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Article

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8-Benzamidochromen-4-one-2-carboxylic Acids - Potent and Selective Agonists for the Orphan G protein-coupled Receptor GPR35

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KEYWORDS

agonist, β-arrestin, chromenone, GPR35, GPR35b, GPR55, G protein-coupled receptor, orphan G protein-coupled receptors, species selectivity, structure-activity relationships

ABSTRACT

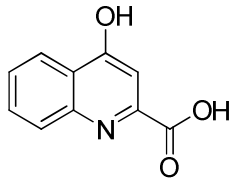
8-Amido-chromen-4-one-2-carboxylic acid derivatives were identified as novel agonists at the G protein-coupled orphan receptor GPR35. They were characterized by a β-arrestin recruitment assay, and optimized to obtain agonists with nanomolar potency for the human GPR35. The compounds were found to exhibit high selectivity versus the related GPR55. The most potent agonists were 6-bromo-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (**85**, EC_{50} 12.1 nM) and 6-bromo-8-(2-chloro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (**90**, EC_{50} 11.1 nM), both of which were >1700-fold selective versus GPR55. Most compounds were considerably less potent at rat and mouse than at human GPR35. 6-Bromo-8-(2-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (**87**) was the only derivative that activated GPR35 of all three species at similar, low micromolar concentration. Compounds **85** and **90** are the most potent agonists at the human GPR35 known to date and might thus serve as powerful pharmacological tools to further elucidate the receptor's (patho)physiological role and its potential as a future drug target.

INTRODUCTION

G protein-coupled receptors (GPCRs) are mediators and modulators of a variety of (patho)physiological processes. Endogenous agonists have been described for about 300 members of this receptor class and many of them are considered as important or at least as promising, potential pharmacotherapeutic targets.[1] However, for nearly 100 (non-olfactory) GPCRs the endogenous agonist remains unknown.[1] Those are designated orphan receptors. One of the orphan GPCRs is the human GPR35, which was first described in 1998.[2] It was reported to be coupled to $G_{i/o}$ and G_{13} proteins.[3-5] Later on a transcript variant was found that encoded for an identical protein but with 31 additional amino acids at its N-terminus.[6] That variant was designated GPR35b.[6] For both, rat and mouse, only one isoform of GPR35 has been described so far.[7, 8] There is only a moderate homology between the orthologs with a sequence identity between human GPR35 and rodent orthologs of 72% and 71% for mouse and rat GPR35, respectively, as it is observed for many other GPCRs.[9, 10] One of the phylogenetically closest relatives of the human GPR35 is GPR55.[11]

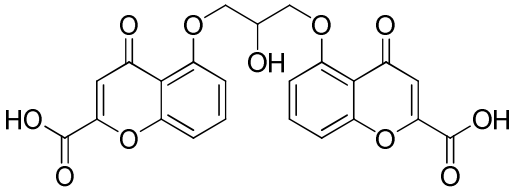
GPR35 mRNA expression was found in the stomach and intestine of humans and rodents, with an increased expression in human gastric cancer cells.[2, 5, 6] Heterologous GPR35 expression was shown to exhibit transforming activity in a mouse fibroblast cell line indicating that the receptor might play a role in cell growth control.[6] The human receptor was also found to be expressed in various immune cells,[5, 12, 13] while a further increase in receptor expression was detected in mast cells upon stimulation with IgE antibodies.[13] Following receptor activation mast cells showed reduced lipopolysaccharide-induced TNFα secretion.[5] For invariant natural killer T cells receptor activation caused a reduction of IL-4 secretion.[12] Thus, GPR35 modulates immune responses. There is also evidence for the involvement of GPR35 in pain perception: receptor mRNA expression was detected in murine neurons of the dorsal root ganglia, which are known to transmit nociceptive signals.[14, 15] Antinociceptive effects were observed after treating mice or rats with GPR35 agonists.[14-16] Furthermore, GPR35 was suggested to play a role in the regulation of blood pressure and in the pathogenesis of cardiovascular diseases.[17, 18]

Although we are only at the very beginning of understanding the physiological role of GPR35, the receptor appears to be a promising target for therapeutic intervention.



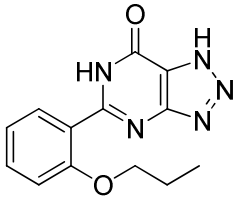
1 Kynurenic acid

EC_{50} (β-arrestin assay)
human GPR35: 217 μM
rat GPR35: 66 μM
AMPA/KainateR (IC_{50}): 10-1900 μM
NMDA receptors (IC_{50}): 15-158 μM
$GABA_AR$ (IC_{50}): 2900 μM
α7 nAChR (IC_{50}): 7-136 μM
AHR (EC_{50}): 0.3 μM



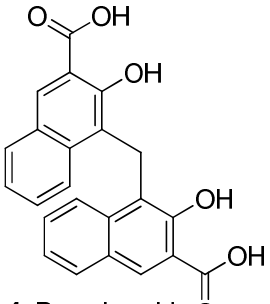
2 Cromolyn

EC_{50} (β-arrestin assay)
human GPR35: 8-17 μM
rat GPR35: 4-5 μM
mouse GPR35: 58 μM
Cl^- channels (IC_{50}): 5-310 μM
PDE (K_i): 0.0008 μM



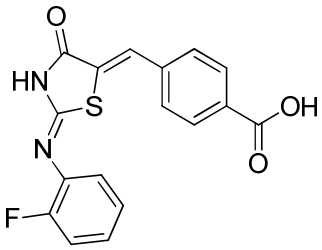
3 Zaprinast

EC_{50} (β-arrestin assay)
human GPR35: 1-8 μM
rat GPR35: 0.002-0.096 μM
mouse GPR35: 0.25-0.98 μM
PDE5 (IC_{50}): 0.76 μM



4 Pamoic acid

EC_{50} (β-arrestin assay)
human GPR35: 0.05-9 μM
rat GPR35: inactive
mouse GPR35: inactive
DNA pol. β (IC_{50}): 9 μM

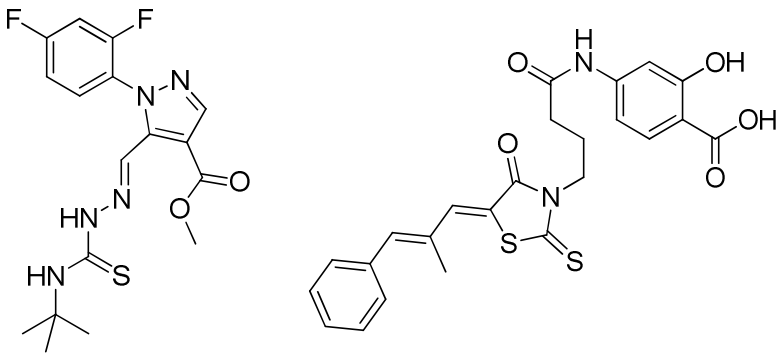


5

EC_{50} (β-arrestin assay)
human GPR35: 0.026 μM
rat GPR35: 8 μM
mouse GPR35: 17 μM

Figure 1. Selected GPR35 agonists with potencies at GPR35 orthologs determined in β-arrestin assays.[3, 19-23] Potencies of compounds at other targets are displayed in gray.[24-28] AMPAR: α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor, NMDA: N-methyl-D-aspartic acid, GABA: γ-aminobutyric acid, nAChR: nicotinic acetylcholine receptor, AHR: aryl hydrocarbon acid receptor, PDE: phosphodiesterase, DNA pol.: DNA polymerase.

The first GPR35 agonist described in the literature was the tryptophane metabolite kynurenic acid (**1**, see Fig. 1),[5] postulated to be the receptor's endogenous agonist. However, its potency - at least at the human ortholog - is relatively low with EC_{50} values typically in the high micromolar range.[5, 23] It is not clear, whether sufficiently high concentrations of **1** are reached under physiological conditions to activate the receptor.[29-31] Among the agonists described so far are several other naturally occurring compounds that might constitute endogenous agonists, including lysophosphatidic acid species,[4] and tyrosine metabolites.[32] However, up to now GPR35 is still considered an orphan receptor, since the physiological agonist has not been unambiguously identified yet.



6 CID 274587 **7** ML 145

Figure 2. Selected GPR35 antagonists.

During the past few years several synthetic GPR35 agonists have been identified, including the antiallergic drug cromolyn (**2**) and zaprinast (**3**).[8, 13] The latter has become the standard agonist for GPR35. While cromolyn and zaprinast are full agonists, pamoic acid (**4**) was shown to be a partial agonist.[3, 23] The recently described and up to now most potent agonist at the human GPR35 is the thiazolidinylidenemethylbenzoic acid derivative **5** (see Fig. 1).[22] So far, only few potent GPR35 antagonists have been described, the most potent ones being CID 2745687 (**6**) and ML 145 (**7**) (see Fig. 2).[23, 33] There are several major drawbacks in investigating the receptor's physiological role using pharmacological tools. On the one hand, although many agonists have been described, only very few show high potency with EC_{50} values in the lower nanomolar range. On the other hand, most known

agonists also bind to other targets, mostly with similar potency: e. g. kynurenic acid inhibits AMPA/kainate receptors, NMDA receptors, $GABA_A$ receptors, α7 nicotinic acetylcholine (α7 nAChR) receptors and potently activates intracellular aryl hydrocarbon acid receptors (AHR),[24] while zaprinast inhibits cGMP-specific phosphodiesterases (PDEs), especially PDE5 and 6,[34] and pamoic acid inhibits DNA polymerase β.[26] Another drawback is the fact that the known GPR35 antagonists are virtually inactive at the rodent orthologs.[21] Thus, there is a need of highly potent and selective GPR35 ligands.

