethane (3 mL) and treated with ethylamine (100 mL of a 70% aqueous solution, 1.26 mol). If no crystallization occurred, water was added to the mixture. The precipitate was filtered off, dried, and recrystallized from ethanol. Yield 0.2 g (65%). Yellow crystals, mp 155–158°C (C_2H_5OH); ms: *m/z* (%) 269 ($M^{+\cdot}$, 100), 254 (27), 240 (30); ^{1}H NMR (deuteriochloroform): δ 1.35 (t, 3H, CH_3, J = 7.2 Hz), 2.63 (s, 3H, CH_3), 3.66–3.75 (m, 2H, CH_2, J = 7.2 Hz), 5.57 (s, br, NH, exchangeable with D_2O), 7.41–7,50 (m, 5H, phenyl-H), 8.49 (s, 1H, CH); ir (potassium bromide): ν 3419 (NH), 2988–2863, 1574–1460, 1128–1012 cm^{-1}. Anal. Calcd. for $C_{15}H_{15}N_3S \times 0.5\ C_2H_5OH$: C, 65.72; H, 6.20; N, 14.37; S, 10.96. Found C, 65.30; H, 5.76; N, 14.75; S, 11.23.

3-Ethyl-5-methyl-6-phenylthieno[2,3-*d*]pyrimidin-4-one (7a). Compound **4a** (0.3 g, 1 mmol) was dissolved in anhydrous chloroform (10 mL). After addition of thionyl chloride (1.19 g, 10 mmol), the mixture was refluxed for 30 min. The solvent was evaporated at room temperature, the precipitate formed was filtered off, and dried. The crude product was purified by column chromatography on silica gel (63–200 μm, Merck) with ethanol. Yield 0.087 g (30%). Red solid, mp 90°C (C_2H_5OH); ms: *m/z* (%) 270 ($M^{+\cdot}$, 100), 242 (42); ESI-HRMS: *m/z* 541.17313 ($[2M+H]^+$)$^\cdot$ ($C_{30}H_{29}N_4O_2S_2^+$ requires 541.17264); 271.08999 ($[M+H]^+$)$^\cdot$ ($C_{15}H_{15}N_2OS^+$ requires 271.08996); 563.15500 ($[2M+Na]^+$)$^\cdot$ ($C_{30}H_{28}N_4NaO_2S_2^+$ requires 563.15459); 293.07208 ($[M+Na]^+$)$^\cdot$ ($C_{15}H_{14}N_4NaOS^+$ requires 293.07190); ^{1}H NMR (deuteriochloroform): δ 1.43 (t, 3H, CH_3, J = 7.2 Hz), 2.64 (s, 3H, CH_3), 4.07 (q, 2H, CH_2, J = 7.3 Hz), 7.39–7.47 (m, 5H, phenyl-H), 7.97 (s, 1H, CH); ^{13}C NMR (deuteriochloroform): δ 14.9 (CH_3), 15.4 (CH_3), 42.1 (NCH_2), 124.4 (C_q), 128.3 (CH, Ph), 128.9 (2 × CH, Ph),

129.9 (2 × CH, Ph), 130.3 (C_q), 133.6 (C_q), 135.5 (C_q), 146.0 (CH), 158.5 (C_q), 163.0 (C_q); ir (potassium bromide): ν 1666, 1658 (C=O), 1600, 1576, 1234–1083 cm^{-1}. Anal. Calcd. for $C_{15}H_{14}N_2OS$: C, 66.64; H, 5.22; N, 10.36. Found: C, 66.32; H, 5.40; N, 10.13.

2,3-Diethyl-5-methyl-6-phenylthieno[2,3-*d*]pyrimidin-4-one (7b). Thionyl chloride (2.38 g, 20 mmol) was added to a solution of **4b** (1.2 g, 3.8 mmol) in anhydrous chloroform (30 mL). The mixture was refluxed for 30 min. The solvent was evaporated at room temperature. The precipitate was filtered off and dried. The crude product was thoroughly washed with acetone and recrystallized from acetonitrile. Yield 0.5 g (40%). Beige solid, mp 175–179°C (CH_3CN); ms: *m/z* (%) 298 ($M^{+\cdot}$, 100), 270 (69), 215 (66); ^{1}H NMR (DMSO-d_6): δ 1.24–1.32 (m, 6H, 2 CH_3, J = 7.2 Hz, J = 6.9 Hz), 2.53 (s, 3H, CH_3), 2.93 (q, 2H, CH_2, J = 7.2 Hz), 4.10 (q, 2H, CH_2, J = 6.9 Hz), 7.44–7.53 (m, 5H, phenyl-H); ir (potassium bromide): ν 3434 (br, traces of water), 1670 (C=O), 1596, 1543 cm^{-1}. Anal. Calcd. for $C_{17}H_{18}N_2OS$: C, 68.43; H, 6.08; N, 9.39, S 10.74. Found C, 68.16; H, 5.83; N, 8.92; S, 10.27.

