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Design, synthesis and pharmacological characterization of coumarin-based fluorescent analogs of excitatory amino acid transporter subtype 1 selective inhibitors, UCPH-101 and UCPH-102

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ABSTRACT

The excitatory amino acid transporters (EAATs) play a pivotal role in regulating the synaptic concentration of glutamate in the mammalian central nervous system. To date, five different subtypes have been identified, named EAAT15 in humans (and GLAST, GLT-1, EAAC1, EAAT4, and EAAT5, respectively, in rodents). Recently, we have published and presented a structure–activity relationship (SAR) study of a novel class of selective inhibitors of EAAT1 (and GLAST), with the analogs UCPH-101 (IC_{50} = 0.66 μM) and UCPH-102 (IC_{50} = 0.43 μM) being the most potent inhibitors in the series. In this paper, we present the design, synthesis and pharmacological evaluation of six coumarin-based fluorescent analogs of UCPH-101/102 as subtype-selective inhibitors at EAAT1. Analogs **1114** failed to inhibit EAAT1 function (IC_{50} values >300 μM), whereas analogs **15** and **UCPH-102F** inhibited EAAT1 with IC_{50} values in the medium micromolar range (17 μM and 14 μM, respectively). Under physiological pH no fluorescence was observed for analog **15**, while a bright blue fluorescence emission was observed for analog **UCPH-102F**. Regrettably, under confocal laser scanning microscopy selective visualization of expression of EAAT1 over EAAT3 was not possible due to nonspecific binding of **UCPH-102F**.

1. Introduction

In the central nervous system (CNS) the excitatory amino acid transporters (EAATs) are responsible for the uptake of the major excitatory neurotransmitter (*S*)-glutamate (Glu) from the synaptic cleft into glial cells and neurons. Thereby, the transporters are crucial players in the maintenance of synaptic as well as extra-synaptic Glu concentrations below levels of neurotoxicity and consequently for the overall firing tone in this neurotransmitter system.[1] To date, five EAAT subtypes have been identified. In humans these subtypes are termed EAAT1–5, whereas they in rodents are termed GLAST, GLT-1, EAAC1, EAAT4, and EAAT5, respectively. The five EAAT subtypes exhibit distinct expression patterns in the CNS: While EAAT1–3 (GLAST, GLT-1, EAAC1) are expressed throughout the CNS, the EAAT4,5 subtypes are expressed exclusively in Purkinje cells of the cerebellum and in the retina, respectively.[2] At the cellular level, EAAT1,2 are expressed predominantly in glia cells and astrocytes, whereas EAAT3,4 are expressed almost exclusively on neurons.[3] Finally, EAAT1–3 are high-capacity Glu transporters, while EAAT4,5 are considered to be low-capacity Glu transporters, functioning primarily as Glu-gated chloride channels.[3] Malfunction of the EAATs has been suggested to be a contributing factor in neurotoxic states and neurodegenerative diseases such as Alzheimer's,[4] Huntington's,[5] amyotrophic lateral sclerosis (ALS),[6] cerebral stroke[7] and epilepsy,[1,8,9] as well as in psychiatric disorders like depression[10] and schizophrenia.[11,12]

Considering the therapeutic potential of the EAATs the available pharmacological tool box for in vitro and in vivo studies is, however, limited with EAAT1 inhibitor UCPH-101 being the most recent disclosure.[3] Thus, as a part of our medicinal chemistry research program within the EAATs, we focus on the discovery of small-molecule fluorescent probes for the potential use in studies of function, expression and trafficking of these transporters. We here report the design and synthesis of a series of coumarin-based fluorescent analogs of the EAAT1-selective inhibitors UCPH-101/102 which may be useful as pharmacological tools.

Abbreviations: ALS, amyotrophic lateral sclerosis; CNS, central nervous system; EAAC1, excitatory amino acid carrier subtype 1; EAAT(s), excitatory amino acid transporter(s); $GABA_A$, γ-aminobutyric acid receptor A; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter subtype 1; SAR, Structure–activity relationship.

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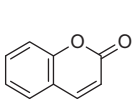
2. Results and discussions

2.1. Coumarins as fluorophores

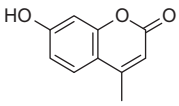
Coumarins comprise the parental skeleton 2*H*-chromen-2-one (**1**) (Table 1) are found in a broad class of natural products, pharmaceutical drugs and fluorophores.[13] While the parental coumarin skeleton **1** is characterized by low fluorogenic properties, addition of an electron rich substituent in the 7-position on the phenyl ring gives rise to electron resonance throughout the fused ring system and thus strong fluorescent characteristics. At physiological pH, 7-hydroxycoumarin (**2**) (Table 1) is equally fluorescent to **1**. However, when placed at pH of 10 or higher, the 7-hydroxy group is fully deprotonated and the phenolate anion participates in resonance, thereby giving rise to fluorescent properties. In contrast, the *N*,*N*-7-diethylamino-coumarin derivative (**3**) (Table 1) is highly fluorescent under physiological pH, which makes this fluorophore attractive for use in biological studies.[14]

Several other coumarin derivatives have been incorporated into compounds and used to study various biologically important targets. For example, the 7-aminocoumarin derivative **4** (Fig. 1) has been used for preparing blue fluorescent conjugates of proteins and nucleic acids,[18] while the 7-diethylaminocoumarin derivative **5** has been incorporated into ligands used for studies of γ-aminobutyric acid type A ($GABA_A$) receptors.[19] The 7-hydroxycoumarin derivative **6**, which comprise three substituents on the benzene ring has been applied as fluorophore for the labeling of recombinant proteins inside living cells and on the cell surface by *Escherichia coli* lipoic acid ligase (LplA) mutants.[20]

Table 1
Chemical structures and absorption and emission maxima for coumarin (**1**) and selected derivatives (**2**–**3**)



1



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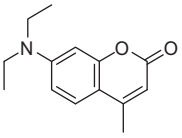
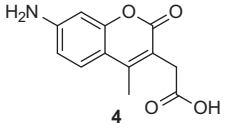


3

	Solvent[a]	λ_{abs} (nm)	λ_{em} (nm)
1[15]	Acetonitrile	320	—
2[16]	0.1 M Phosphate buffer (pH = 10)	360	450
3[14,17]	Abs ethanol	373	451

[a] The experiments were performed at rt.



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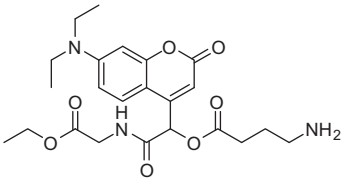

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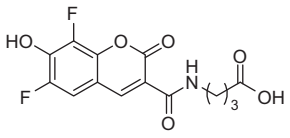


6

Figure 1. Coumarin derivatives **4**–**6** which have found use as fluorophores in biological imaging assays.

2.2. Design and synthesis

In accordance with previous reported structure–activity relationship (SAR) study of UCPH-101 and UCPH-102,[21,22] the presence of an aromatic group in the 7-position is crucial for the inhibitory activity and that a 1-naphthyl group gave the most potent analogs (UCPH-101 and UCPH-102) (Fig. 2). On the other hand, the 4-position was found to be able to accommodate small and larger substituents, not being restricted to aromatics only (compounds **7–10**, Fig. 2).[21,22]

Given this SAR information, our strategy was to incorporate the coumarin moiety into the 4- and 7-positions of the parental scaffold. The coumarin analogs **11** and **12** (Fig. 3) were designed in accordance with **10**, taking advantage of the methyl attachment point of the coumarin skeleton. In the 4-position, the coumarin and 1-naphthyl substituents occupy the same space being flat bicyclic substituents. For **11**, the phenyl group was kept in the 7-position whereas for **12** a 1-naphthyl group was chosen as to mimic UCPH-101/102. The 4-coumarin analogs **13** and **14** (Fig. 3) comprise a phenyl spacer and were designed based on the SAR observation that larger and elongated substituents are allowed (Fig. 2, structure **8** and **9**). As an alternative strategy, the coumarin moiety was incorporated into the 7-position (analogs **15, 16** and **UCPH-102F**) with the methyl group being the point of attachment as to resemble UCPH-101/102. This attachment point is likely essential as compound **7** which comprises a 2-naphthyl group is 10–20 fold less active. A methyl group in the 4-position, as present in UCPH-102, was chosen as to balance solubility properties.