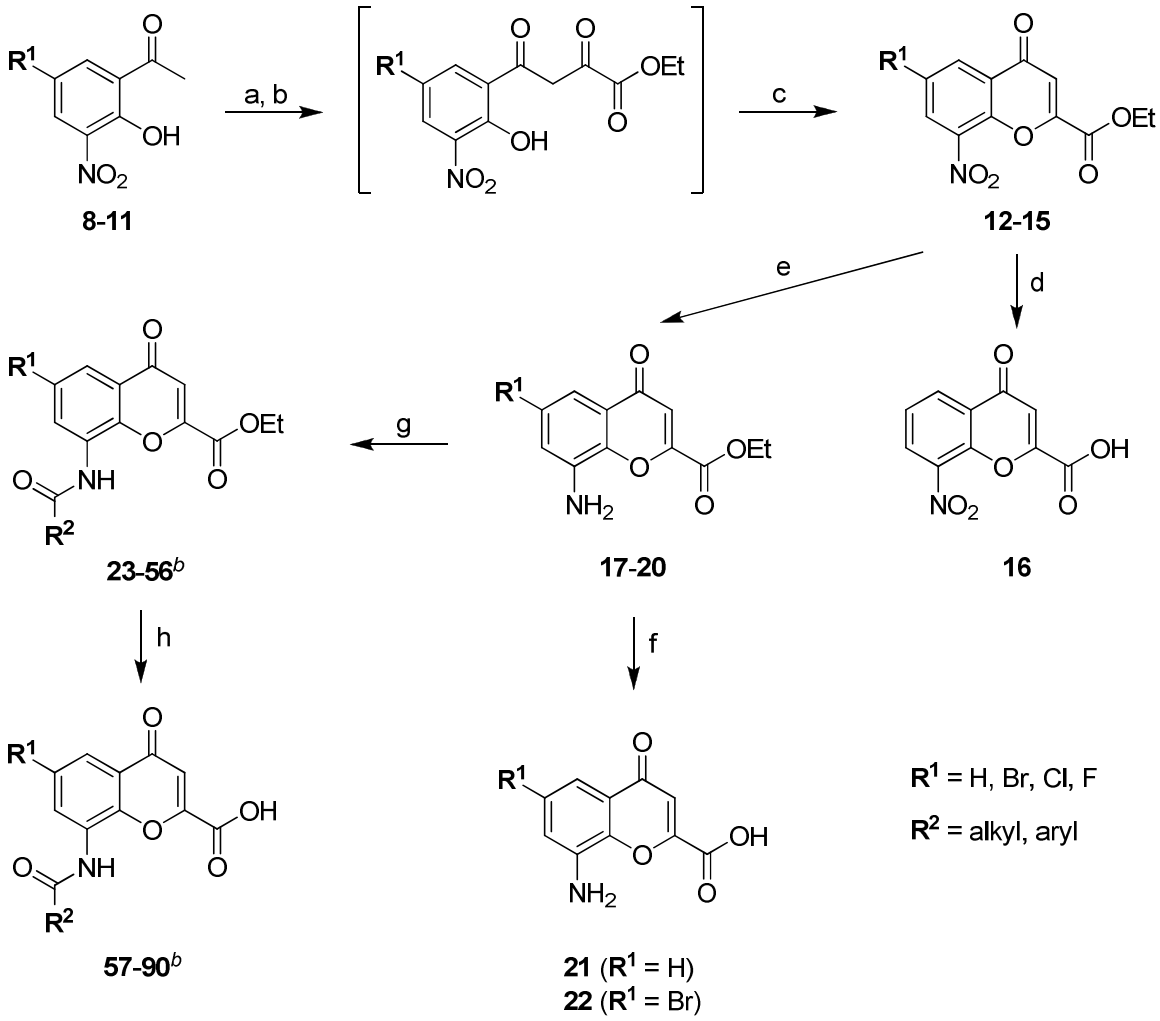
In most published studies focusing on the identification of GPR35 receptor ligands large compound libraries were screened. This led to the discovery of structurally diverse ligands. However, the number of studies on structure-activity relationships (SARs) of GPR35 ligands is very limited. In the present study, we intended to identify a suitable scaffold to develop highly potent and selective GPR35 agonists. Therefore, we started by screening an in-house compound library (http://mueller-group.pharma.uni-bonn.de/mueller-laboratory/compound-library). For this purpose we used a β-arrestin recruitment assay, the most commonly used assay system to pharmacologically characterize GPR35 ligands. We identified a chromen-4-one-2-carboxylic acid, which shared a sub-structure with cromolyn, as a GPR35 agonist with moderate potency. Subsequently, we synthesized a series of mostly novel 8-substituted chromen-4-one-2-carboxylic acid derivatives to study their structure-activity relationships as novel GPR35 agonists.

RESULTS AND DISCUSSION

Chemistry

A large variety of chromen-4-one-2-carboxylic acids with different halogen atoms at the 6-position and various alkyl- or arylamido residues at the 8-position were synthesized (Scheme 1). The compounds were prepared starting from **8**, or the corresponding 5-halogeno derivatives **9**–**11**, respectively. Based on a recently described synthesis of pranlukast derivatives,[35] the acetophenones **8**–**11** were treated with diethyloxalate and potassium *tert*-butoxide as a base in *N*,*N*-dimethylformamide (DMF) at 0–5 °C. The resulting diketone intermediates were isolated after acidification, and subsequently reacted with hydrochloric acid in ethanol under reflux yielding the chromen-4-one derivatives **12**–**15** in very good yields (70–82%). Compounds **12**[36, 37] and **13**[38, 39] had previously been described in the literature using different reaction conditions. The final product **16**[40] was obtained in an excellent yield of 96% by hydrolysis of its ethyl ester **12** using optimized mild basic conditions (potassium carbonate in a mixture of tetrahydrofuran (THF), ethanol and water at room temperature), while strong basic conditions led to a cleavage of the benzopyran-4-one ring. The nitro groups of intermediates **12**–**15** were reduced[41] using tin(II) chloride in diluted hydrochloric acid/ethanol yielding the corresponding amino derivatives **17**,[41] **18**,[38] **19**, and **20** in excellent yields (80–90%). The final chromen-4-one-2-carboxylic acids **21**[42] and **22** were formed by mild basic hydrolysis of **17** and **18**, respectively, under the same conditions as described above for **16**.

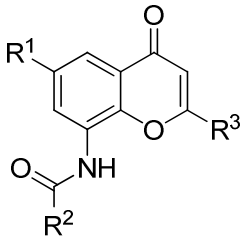
Scheme 1. Synthesis of chromen-4-one-2-carboxylic acids[a]

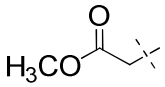


[a] Reagents and conditions: (a) $(COOEt)_2$, KO^{t}Bu, DMF, argon, 0–5 °C, 2–3 h; (b) concd. aq. HCl, ice-water; (c) concd. aq. HCl, EtOH, reflux, overnight, yield 70–82%; (d) K_2CO_3, THF, EtOH, H_2O, rt, overnight; aq. HCl (1 N), H_2O, yield 96%; (e) $SnCl_2 \cdot 2H_2O$, aq. HCl (2 N), EtOH, 65–70 °C, 20–45 min, yield 80–90%; (f) K_2CO_3, THF, EtOH, H_2O, rt, overnight; aq. HCl (1 N), H_2O, yield 96–99%; (g) acetic anhydride, DIPEA, DCM, argon, rt, 2 d for **23**; **R^2**COCl, DIPEA, DCM (THF), argon, rt, 1–4 d for **24**–**56**, yield 21–94%; (h) K_2CO_3, THF, EtOH, H_2O, rt, 2–24 h; aq. HCl (2 N), H_2O, yield 45–99%. [b] For R^2 see Tables 1 and 2.

The amines **17**–**20** were reacted with various acid chlorides in the presence of *N*,*N*-diisopropylethylamine (DIPEA) as a base in dichloromethane (DCM), or in a 6:1 mixture of DCM and THF at room temperature (rt) yielding a series of ethyl 8-amido-5-halogeno-chromen-4-one-2-carboxylates (Scheme 1). A large range of differently substituted benzamido residues were introduced at the 8-position of the chromen-4-one ring system. The structures and yields of the synthesized compounds are shown in Tables 1 and 2. Compound **23**[43] was obtained by treatment of **17** with acetic anhydride in the presence of DIPEA in DCM at rt after a reaction time of two days. In the last step, the ethyl esters **23**–**56** were hydrolyzed yielding the desired chromen-4-one-2-carboxylic acids **57**–**90** (Scheme 1, see Tables 1 and 2 for structures and yields). Mild basic reaction conditions as described above for the hydrolysis of compound **16** were applied.

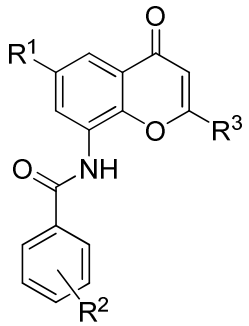
Table 1. Yields of 8-amidochromen-4-one-2-carboxylic acids and their ethyl esters



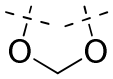
R^1	R^2	compd	yield (%)[a]	compd	yield (%)[a]
		$R^3 = CO_2Et$		$R^3 = CO_2H$	
H	methyl	**23**	82	**57**	77
H	ethyl	**24**	80	**58**	82
H		**25**	85	**59**	90
H	cyclohexyl	**26**	93	**60**	82
H	benzyl	**27**	85	**61**	97
H	2-naphthyl	**28**	85	**62**	94
H	2-quinolinyl	**29**	80	**63**	49

[a] Isolated yields.

Table 2. Yields of substituted 8-benzamidochromen-4-one-2-carboxylic acids and their ethyl esters



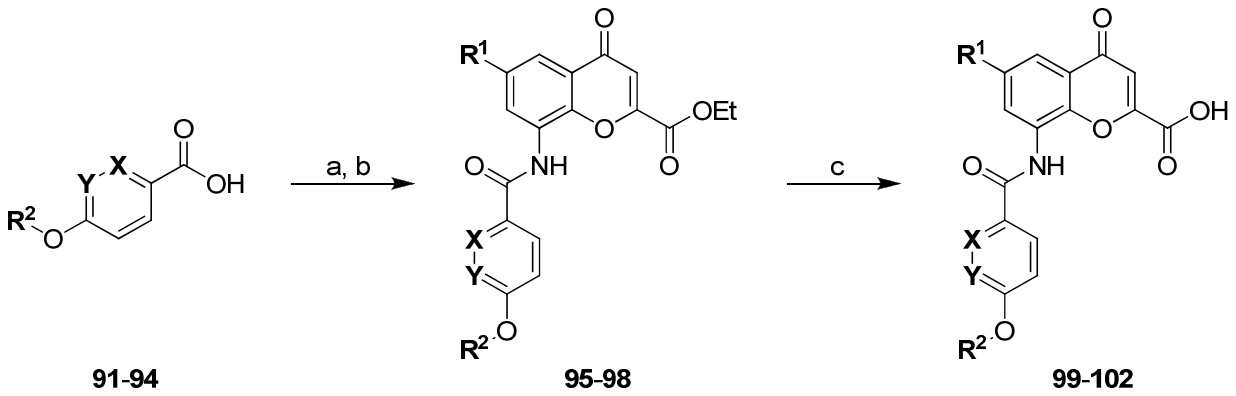
substitution pattern				compd	yield (%)[a]	compd	yield (%)[a]
R^1	R^2			$R^3 = CO_2Et$		$R^3 = CO_2H$	
	ortho	*meta*	*para*				
H	H	H	H	**30**	92	**64**	89
H	H	CH_3	H	**31**	94	**65**	95
H	H	NO_2	H	**32**	83	**66**	79
H	H	H	CH_3	**33**	75	**67**	49
H	H	H	CF_3	**34**	71	**68**	62
H	H	H	OCH_3	**35**	89	**69**	85
H	H	H	Br	**36**	76	**70**	45
F	Cl	Cl	H	**37**	21	**71**	89
F	Cl	H	Cl	**38**	78	**72**	97
F	H	Cl	Cl	**39**	66	**73**	92
F	H	H	OCH_3	**40**	88	**74**	98
Cl	H	H	H	**41**	78	**75**	95
Cl	H	CH_3	H	**42**	91	**76**	82
Cl	H	H	NO_2	**43**	55	**77**	99
Cl	H	H	OCH_3	**44**	88	**78**	98
Br	H	H	H	**45**	81	**79**	73
Br	H	CH_3	H	**46**	71	**80**	99
Br	H	H	Cl	**47**	80	**81**	98
Br	Cl	H	Cl	**48**	73	**82**	91
Br	H	Cl	Cl	**49**	71	**83**	98
Br	H	H	CN	**50**	57	**84**	71
Br	H	H	OCH_3	**51**	89	**85**	95
Br	H	H	ethoxy	**52**	65	**86**	99
Br	OCH_3	H	H	**53**	85	**87**	86

Br	H	OCH_3	OCH_3	**54**	76	**88**	97
Br	H			**55**	79	**89**	96
Br	Cl	H	OCH_3	**56**	81	**90**	95

[a] Isolated yields.