3-Ethyl-2,5-dimethyl-6-phenylthieno[2,3-*d*]pyrimidine-4-thione (8). Ethylamine (3 mL of a 70% aqueous solution, 0.04 mol) was added dropwise to **5** (0.4 g, 1.38 mmol). The mixture was kept at 0°C until the crystallization was finished. The precipitate was separated and dried. Yield 0.1 g (24%). Light yellow crystals, mp 89–91°C (CH_3CN); ms: *m/z* (%) 302 ([$M^{+\cdot}$ + 2], 13), 300 ($M^{+\cdot}$, 100), 272 (51); ^{1}H NMR (DMSO-d_6): δ 1.35 (t, 3H, CH_3, J = 6.9 Hz), 2.74 (s, 3H, CH_3), 2.81 (s, 3H, CH_3), 4.79 (s, br, 2H, CH_2), 7.52–7.59 (m, 5H, phenyl-H), ir (potassium bromide): ν 3440 (br, traces of water), 2974–2926, 1557 (strong, C=S), 1493, 1444, 1191, 1093 cm^{-1}. Anal. Calcd. for $C_{16}H_{16}N_2S_2 \times 0.5\ H_2O$: C, 62.10; H, 5.54; N, 9.05; S, 20.72. Found C, 62.40; H, 5.23; N, 9.11; S, 20.88.

Kainate receptor-aequorin assay. 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 growth medium together with Earle's salts and Glutamax-I (Life Technologies), containing 10% FKS, 1% nonessential amino acids, 100 IU/mL penicillin, 100 μg/mL streptomycin, 600 μg/mL G 418 (Calbiochem) and 500 n*M* ouabaine. One day before the measurement, 60,000 cells per cavity were seeded into white, nontransparent 96-well microtiter plates (Costar). On the test day, cells were incubated with 5 μ*M* 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 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* HEPES, 1 m*M* $MgCl_2$, 10 m*M* glucose, 0.3 mg/mL concanavaline A (ConA) and (for GluR6) 100 m*M* $CaCl_2$ (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 m*M* glutamate-solution in assay buffer was injected (to receive a glutamate concentration of 275 μ*M* per cavity, according to the EC_{50} value of glutamate for GluR6) or 10 μL of 0.8 m*M* glutamate (due to 80 μ*M* = EC_{50} for GluR5). A second injection of 100 μL triton X-100 in assay buffer (without Ca^{2+}) was carried out after 20 s 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 the IC_{50} value, a Hill plot (4-parameter model) was used.

HLE inhibition assay. The spectrophotometric assay for HLE was done on a Varian Cary 50 Bio UV/VIS spectrometer with a cell holder equipped with a constant temperature water bath. HLE was available from a previous study [24]. Reactions were followed at 405 nm at 25°C for 10 min. Stock solutions of the inhibitors were prepared in DMSO. IC_{50} values were calculated from the linear steady-state turnover of the substrate. Assay buffer was 50 m*M* sodium phosphate buffer, 500 m*M* NaCl, pH 7.8. An enzyme stock solution of 50 μg/mL was prepared in 100 m*M* sodium acetate buffer, pH 5.5 and diluted with assay buffer. A 50 m*M* stock solution of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-pNA (Bachem, Bubendorf, Switzerland) was prepared in DMSO and diluted with assay buffer containing 10% DMSO. The final concentration of the substrate was 100 μ*M*, of DMSO was 1.5% and of HLE was 25 ng/mL. Into a cuvette containing 890 μL assay buffer, 10 μL of an inhibitor solution and 50 μL of a substrate solution were added and thoroughly mixed. The reaction was initiated by adding 50 μL of the HLE solution (500 ng/mL).

HLE incubation experiment. Compound **2c**, dissolved in acetonitrile, and a solution of HLE were added to assay buffer and incubated for 4 h at 25°C in a quartz cuvette. The final concentration of **2c** was 20 μ*M*, of acetonitrile was 2% and of HLE was 5 μg/mL. In the control experiment, **2c** was incubated in assay buffer without HLE. In 60-min intervals, 10 μL aliquots were injected into the HPLC system (Dionex P580, Phenomenex Gemini 5 μ, C_{18}, mobile phase A: H_2O/acetonitrile/CF_3CO_2H (475:25:0.3), mobile phase B: H_2O/acetonitrile/tetrahydrofuran/CF_3CO_2H (25:465:10:0.3), gradient 0–25 min: 70–10% A, 30–90% B, 27–30 min: 70% A, 30% B, UV detection, 310 nm). The retention time of **2c** was 17.33–17.35 min.