The synthesis of the compound class profiled by UCPH-101/102 is in general terms carried out by a three-component reaction[21–24] as depicted in Figure 4. The R^1 substituent in the 7-position of the parental skeleton **C** originates from diketone **A**, whereas the R^2 substituent is derived from aldehyde **B**. Thus, to synthesize analogs with a coumarin moeity in the 7-position as in compounds **15**, **16** and **UCPH-102F**, the coumarin is to be part of the diketone fragment. On the other hand, upon its introduction in the 4-position as in compounds **11–14**, the coumarin moiety is to be employed as an aldehyde.

2.3. Synthesis of analogs with a coumarin moiety in the 4-position

The synthesis of analog **11** commenced with the preparation of aldehyde **18** by oxidation of coumarin **17**, using SeO_2 in *p*-xylene.[25,26] The aldehyde was then used in the three-component reaction with 5-phenylcyclohexane-1,3-dione, malononitrile, piperidine in abs EtOH to give the desired product 7-methoxy-coumarin analog **11** in 77% yield (Scheme 1).[21] The synthesis of the *N*,*N*-diethylamino-coumarin analog **12** followed the same pathway starting by oxidation of the diethylamino coumarin **3** with SeO_2 in *p*-xylene but afforded the corresponding aldehyde **19** in only 32% yield (Scheme 1).[25,26]

With aldehyde **19** in hand, it was reacted with 5-(naphthalen-1-yl)cyclohexane-1,3-dione, malononitrile, piperidine in abs EtOH to afford coumarin analog **12** in 57% yield.[21]

The synthesis of analogs **13** and **14** with a coumarin moiety in the 4-position were carried out following the same strategy. The reaction commenced with monobromination of coumarins **17** and **3** using *N*-bromosuccinimide in PEG-400[27] or NH_4OAc in acetonitrile[28] to give 3-monobrominated products **20** and **21** in 41% and 51% yield, respectively (Scheme 2). Subsequently, **20** and **21** underwent palladium cross coupling reaction with 4-formylphenylboronic acid, $Pd(dppf)Cl_2$, Na_2CO_3 in a mixture of DMF/H_2O (4:1) to give the aldehydes **22** and **23** in high yields (86% and 82%, respectively).[28] Finally, the aldehydes were employed in the three component reaction together with 5-phenylcyclohexane-1,3-dione, malononitrile, and piperidine base in abs EtOH to afford the

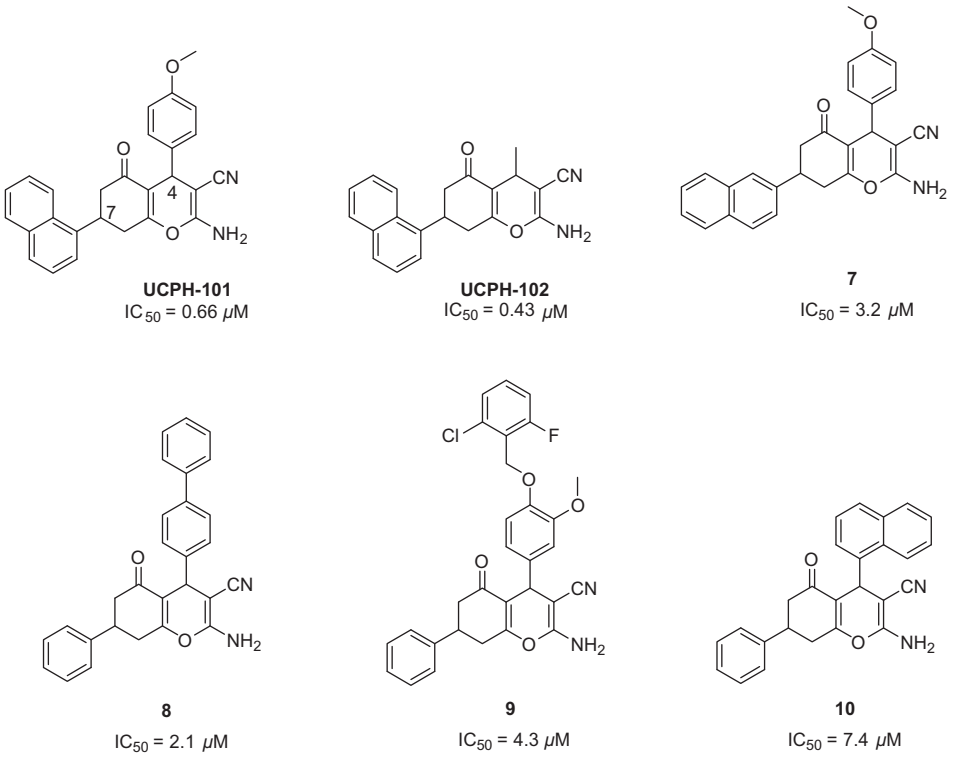


Figure 2. Chemical structures of reported subtype selective inhibitors of EAAT1.[21,22]

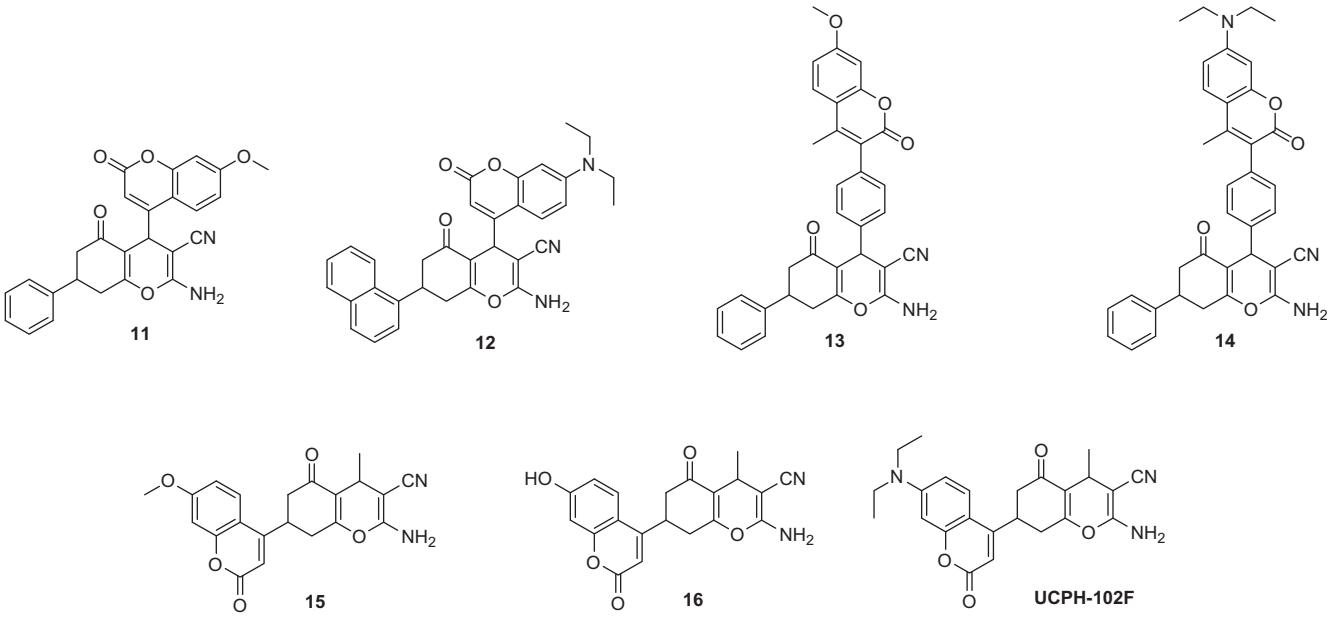


Figure 3. Chemical structures of coumarin-based fluorescent analogs.

4-substituted coumarin analogs **13** and **14** in 61% and 82% yield, respectively.