The final products **99** and **100** were obtained as follows. Treatment of **91** and **92**, respectively, with thionyl chloride in the presence of DMF as a catalyst in DCM at rt for 30 min afforded the corresponding acid chlorides (Scheme 2). Coupling with amine **17**, using the same conditions as described for **23**–**56**, yielded the amides **95** and **96** in good yields. In the last step, the ethyl ester groups of **95** and **96** were hydrolyzed as described above to obtain the desired chromen-4-one-2-carboxylic acids **99** and **100** in excellent yields of 99%. Acid **91** was synthesized starting from methyl 4-hydroxybenzoate, which was converted with cyclopropylmethyl bromide by Williamson ether synthesis (potassium carbonate, DMF, 110–115 °C, overnight).[44] Methyl 4-(cyclopropylmethoxy)benzoate (**103**)[45] was obtained in a good yield (78%). The efficient hydrolysis of the ester, using a KOH solution in methanol under microwave irradiation at 100 °C for 45 min provided the benzoic acid derivative **91**[46] in an excellent yield of 98%. The acids **93** and **94** were converted to their acid chlorides as depicted in Scheme 2, and subsequently reacted with amine **18** in the presence of DIPEA as a base in a 6:1 mixture of DCM and THF at rt for two days yielding the amides **97** or **98**. Good yields of 79–80% were obtained. Ester hydrolysis afforded the target compounds **101** and **102** in yields of 68% and 98%, respectively, using mild basic conditions.

Scheme 2. Synthesis of 8-amidochromen-4-one-2-carboxylic acid derivatives[a]



compd	R^1	R^2	X	Y
99	H	cyclopropylmethyl	CH	CH
100	H	*n*-propyl	CH	CH
101	Br	methyl	N	CH
102	Br	methyl	CH	N

[a]Reagents and conditions: (a) $SOCl_2$, cat. DMF, DCM, argon, rt, 30 min; (b) **17**, DIPEA, DCM, argon, rt, 24 h, yield 74–79% for **95**–**96**; **18**, DIPEA, DCM, THF, argon, rt, 2 d, yield 79–80% for **97**–**98**; (c) K_2CO_3, THF, EtOH, H_2O, rt, overnight; aq. HCl (2 N), H_2O, yield 68–99%.

Altogether, 41 final products (carboxylates) were synthesized, analyzed and tested, 39 of which are new compounds not previously described in the literature. In addition, 37 of the prepared intermediate esters were not previously described and therefore fully analyzed as well. The structures of the synthesized compounds were confirmed by ^1H and ^{13}C NMR spectroscopy, in addition to HPLC analysis coupled to electrospray ionization mass spectrometry (LC/ESI-MS), which was also used to determine the compounds' purity.

Hydrolytic stability

Hydrolytic stability tests were performed for the most active compound of the series, **c**hromen-4-one-2-carboxylic acid **85**, at three different pH values. Therefore, three samples were prepared: (i) a 1.20 mM solution of **85** in 1:1 acetonitrile/water (pH 3.5) containing acetic acid (300 mM) and ammonium acetate (2 mM), (ii) a 1.20 mM solution of **85** in 1:1 acetontrile/water (pH 5.5), and (iii) a 1.20 mM solution of **85** in 1:1 acetontrile/water (pH 8.0) containing ammonia (1 M) and ammonium acetate (2 mM). All samples were analyzed by LC-MS directly, and after 90 min, 4 hours and 2–4 days, respectively. In all cases, no degradation of **85** occurred. These results confirmed the hydrolytic stability of **c**hromen-4-one-2-carboxylic acids over a wide pH range from acidic to basic conditions, which can be observed in the human body.

Pharmacological Evaluation

The Pathhunter® β-arrestin assay (DiscoverX, Fremont, CA) was employed, in which the receptors were C-terminally tagged with a small enzyme fragment. This construct was inserted into a Chinese hamster ovary (CHO) cell line stably expressing β-arrestin fused to an N-terminal deletion mutant of β-galactosidase. Upon activation, receptors recruited β-arrestin fusion proteins, which allowed enzyme complementation. Thus, subsequently measured enzyme activity correlated with agonist-induced β-arrestin recruitment. The compounds were initially tested at a concentration of 10 µM for their agonistic potency at the human GPR35. Effects were normalized to the signal induced by 30 µM zaprinast corresponding to its maximal effect. If compounds showed effects >50%, concentration-response curves were determined. Compounds that did not show agonistic activity were tested for their potency to antagonize receptor activation by 5 µM zaprinast (~ EC_{80}). A selection of compounds was additionally tested at the human GPR35b. Compounds were further evaluated in β-arrestin assays at the mouse and rat GPR35, and, in addition, at the human GPR55 to determine selectivity. Effects were normalized to the signal induced by 30 and 10 µM zaprinast for mouse and rat GPR35, respectively, or by 1 µM lysophospatidylinositol (LPI) for GPR55. The compounds were additionally examined for their potency

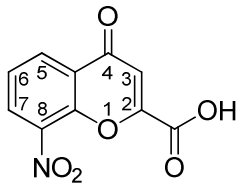
to inhibit GPR55-β-arrestin recruitment induced by 1 μM LPI. Results are summarized in Tables 3, 4, 5 and 6.

Structure-Activity Relationships

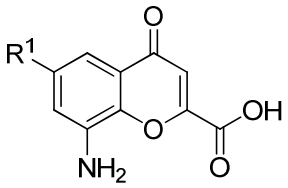
A series of 41 mostly novel chromen-4-one-2-carboxylic acids and one intermediate ethyl ester (compounds **16**, **21**, **22**, **40**, **57–90**, and **99–102**, see Tables 3 and 4) were evaluated at the human GPR35. All compounds were shown to be agonists except for **21**, **99** and **100**, which were inactive. Some compounds showed a somewhat increased or reduced efficacy compared to that of the standard agonist zaprinast. However, there was no obvious correlation pattern between the compounds' structures and their efficacy, and most of the differences were not statistically significant. Thus, the majority of compounds can be considered to be full agonists. The 8-nitro-substituted derivative **16** was the simplest compound that showed weak agonistic properties (EC_{50} 46300 nM). If the nitro group of **16** was reduced to an amino function (in **21**), the resulting compound was virtually inactive in both, agonist and antagonist assays. This indicated that lipophilic residues and hydrogen bond-accepting groups might be favorable at position 8. Due to this observation we chose an amide linker to introduce various larger 8-substituents. The acetamide (**57**) showed a 2-fold increase in potency (EC_{50} 24100 nM) compared to the nitro derivative **16**. Introduction of longer and bulkier alkyl substituents further improved potency: ethyl- (**58**, EC_{50} 13400 nM) and cyclohexyl- (**60**, EC_{50} 4200 nM) substituted compounds had 2- and 6-fold increased potency, respectively, compared to **57**. The introduction of an alkyl substituent with a distal methyl ester (**59**, EC_{50} 20200 nM), thus displaying increased polarity, appeared not to be beneficial. In contrast, compounds with aromatic groups in that position, such as phenyl (**64**, EC_{50} 4910 nM), 2-naphthyl (**62**, EC_{50} 4670 nM) and 2-quinolinyl (**63**, EC_{50} 1340 nM) residues were equally or in case of the latter even more potent than the cyclohexyl derivative **60**. However, elongation of the linker between the chromenone core and the distal aromatic group led to a somewhat decreased potency, as observed for the benzyl derivative **61** (EC_{50} 8960 nM). Bromo-substitution in the 6-position of the

chromenone scaffold appeared to have a strong effect on potency: 6-bromination of the inactive 8-amino derivative **21** led to **22** with an EC_{50} value of 12600 nM (Table 3).