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REFERENCES AND NOTES

[1] Allgeier, H.; Froestl, W.; Koller, M.; Mattes, H.; Nozulak, J.; Ofner, S.; Orain, D.; Rasetti, V.; Renaud, J.; Soldermann, N.; Floersheim, P. Patent (Novartis AG, Switzerland) Appl. WO 2006010591; Chem Abstr 2006, 144, 192265.

[2] Mkrtchyan, A. P.; Noravyan, A. S.; Petrosyan, V. M. Khim Geterotsikl Soedin 2002, 2, 261.

[3] Pinheiro, P. S.; Mulle, C. Nat Rev Neurosci 2008, 9, 423.

[4] Epsztein, J.; Represa, A.; Joquera, I.; Ben, Y.; Crepel, V. J Neurosci 2005, 25, 8229.

[5] Frerking, M.; Nicoll, R. A. Curr Opin Neurobiol 2000, 10, 342.

[6] Lerma, J.; Paternain, A. V.; Rodriguez-Moreno, A.; Lopez-Garcia, J. C. Physiol Rev 2001, 81, 971.

[7] Bräuner-Osborne, H.; Egebjerg, J.; Nielsen, E. O.; Madsen, U.; Krogsgaard-Larsen, P. J Med Chem 2000, 43, 2609.

[8] Christensen, J. K.; Varming, T.; Ahring, P. K.; Jørgensen, T. D.; Nielsen, E. Ø. J Pharmacol Exp Ther 2004, 309, 1003.

[9] Catarzi, D.; Colotta, V.; Varano, F.; Calabri, F. R.; Filacchioni, G.; Galli, A.; Costagli, C.; Carlà, V. J Med Chem 2004, 47, 262.

[10] Dominguez, E.; Iyengar, S.; Shannon, H. E.; Bleakman, D.; Alt, A.; Arnold, B. M.; Bell, M. G.; Bleisch, T. J.; Buckmaster, J. L.; Castano, A. M.; Del Prado, M.; Escribano, A.; Filla, S. A.; Ho, K. H.; Hudziak, K. J.; Jones, C. K.; Martinez-Perez J. A.; Mateo, A.; Mathes, B. M.; Mattiuz, E. L.; Ogden, A. M.; Simmons, R. M.; Stack, D. R.; Stratford, R. E.; Winter, M. A.; Wu, Z.; Ornstein, P. L. J Med Chem 2005, 48, 4200.

[11] Briel, D.; Rybak, A.; Kronbach, C.; Unverferth, K. Eur J Med Chem 2010, 45, 69.

[12] Chua, F.; Laurent, G. J. Proc Am Thorac Soc 2006, 3, 424.

[13] Korkmaz, B.; Moreau, T.; Gauthier, F. Biochimie 2008, 90, 227.

[14] Taggart, C. C.; Greene, C. M.; Carroll, T. P.; O'Neill, S. J.; McElvaney, N. G. Am J Respir Crit Care Med 2005, 171, 1070.

[15] Pham, C. T. Nat Rev Immunol 2006, 6, 541.

[16] Briel, D.; Rybak, A.; Kronbach, C.; Unverferth, K. Pharmazie, 2008, 63, 823.

[17] Briel, D.; Rybak, A.; Mann, S.; Kronbach, C.; Unverferth, K. Curr Med Chem 2009, 6, 4704.

[18] Krantz, A.; Spencer, R. W.; Tam, T. F.; Liak, T. J.; Copp. L. J.; Thomas, E. M.; Rafferty, S. P. J Med Chem 1990, 33, 464.

[19] Stein, R. L.; Strimpler, A. M.; Viscarello, B. R.; Wildonger, R. A.; Mauger, R. C.; Trainor, D. A. Biochemistry 1987, 26, 4126.

[20] Gütschow M.; Neumann, U.; Sieler, J.; Eger, K. Pharm Acta Helv 1998, 73, 95.

[21] Jarvest, R. L.; Parratt, M. J.; Debouck, C. M.; Gorniak, J. G.; Jennings, L. J.; Serafinowska, H. T.; Strickler, J. E. Bioorg Med Chem Lett 1996, 6, 2463.

[22] Gütschow, M.; Neumann, U. J Med Chem 1998, 41, 1729.

[23] Gütschow, M.; Kuerschner, L.; Neumann, U.; Pietsch, M.; Löser, R.; Koglin, N.; Eger, K. J Med Chem 1999, 42, 5437.

[24] Pietsch, M.; Gütschow M. J Biol Chem 2002, 277, 24006.