2.4. Synthesis of analogs with a coumarin moiety in the 7-position

The synthesis of three coumarin analogs **15**, **16** and **UCPH-102F** (Scheme 3and 4) commenced with the building of diketones **26** and **27**. The previously prepared aldehydes **18** and **19** (Scheme 1) were reacted with diethyl-2-oxopropyl-phosphonate, NaH in THF to afford the conjugated ketones **24** and **25** in 91% and 89% yield, respectively. The ketones were subsequently reacted with diethylmalonate, 21% w/w NaOEt in abs EtOH followed by hydrolysis of the ester in 2 M NaOH, H_2O and decarboxylation in 2 M H_2SO_4 (pH≈3) to provide diketones **26** and **27** in 38% and 20% yield, respectively. In the closing three-component reaction, diketones **26** and **27** were reacted with malononitrile, acetaldehyde, *N*-methylmorpholine in abs EtOH to afford the coumarin analogs **15** and **UCPH-102F** in 68% and 77% yield, respectively.

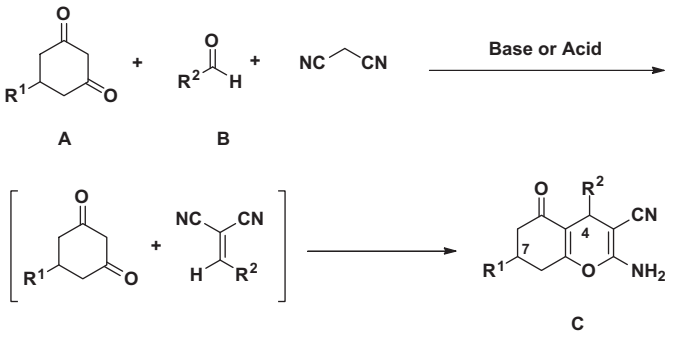
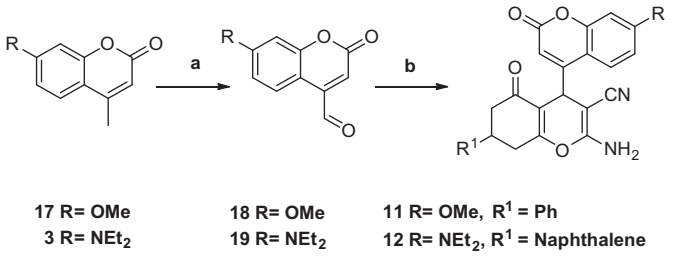
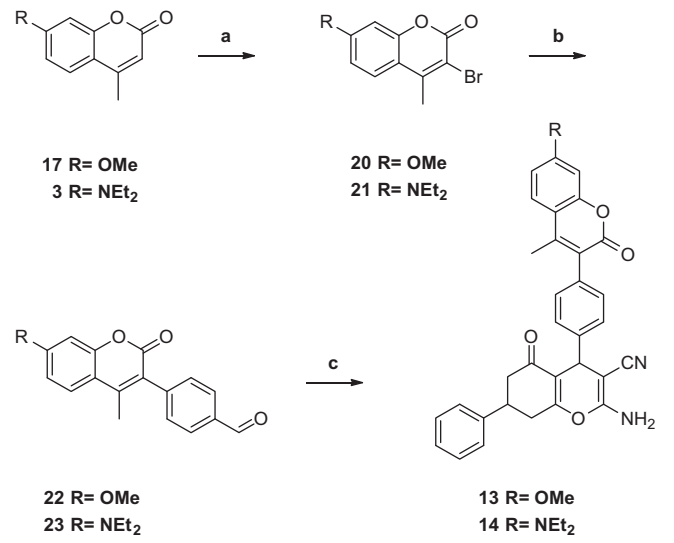


Figure 4. Three component reaction of diketone **A**, aldehyde **B** and malononitrile for the preparation of the 2-amino-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile parental skeleton **C** with various substituents R^1 in the 7-positions and R^2 in the 4-position.

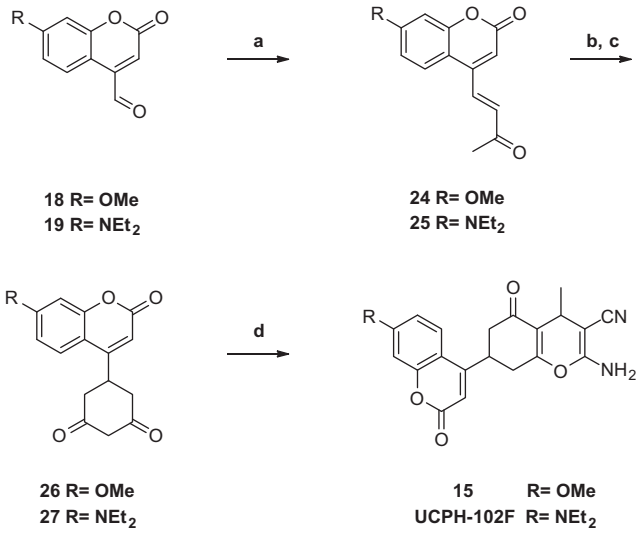


Scheme 1. Synthetic pathway towards the coumarin analogs **11** and **12** reagents and conditions: (a) for **18**: SeO_2, *p*-xylene, 145 °C, 22 h, 84%; for **19**: SeO_2, *p*-xylene, 145 °C, 24 h, 32%; (b) for **11**: 5-phenylcyclohexane-1,3-dione, malononitrile, piperidine, abs EtOH, rt, 21 h, 77%; for **12**: 5-(naphthalen-1-yl)cyclohexane-1,3-dione, malononitrile, piperidine, abs EtOH, rt, 20 h, 57%.



Scheme 2. Reagents and conditions for the synthesis of 4-substituted coumarin analogs **13** and **14** reagents and conditions: (a) for **20**: *N*-bromosuccinimide, PEG-400, rt, 2 h, 46%; for **21**: *N*-bromosuccinimide, NH_4OAc, acetonitrile, rt, 68 h, 51%; (b) for **22**: 4-formylphenylboronic acid, $Pd(dppf)Cl_2$, Na_2CO_3, DMF/H_2O (4:1), 95 °C, 5 h, 86%; for **23**: 4-formylphenylboronic acid, $Pd(dppf)Cl_2$, Na_2CO_3, DMF/H_2O (4:1), 95 °C, 5 h, 82%; (c) for **13**: 5-phenylcyclohexane-1,3-dione, malononitrile, piperidine, abs EtOH, rt, 18 h, 61%; for **14**: 5-phenylcyclohexane-1,3-dione, malononitrile, piperidine, abs EtOH, rt, 19 h, 82%.

However, demethylation of the 7-methoxy group of coumarin analog **15** proved to be troublesome. Using 48% HBr[29] in water, only a trace amount of **16** was observed while pyridine–HCl in dioxane[30] gave a complex reactions mixtures. With BBr_3 in dichloromethane,[31–33] the desired product **16** was identified in low yield but isolation from the product mixture and starting coumarin **15** was unsuccessful and thus this analog was given up.



Scheme 3. Reagents and conditions for the synthesis of coumarin analogs **15** and **UCPH-102F** reagents and conditions: (a) towards **24**: diethyl-2-oxopropyl-phosphonate, NaH, THF, 5 h, 91%; towards **25**: diethyl-2-oxopropyl-phosphonate, NaH, THF, 20 h, 89%; (b) towards **26** and **27**: diethylmalonate, 21% w/w NaOEt, abs EtOH, reflux, 3 h; (c) towards **26** and **27**: 2 M NaOH, H_2O, 100 °C, 5 h, 2 M H_2SO_4 (pH≈3), 100 °C, 1 h, 38% and 20%. (d) towards both **15** and **UCPH-102F**: malononitrile, acetaldehyde, *N*-methylmorpholine, abs EtOH, rt, 4–5 h, 68% and 77%.

3. Characterization of fluorescent and pharmacological properties

The absorption and emission wavelengths of the coumarin-based fluorescent analogs **12**, **14** and **UCPH-102F** were measured using the Jasco FP-6200 Spectrofluorometer, in abs EtOH at rt and reported in Table 2 (fluorescence spectra are available in the Supplementary data). The observed absorption and emission wavelengths are close in range (λ_{abs}: 386 nm, 383 nm and 383 nm, λ_{em}: 465 nm, 468 nm and 461 nm) giving a characteristic bright blue fluorescence.