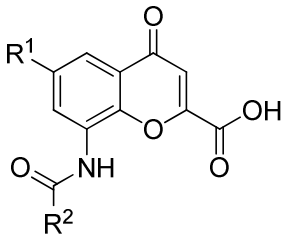
Table 3. Potency of 8-substituted chromen-4-one-2-carboxylic acid derivatives and standard agonists at GPR35



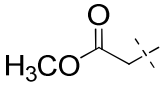
16



21, **22**

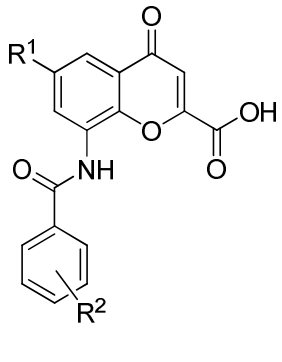


57-63

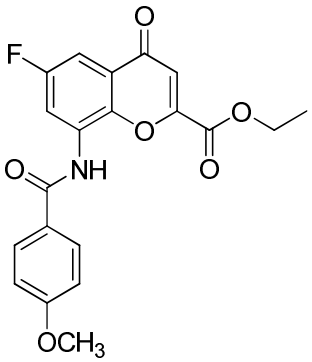
compd	R^1	R^2	human GPR35 EC_{50} ± SEM (μM) (% Effect[a] ± SEM)	human GPR35 E_{max}[a]	mouse GPR35 EC_{50} ± SEM (μM) (% Effect[a] ± SEM)	mouse GPR35 E_{max}[a]	rat GPR35 EC_{50} ± SEM μM (% Effect[a] ± SEM)	rat GPR35 E_{max}[a]
2, cromolyn			**1.26** ± 0.17	119	**4.84** ± 0.60	102	**0.986** ± 0.126	104
3, zaprinast			**1.96** ± 2.4	100	**1.60** ± 0.04	100	**0.0611** ± 0.0061	100
16			**46.3** ± 3.2	101	(4 ± 6)	-	(38 ± 3)	-
21	H		(2 ± 4)	-	(4 ± 2)	-	(40 ± 2)	-
22	Br		**12.6** ± 1.5	84	(18 ± 4)	-	(40 ± 4)	-
57	H	methyl	**24.1** ± 3.3	91	(3 ± 1)	-	(31 ± 3)	-
58	H	ethyl	**13.4** ± 3.4	119	(12 ± 1)	-	(30 ± 5)	-
59	H		**20.2** ± 5.7	87	(15 ± 4)	-	**6.65** ± 0.34	89
60	H	cyclohexyl	**4.20** ± 0.38	118	(38 ± 5)	-	**1.20** ± 0.12	79
64	H	phenyl	**4.91** ± 0.65	107	(13 ± 4)	-	(39 ± 3)	-
61	H	benzyl	**8.96** ± 0.43	107	(19 ± 4)	-	(47 ± 5)	-
62	H	2-naphthyl	**4.67** ± 1.00	125	**2.99** ± 0.51	79	(46 ± 3)	-
63	H	2-quinolinyl	**1.34** ± 0.06	81	**3.59** ± 0.30	65	(41 ± 6)	-

[a]Screening was performed at a concentration of 10 μM. Effects were normalized to the signal induced by 30 μM (human and mouse) and 10 μM zaprinast (rat), corresponding to a maximal response at the respective receptor.

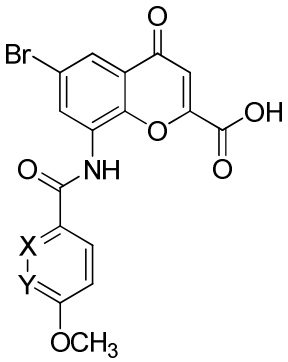
Table 4. Potency of 8-benzamido-chromen-4-one-2-carboxylic acid derivatives at GPR35



64-**90**, **99**, **100**

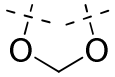


40



101 (X = N, Y = CH)
102 (X = CH, Y= N)

compd	R^1	R^2			human GPR35		mouse GPR35		rat GPR35	
	ortho	*meta*	*para*		EC_{50} ± SEM (μM) (% Effect[a] ± SEM)	E_{max}[a]	EC_{50} ± SEM (μM) (% Effect[a] ± SEM)	E_{max}[a]	EC_{50} ± SEM (μM) (% Effect[a] ± SEM)	E_{max}[a]
65	H	H	CH_3	H	**5.44** ± 1.34	70	(18 ± 4)	-	**2.75** ± 0.35	71
66	H	H	NO_2	H	**6.97** ± 0.30	112	(26 ± 3)	-	(32 ± 6)	-
67	H	H	H	CH_3	**1.38** ± 0.14	104	(43 ± 2)	-	(43 ± 3)	-
68	H	H	H	CF_3	**15.5** ± 3.1	81	(23 ± 2)	-	(38 ± 4)	-
69	H	H	H	OCH_3	**0.346** ± 0.037	104	**5.03** ± 0.67	66	**2.16** ± 0.22	85
70	H	H	H	Br	**0.804** ± 0.148	122	(28 ± 1)	-	(49 ± 6)	-
71	F	Cl	Cl	H	**0.382** ± 0.056	106	n. d.	-	**3.13** ± 0.28	82
72	F	Cl	H	Cl	**0.0170** ± 0.0017	113	(24 ± 4)	-	(46 ± 3)	-
73	F	H	Cl	Cl	**0.117** ± 0.026	131	(37 ± 4)	-	**2.65** ± 0.36	80
74	F	H	H	OCH_3	**0.112** ± 0.011	123	(30 ± 4)	-	**1.76** ± 0.08	78
40					**0.112** ± 0.024	80	(7 ± 2)	-	(1 ± 1)	-
75	Cl	H	H	H	**0.430** ± 0.063	115	(31 ± 3)	-	**2.84** ± 0.41	65
76	Cl	H	CH_3	H	**1.22** ± 0.17	102	(31 ± 5)	-	**2.10** ± 0.21	92
77	Cl	H	H	NO_2	**1.71** ± 0.16	130	(18 ± 1)	-	**3.14** ± 0.65	115
78	Cl	H	H	OCH_3	**0.0168** ± 0.0021	110	(34 ± 3)	-	**1.83** ± 0.27	86
79	Br	H	H	H	**0.303** ± 0.021	110	(40 ± 4)	-	**2.75** ± 0.60	75

80	Br	H	CH_3	H	**0.842** ± 0.165	138	(27 ± 5)	-	**2.08** ± 0.33	79
81	Br	H	H	Cl	**0.0251** ± 0.0019	115	(27 ± 4)	-	(47 ± 1)	-
82	Br	Cl	H	Cl	**0.0164** ± 0.0011	110	(12 ± 4)	-	(32 ± 3)	-
83	Br	H	Cl	Cl	**0.0154** ± 0.0030	110	(29 ± 2)	-	**3.25** ± 0.35	62
84	Br	H	H	CN	**0.399** ± 0.060	108	(41 ± 1)	-	**2.26** ± 0.21	99
85	Br	H	H	OCH_3	**0.0121** ± 0.0010	121	(36 ± 1)	-	**1.40** ± 0.03	88
86	Br	H	H	ethoxy	**0.634** ± 0.076	99	(36 ± 3)	-	**1.36** ± 0.16	74
87	Br	OCH_3	H	H	**1.66** ± 0.28	91	**4.95** ± 0.81	71	**1.10** ± 0.18	73
88	Br	H	OCH_3	OCH_3	**0.425** ± 0.075	102	**6.40** ± 0.48	73	**1.30** ± 0.16	106
89	Br	H			**0.0599** ± 0.0042	107	(34 ± 4)	-	**2.05** ± 0.55	86
90	Br	Cl	H	OCH_3	**0.0111** ± 0.0031	120	(35 ± 2)	-	**4.17** ± 0.33	81
99	H	H	H	cyclo-propyl-methoxy	(10 ± 3)	-	(13 ± 3)	-	(48 ± 1)	-
100	H	H	H	prop-oxy	(4 ± 2)	-	(13 ± 4)	-	**3.61** ± 0.38	80
101					**0.144** ± 0.017	115	(27 ± 5)	-	**1.54** ± 0.21	68
102					**0.0305** ± 0.0016	117	**4.98** ± 0.35	64	**0.991** ± 0.064	94

[a]Screening was performed at a concentration of 10 μM. Effects were normalized to the signal induced by 30 μM (human and mouse) and 10 μM zaprinast (rat), corresponding to a maximal response at the respective receptor.

The phenyl-substituted derivative **64** (8-benzamido-4-oxo-4*H*-chromene-2-carboxylic acid) was selected for further modification due to its high potency, and because a large number of benzoic acid derivatives are easily accessible. In addition, we introduced different halogen atoms in the 6-position due to the initial observation that a bromine atom in that position considerably improved potency (compare **21** to **22**). 8-Benzamidochromen-4-one-2-carboxylic acids with a variety of substituents in the *o*-, *p*-, and *m*-position (mono- and disubstitutions) were investigated. We most thoroughly studied substituents in the *para*-position, since some substituents in that position led to a considerable increase

in potency. The following rank order of potency among compounds with varying substituents in the *para*-position was observed: methoxy (**69**, EC_{50} 346 nM) > bromo (**70**, EC_{50} 804 nM) > methyl (**67**, EC_{50} 1380 nM) > hydrogen (**64**, EC_{50} 4910 nM) > trifluoromethyl (**68**, EC_{50} 15500 nM) > propoxy (**99**) ≈ cyclopropylmethoxy (**100**). *meta*-Substitution with a methyl (**65**, EC_{50} 5440 nM) or a nitro group (**66**, EC_{50} 6970 nM) did not increase potency compared to the unsubstituted benzamidochromenone derivative **64** (EC_{50} 4910 nM). Since *para*-methoxy substitution appeared most favorable so far (**69**, EC_{50} 346 nM) we combined it with halogen substitution in the 6-position in order to investigate whether a combination could further improve potency. After comparing **69** (6-H) with **74** (6-F, EC_{50} 112 nM) **78** (6-Cl, EC_{50} 16.8 nM), and **85** (6-Br, EC_{50} 12.1 nM) it became clear that any of the introduced halogen atoms caused an increase in potency. However, the larger bromine substituent had the greatest impact, followed by the chlorine and fluorine substituents. Although the difference in potencies between bromine- and chlorine-substituted compounds was small, it was consistent (observable for **79**, 6-Br, EC_{50} 303 nM versus **75**, 6-Cl, EC_{50} 430 nM; **85**, 6-Br, EC_{50} 12.1 nM versus **78**, 6-Cl, EC_{50} 16.8 nM; and **80**, 6-Br, EC_{50} 842 nM versus **76**, 6-Cl, EC_{50} 1200 nM). Interestingly, another group also found that the introduction of a bromine atom as a substituent of a bicyclic aromatic core structure greatly increased the compound's potency at the human GPR35.[47] Thus, there seems to be a lipophilic pocket, which is filled by the bromine atom.

Since bromine was discovered to be the best 6-substituent, most further derivatives contained a bromine atom at that position and only few 6-fluoro- and 6-chloro-substituted derivatives were prepared. The *p*-methoxy substituent at the 8-benzamide residue was also found to the best monosubstitution in the 6-bromo-substituted series (**85**, EC_{50} 12.1 nM). It was 2-fold more potent than the *p*-chloro-substituted derivative **81** (EC_{50} 25.1 nM). The rank order of potency for 6-bromo-substituted compounds with varying substituents in the *para*-position was as follows: methoxy (**85**, EC_{50} 12.1 nM) > chloro (**81**, EC_{50} 25.1 nM) > hydrogen (**79**, EC_{50} 303 nM) ≥ cyano (**84**, EC_{50} 399 nM) > ethoxy (**86**, EC_{50} 634 nM).

In the 6-chloro-substituted series, which behaved very similar to the 6-bromo-substituted series, it was shown that a *p*-nitro function was not well tolerated, leading to a reduced potency for compound **77** with an EC_{50} value of 1710 nM (compared to the 6-chloro-substituted benzamidochromenone **75**, EC_{50} 430 nM).

A methoxy substitution in the *o*-position, investigated in the series of 6-bromo-substituted compounds, was not well tolerated reducing potency by a factor of 6 compared to the unsubstituted derivative (**87**, EC_{50} 1660 nM and **79**, EC_{50} 303 nM). A methyl residue in the *m*-position was better tolerated reducing potency only 2-fold (**80**, EC_{50} 842 nM). A similar effect was observed in the 6-chloro-substituted series: 3-fold reduction in potency by an *m*-methyl residue (**76**, EC_{50} 1220 nM compared to unsubstituted **75**, EC_{50} 430 nM).

Furthermore disubstitution of the benzamide ring was investigated. In the 6-fluoro-substituted series *o*,*m*- (**71**), *o*,*p*- (**72**) and *m*,*p*- (**73**) dichloro-substituted derivatives were investigated. The rank order of potency was as follows: *o*,*p*-dichloro (EC_{50} 17.0 nM) >> *m*,*p*-dichloro (EC_{50} 117 nM) > *o*,*m*-dichloro (EC_{50} 382 nM). This result confirmed again the importance of a *para*-substituent such as a chlorine atom (or a methoxy group). Therefore, in the 6-bromo-substituted series, we only investigated the *o*,*p*-dichloro- (**82**) and the *m*,*p*-dichloro- (**83**) substituted derivatives. Here, both substitution patterns led to potent GPR35 agonists with almost the same potency as the mono-methoxy-substituted compound **85** (**82**, EC_{50} 16.4 nM; **83**, EC_{50} 15.4 nM; **85**, EC_{50} 12.1 nM). This result demonstrated the interdependence of the substituents in the 6- and the 8-position. Thus, SARs were not always parallel in the 6-fluoro- and the 6-bromo-substituted series of compounds.

A *m*,*p*-dimethoxy substitution in the 6-bromo-substituted derivative **88** led to a large reduction in activity compared to the *p*-mono-methoxy-substituted compound (**88**, EC_{50} 425 nM; **85**, EC_{50} 12.1 nM). The corresponding, less bulky *m*,*p*-methylendioxy-substitution derivative was better tolerated (**89**, EC_{50} 59.9 nM). A compound in which an *o*-chloro substituent was combined with a *p*-methoxy substituent did not show significantly improved potency, but was well tolerated (**90**, EC_{50} 11.1 nM; **85** EC_{50} 12.1 nM).

Finally, the *p*-methoxybenzamide was replaced by corresponding nitrogen-containing methoxypyridine rings. A nitrogen atom in the 3-position (**102**, EC_{50} 30.5 nM) was better tolerated than in the 2-position (**101**, EC_{50} 144 nM), but both compounds showed reduced potency as compared to the parent methoxybenzamido derivative.

Most, although not all of the GPR35 agonists described in literature are negatively charged under physiological conditions. In order to address the question whether the negative charge of the chromen-4-one-2-carboxylic acid derivatives contributed to their activity the intermediate compound **40**, the ethyl ester of the potent agonist **74**, was also tested. Both compounds were equally potent (EC_{50} 112 nM). This is surprising because ethyl esters of other acidic GPR35 agonists were found to have a strongly reduced potency.[20, 47, 48] Although **40** was equally potent, its efficacy was considerably reduced compared to that of **74** (by 40%) and it thus appeared to be a partial agonist. One explanation might be a cleavage of the ethyl ester during the assay, for example by esterases. However, the ester and the acid clearly differed in their potency at the rat GPR35, the acid being active (EC_{50} 1.76 µM) while the ester was inactive (no activation at 10 µM, see Table 4). Additional studies, including the preparation of stable, non-acidic derivatives, will be performed in the future to address this issue.

All in all, we could show that the potency of the virtually inactive chromen-4-one-2-carboxylic acid **21** was greatly increased when a phenyl residue was attached via an amide linker in the 8-position of the core structure (**64**, EC_{50} 4910 nM). The 6-bromo-substitution at the chromenone core (**79**, EC_{50} 303 nM) and the introduction of a methoxy group in the *para*-position of the phenyl ring (**69**, EC_{50} 346 nM) further increased the compounds' potency. Combination of both substitutions had additive effects on potency and yielded an agonist with an EC_{50} value of 12.1 nM (**85**). Furthermore, we observed that chlorine substituents at the phenyl ring of the 8-benzamido-chromen-4-one-2-carboxylic acid derivatives also caused a significant increase in potency: the *p*-chloro-substituted **81** had an EC_{50} value of 25.1 nM, while the potency of dichloro-substituted **82** and **83** was somewhat higher (EC_{50} values 16.4

and 15.4 nM, respectively; for concentration-response curves of the compounds mentioned above see Fig. 3). Thus, we successively developed a set of novel, highly potent GPR35 agonists with **85** and **90** being the most potent agonists yet described for GPR35, at least when comparing data from β-arrestin recruitment assays.

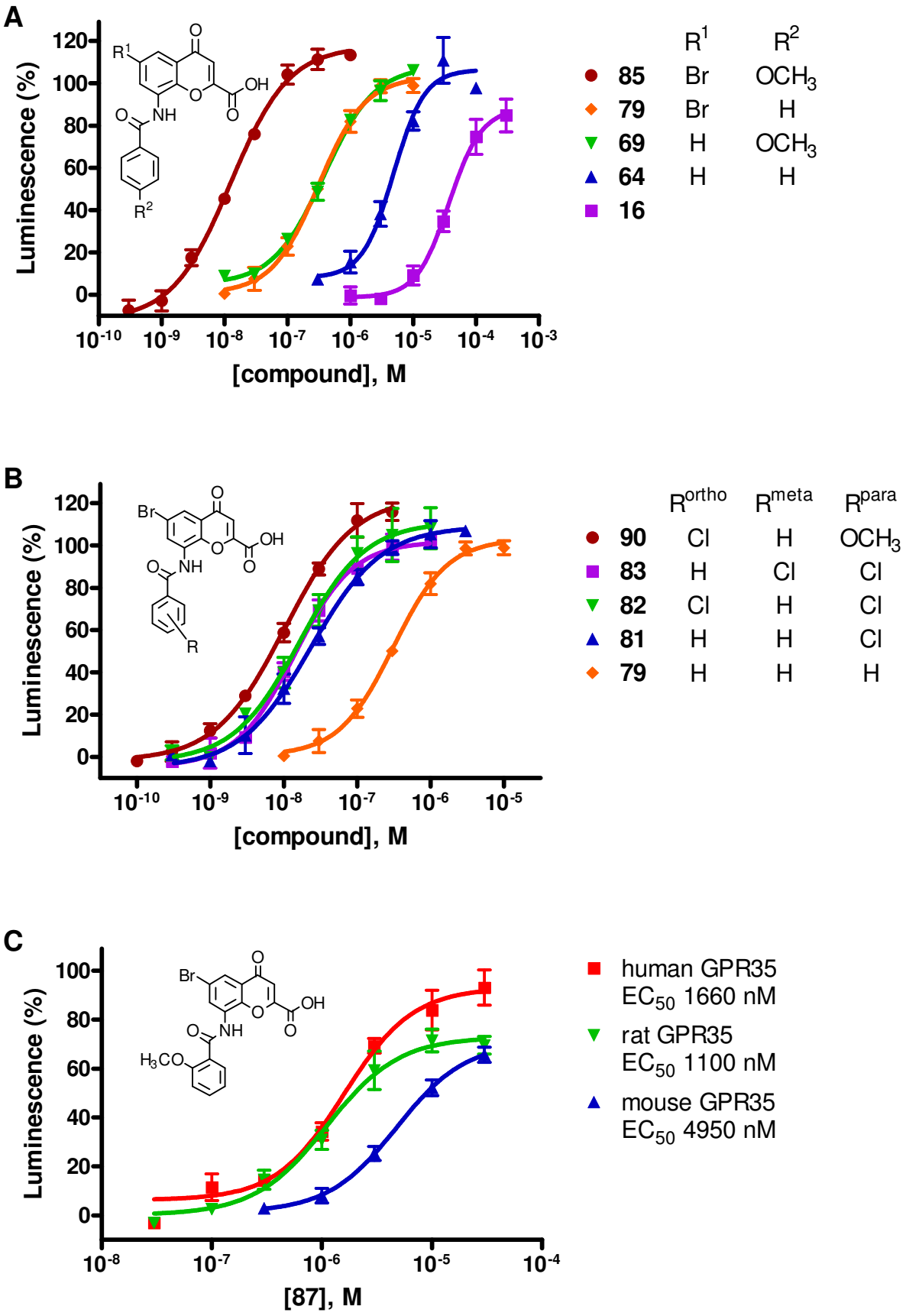

A
Luminescence (%)
[compound], M
R1
R2
OH
NH
85 Br OCH3
79 Br H
69 H OCH3
64 H H
16
B
Luminescence (%)
[compound], M
Br
R
OH
NH
90 Cl H OCH3
83 H Cl Cl
82 Cl H Cl
81 H H Cl
79 H H H
C
Luminescence (%)
[87], M
H3CO
human GPR35
EC50 1660 nM
rat GPR35
EC50 1100 nM
mouse GPR35
EC50 4950 nM

Figure 3. β-Arrestin recruitment assays at GPR35. (A, B) Concentration-response curves of selected, successively optimized compounds at the human GPR35; EC_{50} values, **85**: 12.1 nM, **79**: 303 nM, **69**: 346 nM, **64**: 4910 nM, **87**: 46300 nM, **90**: 11.1 nM, **83**: 15.4 nM, **87**: 16.4 nM, **81**: 25.1 nM. (C) Concentration-response curves of compound **87** at GPR35 orthologs.

Activity on the isotypic human GPR35b

In humans a second receptor isotype was identified, designated GPR35b. This receptor protein has an extended N-terminus (by 31 amino acids) but is otherwise identical to the human GPR35.[6] A series of compounds were tested at the human GPR35b. The results are summarized in Table 5. The potency for almost every compound at GPR35b was not significantly different from that at the shorter GPR35 receptor isotype. This is in agreement with published results by other groups, who had also not found any difference in the two isotypes' pharmacological properties.[23, 49] The physiological role of GPR35b is still unclear. However, it has previously been hypothesized to be a precursor of GPR35 that might be "activated" upon cleavage yielding GPR35.[6] The N-terminus of GPR35b might also be cleaved off to release GPR35 and a signaling peptide. Such signaling peptides are frequently found in class B GPCRs, but have also been suggested to exist in class A GPCRs.[50] Interestingly, GPR35b has only been detected on the mRNA level, but never on the protein level. Possibly, the GPR35b mRNA could partially be translated into the shorter isoform, because the start codon of the GPR35 is included in the sequence and might alternatively be used (a process known as leaky scanning). For future studies, it would be of interest to show whether the GPR35b exists on the protein level, and to address the question whether and under which conditions its N-terminus is cleaved off.