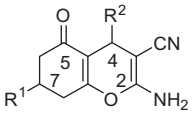
The six coumarin-based analogs **11–15** and **UCPH-102F** were characterized pharmacologically at EAAT1,2,3 stably expressed in HEK293 cells in a [^{3}H]-D-aspartate uptake assay (Table 3).[34] None of the coumarin analogs **11–14**, comprising the coumarin moiety in the 4-position, displayed significant inhibitory activity at EAAT1 at concentrations up to 300 μM. In contrast, analogs **15** and **UCPH-102F** which comprise a coumarin moieties in the 7-position both displayed inhibitory potencies at EAAT1 in medium- micromolar range (IC_{50} values 17 μM and 14 μM, respectively). None of the six analogs displayed significant inhibitory activity at subtypes EAAT2,3 at the highest possible concentrations (IC_{50} > 300 μM).

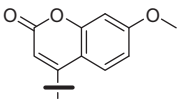
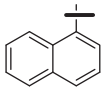
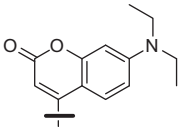
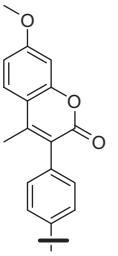
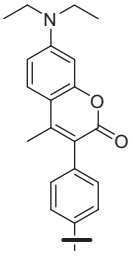
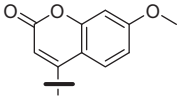
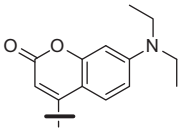
Table 2
Absorption and emission wavelengths for *N*,*N*-diethylamino-coumarin analogs **12**, **14** and **UCPH-102F**

Coumarin analog	Solvent[a]	Absorption λ_{abs} (nm)	Emission λ_{em} (nm)
12	Abs EtOH	386[b]	465[c]
14	Abs EtOH	383[b]	468[c]
UCPH-102F	Abs EtOH	383[b]	461[c]

[a] The experiments were performed at rt.
[b] Measured with a Ultrospec 4300 Pro UV/visible spectrophotometer.
[c] Using a Jasco FP-6200 spectrofluorometer to measure. Emission spectra are available in the Supplementary data.

Table 3
Pharmacological characterization of coumarin analogs of UCPH-101/102 as inhibitors at EAAT1 in a [^{3}H]-D-aspartate uptake asssay[a]



Entry	R^1	R^2	EAAT1 IC_{50} (μM)
11			>300 [<3.5]
12			>300 [<3.5]
13			>300 [<3.5]
14			>300 [<3.5]
15		–CH_3	17 [4.79 ± 0.12]
UCPH-102F		–CH_3	14 [4.85 ± 0.05]

[a] Data are given as IC_{50} values in μM with pIC_{50} ± SEM in brackets. None of the analogs displayed significant inhibitory activity at EAAT2 or EAAT3 when tested at the highest possible concentrations (300 μM).

Finally, **UCPH-102F** was applied to HEK cells expressing EAAT1 over EAAT3 to investigate if selectivity could be detected under confocal laser scanning microscopy. Regrettably, discrimination of EAAT1 expression over EAAT3 could not be shown in this experimental setup due to nonspecific binding of **UCPH-102F**. All attempts to block this were unsuccessful (see Supplementary data).

4. Conclusion

In conclusion, seven coumarin analogs of EAAT1-selective inhibitors UCPH-101/102 were designed on the basis of previously reported SAR for this class of compounds. Of these, six were successfully synthesized and subsequently characterized pharmacologically at EAATs. Analogs **11–14** which comprise a coumarin substituents in the 4-position showed no EAAT1 inhibitory. However, coumarin analogs **15** and **UCPH-102F** holding the coumarin moiety in the 7-position inhibit EAAT1, displaying IC_{50} values in micromolar range. As expected, analog **15** did not display fluorescence properties under physiological pH, whereas a characteristic blue fluorescence emission was observed for analog **UCPH-102F**. Regrettably nonspecific binding prevents its use to visualize EAAT1 expression selectively in a HEK cell setup. Despite this discouraging result, the design and synthesis of fluorescent analogs of UCPH-101/102 remains to be an attractive strategy for the visualization and quantification of EAAT1 expression.

5. Experimental section

5.1. Chemistry

All reactions involving dry solvents or sensitive agents were performed under a nitrogen atmosphere and glassware was dried prior to use. Solvents were dried according to standard procedures and reactions were monitored by analytical thin-layer chromatography (TLC, Merck silica gel 60 F_{254} aluminum sheets). Flash chromatography was carried out using Merck silica gel 60A (35–70 μ. ^{1}H NMR spectra were recorded on a 300 MHz Varian Mercury 300BB or a 400 MHz Avance Bruker and ^{13}C NMR spectra on a 75 MHz Varian Gemini 2000BB or a 100 MHz Avance Bruker. MS spectra were recorded using LC–MS performed using an Agilent 1200 series solvent delivery system equipped with an autoinjector coupled to an Agilent 6400 series triple quadrupole mass spectrometer equipped with an electrospray ionization source. Gradients of 5% aqueous acetonitrile ⩽0.05% formic acid (solvent A) and 95% aqueous acetonitrile +0.043% formic acid (solvent B) were employed. Melting points were measured using a MPA 100 optimelt automatic melting point system. All commercial chemicals were used without further purification.

5.1.1. 2′-Amino-7-methoxy-2,5′-dioxo-7′-phenyl-5′,6′,7′,8′-tetrahydro-2*H*,4′*H*-4,4′-bichromene-3′-carbonitrile (11)

Piperidine (10 μL, 0.10 mmol) was added to a suspension of 7-methoxy-2-oxo-2*H*-chromene-4-carbaldehyde (**18**) (100 mg, 0.49 mmol), 5-phenylcyclohexane-1,3-dione (92 mg, 0.49 mmol) and malononitrile (32 mg, 0.49 mmol) in abs EtOH (7 mL) at rt. The reaction mixture was stirred for 21 h. The white precipitate was filtered off and washed three times with cold abs EtOH. This afforded the titled compound as an off-white solid (166 mg, 0.38 mmol, 77% yield): mp 281–283 °C; ^{1}H NMR (300 MHz, DMSO-d_6) (~3:4 ratio of diastereomers) δ 2.42–2.51 (m, 1H), 2.67–3.24 (m, 3H), 3.46–3.57 (m, 1H), 3.87 (s, 1.5H), 3.88 (s, 1.5H), 4.85 (s, 1H), 5.95 (s, 0.5), 6.12 (s, 0.5H), 7.02–6.95 (m, 2H), 7.38–7.22 (m, 7H), 8.02 (d, J = 9.0 Hz, 0.5H), 8.07 (d, J = 9.0 Hz, 0.5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 33.6, 33.8, 37.2, 37.6, 42.9, 43.0, 55.6, 55.8, 55.9, 100.9, 109.5, 109.7, 111.7, 111.8, 112.0, 118.9, 126.5, 126.6, 126.8, 126.9, 126.9, 128.5, 142.5, 142.6, 155.1, 155.2, 159.0, 159.1, 160.7, 160.8, 162.5, 162.6, 164.9, 165.4, 195.0, 195.1; LC–MS [M+H]$^+$ calcd for $C_{26}H_{20}N_2O_5$: 441.2, found: 441.2; Anal. calcd for $C_{26}H_{20}N_2O_5$: C, 70.90; H, 4.58; N, 6.36, found: C, 70.61, H, 4.64; N, 6.22.

5.1.2. 2′-Amino-7-(diethylamino)-7′-(naphthalen-1-yl)-2,5′-dioxo-5′,6′,7′,8′-tetrahydro-2*H*,4′*H*-4,4′-bichromene-3′-carbonitrile (12)

Piperidine (4 μL, 34 μmol) was added to a solution of 7-(diethylamino)-2-oxo-2*H*-chromene-4-carbaldehyde (**19**) (42 mg, 0.17 mmol) and malononitrile (11.3 mg, 0.17 mmol) in abs EtOH (4 mL) at rt and stirred for 2 min. 5-(Naphthalen-1-yl)cyclohexane-1,3-dione (30 mg, 0.13 mmol) was added and the reaction

mixture was stirred at rt for 20 h. The precipitated solid was filtered off and washed four times with ice-cold abs EtOH to afford the titled compound as a pale-yellow solid (38 mg, 72 μmol, 57% yield); mp 264–266 °C; ^{1}H NMR (300 MHz, $CDCl_3$) (~1:3 ratio of diastereomers) δ 1.21 (t, J = 7.2 Hz, 6H), 2.72–3.10 (m, 4H), 3.41 (q, J = 7.2 Hz, 4H), 4.24–4.36 (m, 1H), 4.86 (s, 1H), 4.69 (s, 2H), 5.90 (s, 1H), 6.50 (d, J = 2.4 Hz, 1H), 6.66 (dd, J = 9.0, 2.4 Hz, 1H), 7.29–7.60 (m, 4H), 7.75–8.07 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.0, 12.5, 29.4, 33.1, 34.2, 41.2, 43.2, 44.7, 61.3, 96.3, 97.7, 106.9, 107.3, 108.9, 113.6, 122.5, 122.6, 125.3, 125.7, 126.0, 126.8, 128.1, 129.2, 130.8, 134.0, 137.1, 151.0, 156.5, 158.4, 158.6, 163.2, 164.3, 195.0; LC–MS $[M+H]^+$ calcd for $C_{33}H_{29}N_3O_4$: 532.2, found: 532.2; Anal. calcd for $C_{33}H_{29}N_3O_4$: C, 74.56; H, 5.50; N, 7.90, found: C, 74.12; H, 5.28; N, 7.84.

5.1.3. 2-Amino-4-(4-(7-methoxy-4-methyl-2-oxo-2*H*-chromen-3-yl)phenyl)-5-oxo-7-phenyl-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (13)

4-(7-Methoxy-4-methyl-2-oxo-2H-chromen-3-yl)benzaldehyde (22) (55 mg, 0.19 mmol), 5-phenylcyclohexane-1,3-dione (35 mg, 0.19 mmol) and malononitrile (12 mg, 0.19 mmol) were stirred in abs EtOH (5 mL) at rt. Piperidine (4 μL, 37 μmol) was added and the reaction mixture was stirred for 16 h at rt. The white precipitate was filtered off and washed three times with cold abs EtOH. This afforded the titled compound as a pale-yellow solid (60 mg, 0.11 mmol, 61% yield): mp 241–243 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) (~1:1 ratio of diastereomers) δ 2.27 (s, 1.5H), 2.29 (s, 1.5H), 2.53–2.95 (m, 4H), 3.35–3.55 (m, 1H), 3.87 (s, 3H), 4.53 (s, 0.5H), 4.55 (s, 0.5H), 4.63 (s, 1H), 4.65 (s, 1H), 6.83–6.89 (m, 2H), 7.15–7.38 (m, 9H), 7.56 (d, J = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.8, 16.9, 34.5, 34.6, 35.0, 35.2, 37.9, 38.8, 43.7, 43.9, 55.8, 62.9, 63.1, 100.6, 112.3, 112.4, 114.2, 115.2, 115.3, 118.7, 118.7, 123.9, 126.2, 126.6, 126.7, 127.4, 127.6, 128.9, 128.9, 130.5, 130.6, 133.4, 133.5, 141.7, 141.8, 142.5, 142.7, 148.1, 148.2, 154.3, 157.9, 158.1, 161.3, 161.4, 161.9, 162.3, 162.4, 162.8, 194.9, 195.2; LC–MS $[M+H]^+$ calcd for $C_{33}H_{26}N_2O_5$: 531.2, found: 531.3; Anal. calcd for $C_{33}H_{26}N_2O_5$: C, 74.7; H, 4.94; N, 5.28, found: C, 74.29; H, 4.62; N, 5.57

5.1.4. 5,6,7,8-Tetrahydro-4*H*-chromene-3-carbonitrile (14)

4-(7-(Diethylamino)-4-methyl-2-oxo-2*H*-chromen-3-yl)benzaldehyde (**23**) (100 mg, 0.30 mmol), 5-phenylcyclohexane-1,3-dione (57 mg, 0.30 mmol) and malononitrile (20 mg, 0.30 mmol) were stirred in abs EtOH (7 mL) at rt. Piperidine (6 μL, 60 μmol) was added and the reaction mixture was stirred for 19 h at rt. The white precipitate was filtered off and washed three times with cold abs EtOH. This afforded the titled compound as a yellow solid (139 mg, 0.25 mmol, 82% yield): mp 241–243 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) (~1:1 ratio of diastereomers) δ 1.21 (t, J = 7.2 Hz, 6H), 2.21 (s, 1.5H), 2.23 (s, 1.5H), 2.52–2.92 (m, 4H), 3.32–3.48 (m, 5H), 4.51 (s, 0.5H), 4.53 (s, 0.5H), 4.68 (s, 1H), 4.69 (s, 1H), 6.51 (t, J = 2.1 Hz, 1H), 6.60 (dd, J = 9.0, 2.1 Hz, 1H), 7.35–7.13 (m, 9H), 7.42 (dd, J = 6.9, 2.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.9, 17.0, 35.0, 35.4, 35.7, 38.3, 39.2, 44.2, 45.2, 63.0, 63.2, 97.8, 109.0, 109.9, 115.5, 115.7, 119.4, 121.0, 126.6, 127.0, 127.1, 127.7, 127.9, 129.2, 129.3, 131.2, 131.3, 134.3, 134.4, 142.1, 142.2, 142.5, 142.7, 149.0, 149.1, 150.6, 155.4, 158.3, 158.5, 162.3, 162.6, 163.2, 195.4, 195.6; LC–MS $[M+H]^+$ calcd for $C_{36}H_{33}N_3O_4$: 572.2, found: 572.2; Anal. calcd for $C_{36}H_{33}N_3O_4$: C, 75.64; H, 5.82; N, 7.35, found: C, 75.44, H, 5.59; N, 7.12.

5.1.5. 2′-Amino-7-methoxy-4′-methyl-2,5′-dioxo-5′,6′,7′,8′-tetrahydro-2*H*,4′*H*-4,7′-bichromene-3′-carbonitrile (15)

N-Methylmorpholine (10 μL, 88 μmol) was added to a solution of 5-(7-methoxy-2-oxo-2*H*-chromen-4-yl)cyclohexane-1,3-dione (**26**) (50 mg, 175 μmol), acetaldehyde (20 μL, 350 μmol) and malononitrile (23 mg, 350 μmol) in abs EtOH (4 mL) at rt and stirred for 5 h. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a yellow solid (53 mg, 140 μmol, 80% yield): R_f = 0.12 (EtOAc/heptane 1:1); mp 193–195 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) (~1:1 ratio of diastereomers) δ 1.25 (d, J = 6.9 Hz, 1.46H), 1.30 (d, J = 6.9 Hz, 1.54H), 2.48–2.89 (m, 4H), 3.39 (q, J = 6.6 Hz, 1H), 3.68–3.84 (m, 1H), 3.89 (s, 3H), 4.54 (s, 2H), 6.13 (s, 0.43H), 6.16 (s, 0.57H), 6.84–6.91 (m, 2H), 7.48 (d, J = 8.7 Hz, 0.45H), 7.53 (d, J = 8.7 Hz, 0.55H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.3, 22.9, 24.8, 24.9, 31.5, 31.6, 32.3, 32.8, 41.3, 55.9, 56.0, 57.5, 57.8, 101.1, 101.2, 109.3, 111.1, 111.2, 112.3, 114.7, 114.8, 119.9, 126.0, 126.1, 155.1, 156.5, 156.7, 158.6, 158.7, 160.2, 160.3, 162.4, 162.5, 162.8, 194.7, 194.9; LC–MS $[M+H]^+$ calcd for $C_{21}H_{18}N_2O_5$: 379.1, found: 379.1. Anal. calcd C, 66.66; H, 4.79; N 7.40, for $C_{21}H_{18}N_2O_5$: found: C, 66.23; H, 4.90; N, 7.03.

5.1.6. 7-Methoxy-2-oxo-2*H*-chromene-4-carbaldehyde (18)

7-Methoxy-4-methyl-2*H*-chromen-2-one (**17**) (1 g, 5.26 mmol) and selenium dioxide (875 mg, 7.89 mmol) were stirred in *p*-xylene (50 mL) at 145 °C for 18 h. The reaction mixture was cooled to rt and the white precipitate was filtered and purified by column chromatography on silica gel. This afforded the titled compound as a yellow solid (902 mg, 4.42 mmol, 84% yield): R_f = 0.59 (100% EtOAc); mp 200–202 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.89 (s, 3H), 6.69 (s, 1H), 6.85 (d, J = 2.4 Hz, 1H), 6.90 (dd, J = 9.0, 2.4 Hz, 1H), 8.47 (d, J = 9.0 Hz, 1H), 10.05 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 55.8, 101.1, 108.1, 113.3, 122.2, 127.3, 143.7, 156.5, 160.7, 163.3, 191.7; LC–MS $[M+H]^+$ calcd for $C_{11}H_8O_4$: 205.1, found: 205.1.