Table 5. Potency of selected derivatives at GPR35b

compd	human GPR35b EC_{50} ± SEM (μM) (% Effect[a] ± SEM)[b]	E_{max}[a]
3	2.24 ± 0.47[ns]	100
69	0.472 ± 0.057[ns]	115
70	0.678 ± 0.100[ns]	113
71	0.873 ± 0.100*	104
72	0.0205 ± 0.0010[ns]	110
73	0.106 ± 0.004[ns]	136
74	0.143 ± 0.028[ns]	113
78	0.0286 ± 0.0041[ns]	125
80	0.699 ± 0.061[ns]	111
81	0.0405 ± 0.0092[ns]	130
82	0.0346 ± 0.0093[ns]	116
83	0.0272 ± 0.0054[ns]	109
85	0.0221 ± 0.0036[ns]	136
88	0.628 ± 0.047[ns]	101
89	0.128± 0.025[ns]	118
90	0.0147± 0.0014[ns]	129

[a]Effects were normalized to the effect induced by 30 μM zaprinast. [b]Results of a two-tailed t test: [ns]data not significantly different from those at the human GPR35, * $p > 0.05$.

Species selectivity

To investigate species differences of the chromenone derivatives, we additionally determined their potency to induce β-arrestin recruitment at mouse and rat GPR35. The results are displayed in Tables 3 and 4. Most compounds appeared to be agonists at the rodent receptors as well. However, they were

mostly far less potent than at the human GPR35. Moreover, if concentration-response curves were determined, observed maximal effects for many compounds were below that of zaprinast. Thus, most of these compounds are partial agonists at the rodent GPR35.

At the mouse receptor, compounds with long and bulky substituents introduced via an amide linker in the 8-position (e. g. 2-naphthyl in **62**, EC_{50} 2990 nM, 2-quinolinyl in **63**, EC_{50} 3590 nM) were much more potent than simpler derivatives like the 8-amino-substituted **21**. This is in agreement with the results obtained for the human receptor. However, the increase in potency could only be found for compounds with bicyclic substituents or (very few) substituted phenyl derivatives including *p*-methoxyphenyl (**69**, EC_{50} 5030 nM), *o*-methoxyphenyl (**87**, combined with 6-bromo-substitution, EC_{50} 4950 nM), and *m*,*p*-dimethoxyphenyl (**88**, combined with 6-bromo-substitution, EC_{50} 6400 nM). However, the simple benzamido-chromenone **64**, which we used as a lead structure for developing agonists for the human receptor, was nearly inactive at the mouse GPR35. Another major difference in the SARs between mouse and human is the fact that halogen substituents in the 6-position of the chromenone core caused a reduction in potency at the mouse receptor, which becomes evident by comparison of the 6-unsubstituted *p*-methoxybenzamido derivative **69** (EC_{50} 5030 nM) with the corresponding halogenated analogs **74** (6-F), **78** (6-Cl), and **85** (6-Br) (EC_{50} >10000 nM). Furthermore, chloro-substitution in different positions of the phenyl ring of the 8-benzamido residue did not lead to potent agonists. In agreement with results at the human receptor, substitution of the phenyl ring of **64** with a *p*-methoxy residue (**69**, EC_{50} 5030 nM) increased potency at the mouse receptor. Exchange of the phenyl ring of **85** for a 3-pyridyl residue (**102**, EC_{50} 4980 nM), but not for a 2-pyridyl ring (**101**, EC_{50} >10000 nM), led to an increase in potency.

At the rat GPR35 chromenone derivatives with small substituents in the 8-position like the 8-amino derivative **21** displayed low potency. Similar as for the human receptor, introduction of a cyclohexyl moiety at the 8-position of the core structure connected by an amide linker increased potency (**60**, EC_{50}

1200 nM). However, this effect could not be observed for compounds with aromatic residues in the same position (phenyl in **64**, benzyl in **61**, 2-naphthyl in **62** or 2-quinolinyl in **63**, all with EC_{50} >10000 nM) in contrast to the human GPR35, and in contrast to the mouse receptor regarding the derivatives with bicyclic residues (**62**, **63**). We next examined derivatives of 8-benzamido-4-oxo-4*H*-chromene-2-carboxylic acid (**64**) with various substituents at the phenyl ring. The *p*-methoxy substitution (**69**, EC_{50} 2160 nM) caused an increase in potency, which appeared to be a general effect observed for all orthologs. We also observed that bioisosteric replacement of the phenyl ring in **85** (EC_{50} 1400 nM) by a 2-pyridyl residue (**102**, EC_{50} 991 nM), or a 3-pyridyl residue (**101**, EC_{50} 1540 nM) was tolerated. These observations are consistent with those for the mouse receptor. However, there are a few SARs unique for the rat GPR35: halogen substituents in the 6-position of the chromenone core structure barely affected the compounds' potency at the rat receptor, observable e. g. for the *p*-methoxybenzamido derivatives: **69** (6-H, EC_{50} 2160 nM), **74** (6-F, EC_{50} 1760 nM), **78** (6-Cl, EC_{50} 1830 nM), and **85** (6-Br, EC_{50} 1400 nM). Comparison of the compounds with chloro substituents (**71**, *o,m*-dichloro (6-F), EC_{50} 3130 nM; **72**, *o,p*-dichloro (6-F), EC_{50} >10000 nM; **73**, *m,p*-dichloro (6-F), EC_{50} 2650 nM; **81**, *p*-chloro (6-Br), EC_{50} >10000 nM; **82**, *o,p*-dichloro (6-Br), EC_{50} >100000 nM, and **83**, *m,p*-dichloro (6-Br), EC_{50} 3250 nM) showed that mainly a chlorine atom in the *meta*-position increased potency. Contrary to the human receptor, the negative charge appeared to be important: the ethyl ester **40** was inactive in contrast to the corresponding acid **74** (EC_{50} 1760 nM). Interestingly, apart from **88** (*m,p*-dimethoxy-substituted, EC_{50} 1300 nM), **77** (with a nitro group in the *para*-position, EC_{50} 3140 nM) and **84** (cyano-substituted in the *para*-position, EC_{50} 2260 nM) the efficacies of the agonists appeared to be somewhat lower than that of the standard compound zaprinast.

We showed that most compounds are highly species-selective for the human GPR35, e. g. **85** (>100-fold compared to rat GPR35, >1000-fold compared to mouse GPR35). However, we could also identify several compounds that activated two of the three investigated orthologs in a nearly equipotent manner: (i) human and rat GPR35: **60** (EC_{50}, human: 4200 nM, rat: 1200 nM), **65** (EC_{50}, human: 5440 nM, rat:

2750 nM), **76** (EC_{50}, human: 1220 nM, rat: 2100 nM), **77** (EC_{50}, human: 1710 nM, rat: 3140 nM), and **80** (EC_{50}, human: 842 nM, rat: 2080 nM); (ii) human and mouse GPR35: **62** (EC_{50}, human: 4670 nM, mouse: 2990 nM) and **63** (EC_{50}, human: 1340 nM, mouse: 3590 nM); (iii) rat and mouse GPR35: **69** (EC_{50}, rat: 2160 nM, mouse: 5030 nM). Compound **87** is the only compound that was shown to activate all three investigated orthologs equipotently with EC_{50} values in the low micromolar range (human: 1660 nM, rat: 1100 nM, mouse: 4950 nM, see Figure 3C).

Receptor selectivity

In order to assess the selectivity of the new GPR35 agonists versus the closely related human orphan receptor GPR55, we tested the compounds' potency to induce β-arrestin recruitment by GPR55. We found that none of the chromenone derivates activated GPR55 at a high concentration of 10 μM. Thus, the investigated compounds are selective GPR35 agonists versus GPR55. Moreover, these results showed that the effects observed for GPR35 cannot represent assay artifacts, since the same assay system (Pathhunter® β-arrestin assay) was used for assaying the receptors, employing CHO cell lines recombinantly expressing either GPR35 or GPR55. Subsequently, the compounds were evaluated as potential GPR55 antagonists. Several compounds were found to inhibit LPI-induced β-arrestin recruitment with moderate potency. For the most potent antagonists full concentration-inhibition curves were determined. We identified several moderately potent GPR55 antagonists with IC_{50} values in the lower micromolar range. Thus, we discovered some novel, however non-selective GPR55 antagonists. Interestingly, **85** and **90** were not only among the most potent GPR55 antagonists, but were also the most potent GPR35 agonists. However, both compounds were still more than 1700-fold selective for GPR35.

Table 6. Potency of chromen-4-one-2-carboxylic acid derivatives at human GPR55

compd	human GPR55

	EC_{50} ± SEM (μM) (% Effect[a] ± SEM)	IC_{50} ± SEM (μM) (% Inhibition ± SEM)[b]
16	(-6 ± 2)	(32 ± 8)
21	(-11 ± 4)	(19 ± 2)
22	(4 ± 2)	(17 ± 3)
40	(-1 ± 5)	(26 ± 1)
57	(1 ± 7)	(-41 ± 9)
58	(15 ± 4)	(16 ± 4)
59	(-4 ± 7)	(31 ± 8)
60	(17 ± 7)	(16 ± 6)
61	(-11 ± 3)	(26 ± 10)
62	(6 ± 5)	**29.1** ± 17.3
63	(-6 ± 4)	(31 ± 3)
64	(3 ± 2)	(24 ± 2)
65	(10 ± 6)	(25 ± 3)
66	(12 ± 7)	(20 ± 3)
67	(6 ± 6)	(16 ± 8)
68	(1 ± 3)	(5 ± 14)
69	(0 ± 6)	(-3 ± 3)
70	(8 ± 7)	(9 ± 4)
71	(5 ± 4)	(40 ± 8)
72	(-8 ± 5)	(18 ± 9)
73	(3 ± 0)	(54 ± 5)
74	(7 ± 3)	(34 ± 7)
75	(20 ± 4)	(23 ± 1)
76	(4 ± 4)	(40 ± 6)
77	(8 ± 2)	(14 ± 9)
78	(-2 ± 3)	(47 ± 6)
79	(23 ± 7)	(21 ± 6)
80	(-8 ± 4)	(46 ± 6)

81	(12 ± 4)	(40 ± 5)
82	(10 ± 9)	(43 ± 5)
83	(16 ± 5)	(47 ± 7)
84	(0 ± 2)	(38 ± 10)
85	(0 ± 5)	**21.7** ± 6.2
86	(-1 ± 7)	(39 ± 7)
87	(9 ± 5)	(44 ± 2)
88	(-12 ± 1)	(45 ± 5)
89	(9 ± 3)	(46 ± 4)
90	(10 ± 2)	**21.7** ± 1.9
99	(-5 ± 7)	(24 ± 6)
100	(10 ± 1)	(21 ± 9)
101	(4 ± 4)	**8.92** ± 0.64
102	(9 ± 6)	**6.20** ± 0.36

[a]Screening was performed at a concentration of 10 μM. Effects were normalized to the signal induced by 1 μM LPI. [b]Inhibition of β-arrestin recruitment induced by 1 μM LPI.