5.1.7. 7-(Diethylamino)-2-oxo-2*H*-chromene-4-carbaldehyde (19)

Selenium dioxide (720 mg, 6.48 mmol) was added to a solution of 7-(diethylamino)-4-methylcoumarine (**3**) (1 g, 4.32 mmol) in *p*-xylene (50 mL) at rt under a N_2 atmosphere. The reaction mixture was stirred at 145 °C for 21 h and cooled to rt. The dark suspension was filtered through celite and concentrated in vacuo. The crude product was purified by column chromatography on silica gel to afford the titled compound as a dark red solid (320 mg, 1.38 mmol, 32% yield): R_f = 0.20 (100% dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (t, J = 7.2 Hz, 6H), 3.42 (q, J = 7.2 Hz, 4H), 6.42 (s, 1H), 6.49 (d, J = 2.7 Hz, 1H), 6.60 (dd, J = 9.0, 2.7 Hz, 1H), 8.26 (d, J = 9.0 Hz, 1H), 9.99 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.4, 44.8, 97.5, 103.7, 109.5, 117.2, 126.9, 143.8, 150.9, 157.3, 161.8, 192.5; LC–MS $[M+H]^+$ calcd for $C_{14}H_{15}NO_3$: 246.1, found: 246.1.

5.1.8. 3-Bromo-7-methoxy-4-methyl-2*H*-chromen-2-one (20)

N-Bromosuccinimide (224 mg, 1.26 mmol) was added slowly to a suspension of 7-methoxy-4-methyl-2*H*-chromen-2-one (**17**) (200 mg, 1.05 mmol) in PEG-400 (6 mL). The reaction mixture was stirred at rt for 2 h. The reaction mixture was extracted with H_2O (25 mL) and EtOAc (3 × 25 mL) and the combined organic phases were washed with brine (30 mL). The organic phase was dried over Na_2SO_4. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a white solid (130 mg, 0.48 mmol, 46% yield); R_f = 0.35 (EtOAc/heptane 1:2); mp 144–146 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.60 (s, 3H), 3.88 (s, 3H), 6.81 (d, J = 2.7 Hz, 1H), 6.88 (dd, J = 9.0, 2.7 Hz, 1H), 7.54 (d, J = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.5, 55.8, 100.7, 109.7, 112.9, 113.4, 126.0, 151.1, 153.6, 157.3, 162.7; LC–MS $[M+H]^+$ calcd for $C_{11}H_9BrO_3$: 269.0, found: 269.0; Anal. calcd for $C_{11}H_9BrO_3$: C, 49.10, H, 3.37, found: C, 48.76; H, 3.13.

5.1.9. 3-Bromo-7-(diethylamino)-4-methyl-2*H*-chromen-2-one (21)

7-(Diethylamino)-4-methyl-2*H*-chromen-2-one (**3**) (1.5 g, 6.48 mmol), ammonium acetate (500 mg, 6.48 mmol) and *N*-bromosuccinimide (1.16 g, 6.48 mg) were stirred in dry acetonitrile (25 mL) at rt under a N_2 atmosphere. The reaction mixture was stirred at rt for 68 h. The crude reaction was concentrated and H_2O (50 mL) was added. The water phase was extracted with EtOAc (3 × 50 mL) and the combined organic phases were washed with brine (30 mL). The organic phase was dried over $MgSO_4$. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a yellow solid (1.02 g, 3.31 mmol, 51% yield): R_f = 0.07 (EtOAc/heptane 1:10); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (t, J = 7.2 Hz, 6H), 2.51 (s, 3H), 3.40 (q, J = 7.2 Hz, 4H), 6.45 (d, J = 2.7 Hz, 1H), 6.58 (dd, J = 9.0, 2.7 Hz, 1H), 7.40 (d, J = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.9, 19.5, 45.2, 97.6, 105.9, 109.3, 109.4, 126.5, 151.0, 151.9, 154.8, 158.5; LC–MS $[M+H]^+$ calcd for $C_{14}H_{16}BrNO_2$: 310.1, found: 310.1; Anal. calcd for $C_{14}H_{16}NO_2$: C, 54.21; H, 5.20; N, 4.52, found: C, 53.80; H, 4.90; N, 4.90.

5.1.10. 4-(7-Methoxy-4-methyl-2-oxo-2*H*-chromen-3-yl)benzaldehyde (22)

3-Bromo-7-methoxy-4-methyl-2*H*-chromen-2-one (**20**) (120 mg, 0.45 mmol), 4-formyl-phenylboronic acid (67 mg, 0.45 mmol), $PdCl_2$(dppf) (6.5 mg, 9 μmol) and Na_2CO_3 (123 mg, 1.16 mmol) were stirred in a mixture of DMF/H_2O (5 mL, 4:1) at 90 °C under a N_2 atmosphere for 5 h. The reaction mixture was cooled to rt and H_2O (30 mL) was added. The reaction mixture was extracted with dichloromethane (3 × 30 mL) and the combined organic phases were washed with H_2O (3 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as an off-white solid (113 mg, 0.39 mmol, 86% yield): R_f = 0.36 (EtOAc/heptane 1:1); mp 188–190 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.89 (s, 3H), 2.30 (s, 3H), 6.85 (d, J = 2.7 Hz, 1H), 6.90 (dd, J = 9.0, 2.7 Hz, 1H), 7.48 (d, J = 8.1 Hz, 2H), 7.58 (d, J = 9.0 Hz, 1H), 7.95 (d, J = 8.1 Hz, 2H), 10.04 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.7, 55.8, 100.7, 112.6, 113.7, 123.0, 126.2, 129.6, 129.7, 131.1, 131.2, 135.7, 141.1, 148.6, 154.5, 160.8, 162.7, 191.8; LC–MS $[M+H]^+$ calcd for $C_{18}H_{14}O_4$: 295.1, found: 295.2; Anal. calcd for $C_{18}H_{14}O_4$: C, 73.46; H, 4.79, found: C, 73.64, H, 4.61.

5.1.11. 4-(7-(Diethylamino)-4-methyl-2-oxo-2*H*-chromen-3-yl)benzaldehyde (23)

3-Bromo-7-(diethylamino)-4-methyl-2*H*-chromen-2-one (**21**) (400 mg, 1.29 mmol), 4-formyl-phenylboronic acid (194 mg, 1.29 mmol), $PdCl_2$(dppf) (19 mg, 26 μmol) and Na_2CO_3 (355 mg, 3.35 mmol) were stirred in a mixture of DMF/H_2O (10 mL, 4:1) at 90 °C under a N_2 atmosphere for 5 h. The reaction mixture was cooled to rt and H_2O (30 mL) was added. The reaction mixture was extracted with dichloromethane (3 × 30 mL). The combined organic phases were washed with H_2O (3 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a yellow solid (354 mg, 1.06 mmol, 82% yield): R_f = 0.30 (EtOAc/heptane 8:10); mp 140–142 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (t, J = 6.9 Hz, 6H), 2.25 (s, 3H), 3.44 (q, J = 6.9 Hz, 4H), 6.55 (d, J = 2.4 Hz, 1H), 6.64 (dd, J = 9.0, 2.4 Hz, 1H), 7.46–7.52 (m, 3H), 7.92–7.96 (m, 2H), 10.10 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.9, 16.8, 45.2, 97.8, 109.2, 109.5, 120.1, 126.7, 130.0, 131.9, 135.8, 142.4, 149.4, 151.0, 155.7, 161.9, 192.3; LC–MS $[M+H]^+$ calcd for $C_{21}H_{21}NO_3$: 336.2, found: 336.2; Anal. calcd for $C_{21}H_{21}NO_3$: C, 75.20; H, 6.31; N, 4.52, found: C, 74.92; H, 5.92; 4.24.

5.1.12. (*E*)-7-Methoxy-4-(3-oxobut-1-enyl)-2*H*-chromen-2-one (24)

A solution of diethyl-2-oxopropylphosphonate (518 μL, 2.45 mmol) in dry THF (4 mL) was added dropwise over 40 min to a solution of NaH (60% dispersion in mineral oil, 108 mg, 2.70 mmol) in dry THF (20 mL) at rt under a N_2 atmosphere. The lightly yellow solution was stirred for an additional 30 min at rt, after which a solution of 7-methoxy-2-oxo-2*H*-chromene-4-carbaldehyde (**18**) (500 mg, 2.45 mmol) in dry THF (30 mL) was added dropwise over 40 min at 0 °C. The reaction mixture was stirred at rt for 3 h. The reaction mixture was quenched with H_2O (10 mL) and extracted with EtOAc (3 × 30 mL) and the combined organic phases were washed with brine (30 mL). The organic phase was dried over Na_2SO_4. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a yellow solid (542 mg, 2.23 mmol, 91% yield): R_f = 0.25 (EtOAc/heptane 1:1); mp 144–146 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.44 (s, 3H), 3.89 (s, 3H), 6.38 (s, 1H), 7.80 (d, J = 16.2 Hz, 1H), 6.83–6.89 (m, 2H), 7.56 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 16.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.8, 55.9, 101.3, 110.0, 111.3, 112.7, 125.4, 133.7, 134.8, 148.2, 155.6, 160.7, 163.1, 196.7; LC–MS $[M+H]^+$ calcd for $C_{14}H_{12}O_4$: 245.1, found: 245.1; Anal. calcd for $C_{14}H_{12}O_4$: C, 68.85, H, 4.94, found: C, 68.51; H, 4.80.

5.1.13. (*E*)-7-(Diethylamino)-4-(3-oxobut-1-enyl)-2*H*-chromen-2-one (25)

A solution of diethyl-2-oxopropylphosphonate (259 μL, 1.34 mmol) in dry THF (4 mL) was added dropwise over 1 h to a solution of NaH (60% dispersion in mineral oil, 54 mg, 1.34 mmol) in dry THF (10 mL) at rt under a N_2 atmosphere. The light yellow solution was stirred for an additional 45 min at rt, after which a solution of 7-(diethylamino)-2-oxo-2*H*-chromene-4-carbaldehyde (**19**) (300 mg, 1.22 mmol) in dry THF (5 mL) was added dropwise over 30 min at 0 °C. The reaction mixture was stirred at rt for 2 days. The crude reaction was quenched with H_2O (5 mL) and extracted with dichloromethane (3 × 25 mL) and the combined organic phases were washed with brine (25 mL). The organic phase was dried over $MgSO_4$. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a red solid (312 mg, 1.08 mmol, 89% yield): R_f = 0.38 (dichloromethane/EtOAc 10:1); mp 143–144 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (t, J = 6.9 Hz, 6H), 2.43 (s, 3H), 3.42 (q, J = 6.9 Hz, 4H), 6.16 (s, 1H), 6.50 (d, J = 2.1 Hz, 1H), 6.58 (dd, J = 9.0, 2.1 Hz, 1H), 6.77 (d, J = 15.6 Hz, 1H), 7.44 (d, J = 9.0 Hz, 1H), 7.69 (d, J = 15.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.4, 28.7, 44.8, 97.9, 106.0, 106.8, 108.7, 125.3, 133.1, 135.6, 148.1, 150.9, 156.4, 161.8, 197.2; LC–MS $[M+H]^+$ calcd for $C_{17}H_{19}NO_3$: 286.1, found: 286.1; Anal. calcd for $C_{17}H_{19}NO_3$: C, 71.56; H, 6.71; N, 4.91, found: C, 71.40; H, 6.48; N, 4.85.

5.1.14. 5-(7-Methoxy-2-oxo-2*H*-chromen-4-yl)cyclohexane-1,3-dione (26)

A solution of NaOEt (688 μL, 1.84 mmol) in abs EtOH (3 mL) was added dropwise over 40 min to a stirred solution of diethylmalonate (290 μL, 1.84 mmol) in abs EtOH (7 mL) at rt under a N_2 atmosphere. This yellow solution was stirred at rt for an additional 1 h, after which it was added dropwise over 1 h to a yellow suspension of (*E*)-7-methoxy-4-(3-oxobut-1-enyl)-2*H*-chromen-2-one (**24**) (300 mg, 1.22 mmol) in abs EtOH (15 mL). The dark red solution was left to stir at rt for 1 h and refluxed for 3 h at 90 °C. The reaction mixture was concentrated and the brown sticky residue was dried in vacuo. To this residue H_2O (3 mL) and 2 M NaOH (3 mL, 6.1 mmol) were added and the reaction mixture was stirred at 100 °C for 5 h, where after it was cooled to rt. The pH was adjusted

to 2 by adding 2 M H_2SO_4 and stirred at 100 °C for 1 h. The crude reaction was extracted with EtOAc (3 × 30 mL) and the combined organic phases were washed with H_2O (20 ml) and brine (30 mL). The organic phase was dried over Na_2SO_4. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a pale-yellow solid (132 mg, 0.46 mmol, 38% yield): R_f = 0.28 (EtOAc/heptane (10:1) → EtOAc/MeOH (10:1)); mp 200–202 °C (decomposed); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.24–2.82 (m, 4H), 3.76–3.92 (m, 1H), 3.85 (s, 3H), 5.30 (s, 1H), 6.26 (s, 1H), 6.94 (dd, J =9.0, 2.7 Hz, 1H), 7.00 (d, J = 2.7 Hz, 1H), 7.84 (d, J = 9.0 Hz, 1H), 11.30 (s, 1H); (100 MHz, DMSO-d_6) δ 33.3, 55.9, 56.0, 101.2, 103.4, 109.1, 111.3, 112.3, 125.9, 155.1, 157.3, 160.4, 162.3; LC–MS $[M+H]^+$ calcd for $C_{16}H_{14}O_5$: 287.1, found: 287.1; Anal. calcd C, 67.13; H, 4.93, for $C_{16}H_{14}O_5$: C, 67.40; H, 4.97.

5.1.15. 5-(7-(Diethylamino)-2-oxo-2*H*-chromen-4-yl)cyclohexane-1,3-dione (27)

A solution of NaOEt (491 μL, 1.31 mmol) in abs EtOH (3 mL) was added dropwise over 40 min to a stirred solution of diethylmalonate (206 μL, 1.31 mmol) in abs EtOH (7 mL) at rt under a N_2 atmosphere. This yellow solution was stirred at rt for an additional 1 h, after which it was added dropwise over 50 min to a red solution of (*E*)-7-(diethylamino)-4-(3-oxobut-1-enyl)-2*H*-chromen-2-one (**25**) (250 mg, 0.88 mmol) in a mixture of abs EtOH and THF (20 mL, 3:1). The dark red solution was left to stir at rt for 1 h and refluxed for 3 h at 90 °C. The reaction mixture was concentrated and the brown sticky residue was dried in vacuo. To this residue H_2O (3 mL) and 2 M NaOH (2.2 mL, 4.38 mmol) were added and the reaction mixture was stirred at 100 °C for 6 h, where after it was cooled to rt. The pH was adjusted to 2 by adding 2 M H_2SO_4 and stirred at 100 °C for 1 h. The crude reaction was quenched with sat NH_4Cl (10 mL) and extracted with EtOAc (3 × 30 mL) and the combined organic phases were washed with H_2O (20 ml) and brine (30 mL). The organic phase was dried over Na_2SO_4. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a yellow solid (56 mg, 0.18 mmol, 20% yield): R_f = 0.10 (EtOAc/heptane (10:1) → EtOAc/MeOH (10:1)); mp 132–134 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (t, J = 6.9 Hz, 6H), 2.48–2.73 (m, 4H), 3.44 (q, J = 6.9 Hz, 4H), 3.65–3.76 (m, 1H), 5.51(s, 1H), 6.00 (s, 1H), 6.56 (d, J = 2.4 Hz, 1H), 6.62 (dd, J = 9.3, 2.4 Hz, 1H), 7.41 (d, J = 9.3 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 12.3, 24.9, 29.5, 33.1, 41.9, 43.9, 97.1, 103.4, 105.2, 106.4, 108.8, 125.5, 150.2, 155.1, 157.6, 161.0, 162.9; LC–MS $[M+H]^+$ calcd for $C_{19}H_{21}NO_4$: 328.1, found: 328.1.