Physicochemical properties of selected compounds

Several physicochemical parameters can be calculated to evaluate the druglikeness of a given compound, including the partition coefficient (clogP), the polar surface area (PSA), the ligand efficiency (LE) and the ligand-lipophilicity efficiency (LLE or LipE).[51-53] We calculated these parameters for selected 8-benzamidochromen-4-one-2-carboxylic acids and compared them to the previously described most potent agonist **5** (see Table 7). The clogP value was in all cases lower than 5, which is consistent with Lipinski's rule of 5 for druglikeness.[54, 55] Compounds **69**, **88** and **102** exhibit a logP value between 2 and 3, which is often considered optimal for perorally administered drugs. The PSA is defined as the sum over all polar atoms, mainly oxygen and nitrogen including the hydrogen atoms attached to them. This parameter is used to assess the compound's ability to permeate cells. For the most potent

compounds of the present series the PSA values were ranging between 93 Å^2 and 115 Å^2. Compounds with a PSA value of lower than 140 Å^2 are likely to penetrate cell membranes. Thus the developed compounds may be perorally bioavailable. However, for brain penetration, their PSA may be too large since for brain permeability a PSA of 60 Å^2 should not be exceeded. The LE represents the binding energy per atom for a ligand to its receptor. It combines physicochemical with pharmacological properties and is calculated as follows: LE = pEC_{50}/N (N = non-hydrogen atoms). For most of the selected compounds a LE value of about 0.3 was calculated. In contrast, compound **5** showed a lower LE value of 0.22. A further useful parameter is the LLE (or LipE) that links potency and lipophilicity (LLE = pEC_{50} – clogP). For compounds **78**, **85** and **102** the LLE value was found to be about 5, while all other compounds, including compound **5,** possessed lower ligand-lipophilicity efficiency. Compounds with a LE > 0.3 and LLE > 5 can be considered as suitable drug candidates. In case of the new compounds, **85** and **102** showed very good LLE and LE values combined with high potency and may thus serve as lead structures for further optimizing this novel class of GPR35 agonists not only with regard to potency, but also regarding physicochemical properties.

Table 7. Physicochemical properties of selected compounds

compd	pEC_{50}	clogP[a]	PSA (Å^2)[a]	LLE	LE
5	7.59	3.71	78.8	3.88	0.22
69	6.46	2.23	102	4.23	0.26
72	7.77	3.74	92.7	4.03	**0.30**
78	7.77	2.84	102	**4.93**	**0.30**
82	7.79	4.37	92.7	3.42	**0.30**
83	7.81	4.37	92.7	3.44	**0.30**
85	7.92	3.00	102	**4.92**	**0.30**
87	5.78	3.00	102	2.78	0.22
88	6.37	2.84	111	3.53	0.23

90	7.95	3.60	102	4.35	0.29
102	7.52	2.38	115	**5.14**	0.29

[a]Calculated by the MarvinSketch program (5.12.3), 2013, ChemAxon (http://www.chemaxon.com)

CONCLUSION

A series of 41 8-substituted chromen-4-one-2-carboxylic acids and one intermediate ethyl ester was evaluated as human GPR35 agonists in β-arrestin recruitment assays. Analysis of structure-activity relationships allowed chemical optimization and thus the development of highly potent agonists. Especially, a bromine substituent in 6-position and a *p*-methoxy-benzamide moiety in the 8-position of the core structure had major impacts on the compounds' potency. Combination of both substitutions yielded the highly potent agonist **85** and its derivative **90** (EC_{50} values 12.1 nM, and 11.1 nM, respectively). Regarding their potency determined in β-arrestin assays, both compounds represent the most potent agonists at the human GPR35 known to date. Both compounds, **85** and **90,** were species-selective (EC_{50} values at the rat GPR35: 1400 nM, and 4170 nM, respectively; nearly inactive at the mouse ortholog at 10 μM). Also both compounds were found to be GPR55 antagonists, however, their potency was weak (IC_{50} values for both compounds: 21700 nM). Hence, **85** and **90** can be considered highly selective (> 1700-fold) for GPR35 compared to the closely related GPR55. Furthermore, this study provides valuable information for the further improvement of this new class of GPR35 agonists at rodent GPR35 receptors. Optimized agonists at the rodent receptor, as well as the developed highly potent human GPR35 agonists, such as **85** and **90**, may become useful pharmacological tools to further elucidate the receptors' (patho)physiological role. Additionally, these compounds should have sufficiently high affinity for human GPR35 to be labeled and utilized as radioligand.

EXPERIMENTAL SECTION

General

All commercially available reagents were used as purchased (Acros, Alfa Aesar, Sigma-Aldrich or Fluorochem). Solvents were used without additional purification or drying except for dichloromethane, which was distilled over calcium hydride. The reactions were monitored by thin layer chromatography (TLC) using aluminum sheets with silica gel 60 F_{254} (Merck). Column chromatography was performed with silica gel 0.060-0.200 mm, pore diameter ca. 6 nm. For microwave reactions a CEM Focused Microwave Synthesis type Discover apparatus was used. Preparative HPLC was performed on a Knauer HPLC system using a Wellchrome K-1800 pump, a WellChrome K-2600 spectrophotometer, and a Eurospher 100 C18 column (250 mm x 20 mm, particle size 10 μm). A gradient of methanol in water was used as indicated below with a flow rate of 20 mL/min. A freeze-dryer (CHRIST ALPHA 1–4 LSC) was utilized for lyophilization. All synthesized compounds were finally dried in vacuum at 8–12 Pa (0.08–0.12 mbar) using a sliding vane rotary vacuum pump (Vacuubrand GmbH). ^{1}H- and ^{13}C NMR data were collected on a Bruker Avance 500 MHz NMR spectrometer at 500 MHz (^{1}H), or 126 MHz (^{13}C), respectively. If indicated, NMR data were collected on a Bruker Ascend 600 MHz NMR spectrometer at 600 MHz (^{1}H), or 151 MHz (^{13}C), respectively. DMSO-d_6 was employed as a solvent at 303 K, unless otherwise noted. Chemical shifts are reported in parts per million (ppm) relative to the deuterated solvent; that is, DMSO, δ ^{1}H: 2.49 ppm; ^{13}C: 39.7 ppm. Coupling constants J are given in Hertz and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), sext. (sextet), m (multiplet), br (broad). Melting points were determined on a Büchi 530 melting point apparatus and are uncorrected. The purities of isolated products were determined by ESI-mass spectra obtained on an LCMS instrument (Applied Biosystems API 2000 LCMS/MS, HPLC Agilent 1100) using the following procedure: the compounds that contained a chromen-4-one ring system were dissolved at a concentration of 0.5 mg/mL in acetonitrile/water containing 2 mM ammonium acetate. Then, 10 μL of the sample were injected into an HPLC column (Macherey-Nagel Nucleodur® 3 μ C18, 50 x 2.00 mm). Elution was performed with a gradient of water/acetonitrile (containing 2 mM ammonium acetate) from

90:10 to 0:100 for 20 min at a flow rate of 300 μL/min, starting the gradient after 10 min. UV absorption was detected from 200 to 950 nm using a diode array detector. For all other compounds the same method was used, but acetonitrile was replaced by methanol. Purity of all compounds was determined at 254 nm. The purity of the compounds was generally ≥95%.

Compounds **17**[41] and **18**[38] were synthesized as described in literature. Compounds **12**,[33,34] **13**,[38, 39] **16**,[40] **21**,[42] **23**,[43] **91**[46] and **103**[45] have previously been described but were obtained by new methodologies, and additional structural characterization data is provided in the present study. The synthesis and structural characterization data of the most potent compounds and their intermediates are described below. For detailed synthetic and structural characterization data of all other prepared compounds see Supporting Information.

General procedure for the synthesis of compounds 12–15. The appropriate (5'-halogeno-)2'-hydroxy-3'-nitroacetophenone (12.0 mmol) and diethyl oxalate (4.0 mL, 29.5 mmol) were dissolved in 50–70 mL of anhydrous *N*,*N*-dimethylformamide (DMF) under an argon atmosphere and were cooled to 0–5 °C. Potassium *tert*-butoxide (5.37 g, 47.9 mmol) was added and the reaction mixture was stirred under an argon atmosphere for 2 - 3 hours at 0–5 °C. Then a cooled solution of concd. (12 M) aq. HCl (7.76 mL, 93.1 mmol) in 80 mL of water was added and the mixture was extracted with EtOAc (3 x 60 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in 60 mL of EtOH and concd. (12 M) aq. HCl (3.85 mL, 46.2 mmol) was subsequently added. The reaction mixture was refluxed overnight under argon atmosphere. The mixture was concentrated under reduced pressure until the product started to crystallize and was subsequently cooled to 0–5 °C for completion of crystallization. The product was filtered off, washed with ice-cold EtOH in small portions, and dried in vacuum at 50 °C.

General procedure for the synthesis of compounds 17–20. The appropriate nitro compound (8.4 mmol) was suspended in a mixture of EtOH (25 mL) and diluted aq. HCl (2 N, 25 mL). Tin(II) chloride dihydrate (7.58 g, 33.6 mmol) was subsequently added and the reaction mixture was heated to 65 °C until the solid was completely dissolved. The mixture was stirred for additional 20 min at this temperature. The EtOH was removed under reduced pressure and the resulting suspension was added to 200 mL of EtOAc containing sodium carbonate (25 g). The precipitated tin complex was filtered off and washed with 100 mL of EtOAc. The filtrate was washed with brine (100 mL), dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The resulting solid was dried in vacuum at 50 °C.

General procedure for the synthesis of compounds 24–30, 32–36. Amine **17** (163 mg, 0.7 mmol) and DIPEA (0.23 mL, 1.4 mmol) were dissolved in DCM (5 mL). Then a solution of the appropriate acid chloride (1.4 mmol) in DCM (1 mL) was added and the reaction mixture was stirred at rt for 24 h under an argon atmosphere, during which part of the amide precipitated. The products, which were soluble in DCM containing some drops of DMF at 40 °C, were chromatographed on a column of silica gel (for conditions see the individual compounds) to yield the pure amide (*work-up A*). The hardly soluble amides were filtered off and washed twice with 1 mL of DCM each. If necessary, the filtrate was concentrated to induce crystallization of further product, and the procedure was repeated, or separation by column chromatography (9:1 DCM/EtOAc) was performed (*work-up B*). All products were finally dried in vacuum at 50 °C.