5.1.16. 2′-Amino-7-(diethylamino)-4′-methyl-2,5′-dioxo-5′,6′,7′,8′-tetrahydro-2*H*,4′*H*-4,7′-bichromene-3′-carbonitrile (UCPH-102F)

N-Methylmorpholine (4 μL, 31 μmol) was added to a solution of 5-(7-(diethylamino)-2-oxo-2*H*-chromen-4-yl)cyclohexane-1,3-dione (**27**) (20 mg, 61 μmol), acetaldehyde (7 μL, 122 μmol) and malononitrile (8.1 mg, 122 μmol) in abs EtOH (4 mL) at rt and stirred for 4 h. After concentration in vacuo the crude product was purified by column chromatography on silica gel. This afforded the titled compound as a yellow solid (20 mg, 77% yield): R_f = 0.13 (EtOAc/heptane 1:1) mp 178–180 °C (decomposed); ^{1}H NMR (300 MHz, $CDCl_3$) (~1:1 ratio of diastereomers) δ 1.19–1.31 (m, 9H), 2.47–2.89 (m, 4H), 3.42 (q, J = 7.2 Hz, 5H), 3.60–3.81 (m, 1H), 4.60 (s, 1H), 4.61 (s, 1H), 5.93 (s, 0.5H), 5.95 (s, 0.5H), 6.51 (t, J = 2.4 Hz, 1H), 6.57 (t, J = 2.4 Hz, 0.5H), 6.60 (t, J = 2.4 Hz, 0.5H), 7.35 (d, J = 9.0 Hz, 0.5H), 7.40 (d, J = 9.0 Hz, 0.5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.4, 22.2, 22.7, 22.9, 24.9, 25.0, 32.0, 32.2, 33.0, 33.5, 42.2, 42.3, 42,4, 44.8, 63.1, 63.4, 98.1, 105.7, 105.8, 106.3, 106.4, 108.7, 108.8, 116.4, 116.5, 118.7, 118.8, 124.3, 124.4, 150.7, 150.8, 155.3, 155.4, 156.5, 157.6, 157.7, 161.4, 161.9, 162.0, 162.1, 194.4, 194.6; LC–MS $[M+H]^+$ calcd for $C_{24}H_{25}N_3O_4$: 420.2, found: 420.2; Anal. calcd for $C_{24}H_{25}N_3O_4$: C, 68.72; H, 6.01; N, 10.02, found: C, 68.35; H, 6.10; N, 9.67.

5.2. Pharmacology

Cell culture of the EAAT1,2,3-HEK293 cell lines and the [^{3}H]-D-Aspartate uptake assay were performed essentially as previously described.[34] The experimental procedures are described in detail in Supplementary data.

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Supplementary data

Supplementary data associated (the emission spectra for the coumarin analogs **12**, **14** and **UCPH-102F** are available) with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.049.

References and notes

1. Beart, P. M.; O'shea, R. D. *Br. J. Pharmacol.* **2007**, *150*, 5.
2. Foster, A. C.; Kemp, J. A. *Curr. Opin. Pharmacol.* **2006**, *6*, 7.
3. Bunch, L.; Erichsen, M. N.; Jensen, A. A. *Expert Opin. Ther. Targets* **2009**, *13*, 719.
4. Lee, S. G.; Su, Z. Z.; Emdad, L.; Gupta, P.; Sarkar, D.; Borjabad, A.; Volsky, D. J.; Fisher, P. B. *J. Biol. Chem.* **2008**, *283*, 13116.
5. Estrada-Sanchez, A. M.; Mejia-Toiber, J.; Massieu, L. *Arch. Med. Res.* **2008**, *39*, 265.
6. Corona, J. C.; Tovar-y-Romo, L. B.; Tapia, R. *Expert Opin. Ther. Targets* **2007**, *11*, 1415.
7. Muir, K. W. *Curr. Opin. Pharmacol.* **2006**, *6*, 53.
8. Sheldon, A. L.; Robinson, M. B. *Neurochem. Int.* **2007**, *51*, 333.
9. Alexander, G. M.; Godwin, D. W. *Epilepsy Res.* **2006**, *71*, 1.
10. Sanacora, G.; Treccani, G.; Popoli, M. *Neuropharmacology* **2011**, 1.
11. Karlsson, R. M.; Tanaka, K.; Saksida, L. M.; Bussey, T. J.; Heilig, M.; Holmes, A. *Neuropsychopharmacology* **2009**, *34*, 1578.
12. Karlsson, R. M.; Tanaka, K.; Heilig, M.; Holmes, A. *Biol. Psychiatry* **2008**, *64*, 810.
13. Lavis, L. D.; Raines, R. T. *ACS Chem. Biol.* **2008**, *3*, 142.
14. Reynolds, G. A.; Drexhage, K. H. *Opt. Commun.* **1975**, *13*, 222.
15. Fisher, J. F.; Trama, L. A. *J. Agric Food Chem.* **1979**, *27*, 1334.
16. Sun, W. C.; Gee, K. R.; Haugland, R. P. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3107.
17. Jones, G.; Jackson, W. R.; Choi, C. *J. Phys. Chem.* **1985**, *89*, 294.
18. Khalfan, H.; Abuknesha, R.; Randweaver, M.; Price, R. G.; Robinson, D. J. *J. Histochem.* **1986**, *18*, 497.
19. Fan, L.; Lewis, R. W.; Hess, G. P.; Ganem, B. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3932.
20. Cohen, J. D.; Thompson, S.; Ting, A. Y. *Biochemistry* **2011**, *50*, 8221.
21. Erichsen, M. N.; Huynh, T. H. V.; Abrahamsen, B.; Bastlund, J. F.; Bundgaard, C.; Monrad, O.; Jensen, A. B.; Nielsen, C. W.; Frydenvang, K.; Jensen, A. A.; Bunch, L. *J. Med. Chem* **2010**, *53*, 7180.
22. Jensen, A. A.; Erichsen, M. N.; Nielsen, C. W.; Stensbøl, T. B.; Kehler, J.; Bunch, L. *J. Med. Chem* **2009**, *52*, 912.
23. Balalaie, S.; Bararjanian, M.; Sheikh-Ahmadi, M.; Hekmat, S.; Salehi, P. *Synth. Commun.* **2007**, *1097*, 37.
24. Devi, I.; Bhuyan, P. J. *Tetrahedron Lett.* **2004**, *45*, 8625.
25. Ito, K.; Maruyama, J. *Chem. Pharm. Bull.* **1983**, *31*, 3014.
26. Lin, W.; Long, L.; Feng, J.; Wang, B.; Guo, C. *Eur. J. Org. Chem.* **2007**, 4301.
27. Venkateswarlu, K.; Suneel, K.; Das, B.; Reddy, K. N.; Reddy, T. S. *Synth. Commun.* **2009**, *39*, 215.
28. Guo, H.; Tanaka, F. *J. Org. Chem.* **2009**, *74*, 2417.

29. Perrone, R.; Berardi, F.; Colabufo, N. A.; Lacivita, E.; Leopoldo, M.; Tortorella, V. *J. Med. Chem.* **2003**, *46*, 646.
30. Rizzi, E.; Dallavalle, S.; Merlini, L.; Pratesi, G.; Zunino, F. *Synth. Commun.* **2006**, *36*, 1117.
31. Belluti, F.; Fontana, G.; Carenini, N.; Giommarelli, C.; Zunino, F. *Bioorg. Med. Chem.* **2010**, *18*, 3543.
32. Lang, M.; Mühlbauer, A.; Gräf, C.; Beyer, J.; Lang-Fugmann, S.; Polborn, K.; Steglich, W. *Eur. J. Org. Chem.* **2008**, 816.
33. Shi, Q.; Lee, Y.; Song, H.; Cheng, M.; Jew, S.; Park, H.; Jeong, B. *Chem. Lett.* **2008**, *37*, 436.
34. Jensen, A. A.; Bräuner-Osborne, H. *Biochem. Pharmacol.* **2004**, *67*, 2115.