General procedure for the synthesis of compounds 31, 37–55. The appropriate ethyl 8-amino-4-oxo-4*H*-chromene-2-carboxylate (0.9 mmol) and DIPEA (0.20 mL, 1.2 mmol) were dissolved in DCM (4.5 mL) and anhydrous THF (1.5 mL). In case of amine **17**, 6 mL of DCM instead of a DCM/THF mixture were used for dissolution. Then a solution of the appropriate acid chloride (1.2 mmol) in DCM (3 mL) was added and the reaction mixture was stirred at rt over a period from 1 to 4 days under an argon

atmosphere. *Work-up A*: If a precipitation of the product occurred, the solid was filtered off, washed twice or three times with 1 mL of DCM each, and dried in vacuum at 50 °C. If necessary, the filtrate was concentrated to achieve the crystallization of further product, or column chromatography was performed, respectively (for conditions see individual compounds); *Work-up B*: If no or little precipitation of the product occurred, the reaction mixture was concentrated under reduced pressure and chromatographed on a column of silica gel to yield the pure product (for conditions see individual compounds).

General procedure for the synthesis of compounds 56, 95–98. To form the acid chloride, the appropriate acid (1.2 mmol) and three drops of anhydrous DMF were dissolved in DCM (5 mL). Then 1 mL of freshly distilled thionyl chloride was added and the reaction mixture was stirred under an argon atmosphere at rt for 30 min. The DCM and excess of thionyl chloride were distilled off under reduced pressure using a glass filter pump. Cooling by an ice-bath induced the crystallization of the desired acid chloride. In the meantime, the appropriate ethyl 8-amino-4-oxo-4*H*-chromene-2-carboxylate (0.9 mmol) and DIPEA (0.20 mL, 1.2 mmol) were dissolved in DCM (4.5 mL) and anhydrous THF (1.5 mL). In case of amine **17**, 6 mL of DCM instead of a DCM/THF mixture were used for dissolution. This solution was added to the formed acid chloride, which had been dissolved in 4 mL of DCM. The reaction mixture was then stirred at rt from 1 to 2 days under an argon atmosphere. For work-up conditions, see individual compounds.

General procedure for the synthesis of compounds 16, 21, 22, 57–90. A solution of potassium carbonate (79 mg, 0.57 mmol) in water (4.5 mL) was slowly added to a suspension of the appropriate ethyl 4-oxo-4*H*-chromene-2-carboxylate (0.44 mmol) in THF (12 mL) and EtOH (3 mL). The reaction mixture was stirred at rt for 16–24 h until a clear solution was obtained. After addition of water (4 mL), the mixture was acidified with diluted aq. HCl solution (2 N) until a pH $\leq$ 2 was reached. The solvents

THF and EtOH were removed under reduced pressure. The obtained precipitate was filtered off, washed with 15 mL of water, and dried in vacuum at 45 °C. In some cases, further purification was necessary (see individual compounds).

Ethyl 6-chloro-8-nitro-4-oxo-4*H*-chromene-2-carboxylate (14). The compound was synthesized using 5'-chloro-2'-hydroxy-3'-nitroacetophenone (2.59 g, 12.0 mmol) and was isolated as a beige-colored solid (2.86 g, 80% yield). ^{1}H NMR: δ 1.34 (t, *J* = 7.1 Hz, 3H, CH_3), 4.40 (q, *J* = 7.1 Hz, 2H, CH_2), 7.10 (s, 1H, 3-H), 8.30 (d, *J* = 2.5 Hz, 1H, 5-H or 7-H), 8.69 (d, *J* = 2.5 Hz, 1H, 5-H or 7-H). ^{13}C NMR: δ 13.9 (CH_3), 63.0 (CH_2), 114.5 (C-3), 126.5 (C-4a), 129.6 (C-7), 129.8 (C-6), 130.6 (C-5), 140.0 (C-8), 146.4 (C-8a), 152.4 (C-2), 159.2 ($\underline{C}O_2Et$), 175.1 (C-4). LC-MS (*m*/*z*): positive mode 298 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 126–127 °C.

Ethyl 6-fluoro-8-nitro-4-oxo-4*H*-chromene-2-carboxylate (15). The compound was synthesized using 5'-fluoro-2'-hydroxy-3'-nitroacetophenone (1.99 g, 10.0 mmol) and was isolated as a beige-colored solid (1.99 g, 71% yield). ^{1}H NMR: δ 1.34 (t, *J* = 7.1 Hz, 3H, CH_3), 4.40 (q, *J* = 7.1 Hz, 2H, CH_2), 7.08 (s, 1H, 3-H), 8.14 (dd, *J* = 7.7 Hz, *J* = 3.3 Hz, 1H, 5-H), 8.62 (dd, *J* = 7.9 Hz, *J* = 3.2 Hz, 1H, 7-H). ^{13}C NMR: δ 13.9 (CH_3), 63.1 (CH_2), 113.7 (C-3), 116.0 (d, *J* = 23.8 Hz, C-5), 119.6 (d, *J* = 30.2 Hz, C-7), 126.7 (d, *J* = 7.3 Hz, C-4a), 140.0 (d, *J* = 9.2 Hz, C-8), 144.4 (C-8a), 152.4 (C-2), 157.4 (d, *J* = 249.3 Hz, C-6), 159.4 ($\underline{C}O_2Et$), 175.4 (C-4). LC-MS (*m*/*z*): positive mode 282 $[M+H]^+$, 299 $[M+NH_4]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 94.5%. mp 115 °C.

Ethyl 8-amino-6-chloro-4-oxo-4*H*-chromene-2-carboxylate (19). The compound was synthesized using **14** (2.50 g, 8.4 mmol) and was isolated as a yellow solid (1.39 g, 80% yield). ^{1}H NMR: δ 1.35 (t, *J* = 7.1 Hz, 3H, CH_3), 4.39 (q, *J* = 7.1 Hz, 2H, CH_2), 5.87 (br, 2H, NH_2), 6.89 (s, 1H, 3-H), 7.05 (d, *J* = 2.5 Hz, 1H, 5-H or 7-H), 7.07 (d, *J* = 2.5 Hz, 1H, 5-H or 7-H). ^{13}C NMR: δ 14.0 (CH_3), 62.8 (CH_2), 108.8 (C-7), 113.3 (C-3), 116.3 (C-5), 125.2 (C-4a), 131.0 (C-6), 140.8 (C-8), 142.7 (C-8a), 151.9 (C-

2), 160.0 ($\underline{C}O_2Et$), 176.8 (C-4). LC-MS (*m/z*): negative mode 266 $[M-H]^-$, positive mode 268 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 98.9%. mp 150 °C.

Ethyl 8-amino-6-fluoro-4-oxo-4*H*-chromene-2-carboxylate (20). The nitro compound **15** (1.94 g, 6.9 mmol) was suspended in a mixture of ethanol (40 mL) and diluted HCl (2 N, 30 mL). Tin (II) chloride dihydrate (6.23 g, 27.6 mmol) was subsequently added and the reaction mixture was heated to 65 °C until the solid was completely dissolved. The mixture was stirred for additional 40 min at this temperature. The work-up was performed as described in the general procedure. The product was isolated as a yellow solid (1.39 g, 80% yield). ^{1}H NMR: δ 1.35 (t, *J* = 7.1 Hz, 3H, CH_3), 4.39 (q, *J* = 7.1 Hz, 2H, CH_2), 5.90 (br, 2H, NH_2), 6.77 (dd, *J* = 8.5 Hz, *J* = 3.2 Hz, 1H, 5-H), 6.86-6.89 (m, 2H, 3-H, 7-H). ^{13}C NMR: δ 14.0 (CH_3), 62.8 (CH_2), 94.5 (d, *J* = 24.7 Hz, C-5), 104.5 (d, *J* = 28.4 Hz, C-7), 112.6 (C-3), 125.2 (d, *J* = 9.2 Hz, C-4a), 140.8 (C-8a), 141.4 (d, *J* = 12.8 Hz, C-8), 151.8 (C-2), 160.0 ($\underline{C}O_2Et$), 160.2 (d, *J* = 241.9 Hz, C-6), 177.2 (C-4). LC-MS (*m/z*): negative mode 250 $[M-H]^-$, positive mode 252 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.7%. mp 162–163 °C.

Ethyl 8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (35). The compound was synthesized using 4-methoxybenzoyl chloride (239 mg, 1.4 mmol). The formed amide was filtered off and washed twice with 1 mL of DCM each. The filtrate was concentrated and the residue was recrystallized from 1:1 acetone/ethanol with addition of 4 drops of DMF. Both isolated solids were combined and dried in vaccum at 50 °C. The amide was isolated as a white powder (228 mg, 89% yield). ^{1}H NMR: δ 1.24 (t, *J* = 7.1 Hz, 3H, CH_2-$C\underline{H}_3$), 3.85 (s, 3H, OCH_3), 4.33 (q, *J* = 7.1 Hz, 2H, $C\underline{H}_2$-CH_3), 6.98 (s, 1H, 3-H), 7.09 (d, *J* = 8.8 Hz, 2H, 3'-H, 5'-H), 7.55 (dd, *J* = 7.9 Hz, 1H, 6-H), 7.89 (dd, *J* = 8.0 Hz, *J* = 1.6 Hz, 1H, 5-H), 8.01 (d, *J* = 8.8 Hz, 2H, 2'-H, 6'-H), 8.10 (dd, *J* = 7.6 Hz, *J* = 1.6 Hz, 1H, 7-H), 10.06 (s, 1H, NH). ^{13}C NMR: δ 13.8 (CH_2-$\underline{C}H_3$), 55.7 (OCH_3), 62.8 ($\underline{C}H_2$-CH_3), 113.7 (C-3), 114.0 (C-3', C-5'), 121.4 (C-5), 124.5 (C-4a), 125.9 (C-7), 126.1 (C-1'), 128.6 (C-8), 129.8 (C-2', C-6'), 130.8 (C-6), 149.3 (C-8a), 151.9 (C-2), 160.0 ($\underline{C}O_2Et$), 162.4 (C-4'), 165.0 (CO-NH), 177.4 (C-4).

LC-MS (*m*/*z*): negative mode 366 $[M-H]^-$, positive mode 368 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 96.5%. mp 202–203 °C.

Ethyl 8-(2,4-dichlorobenzamido)-6-fluoro-4-oxo-4*H*-chromene-2-carboxylate (38). The compound was synthesized using 2,4-dichlorobenzoyl chloride (251 mg, 1.2 mmol) and **20** (226 mg, 0.9 mmol) in a reaction time of 2 days (*work-up A*: 95:5 DCM/EtOAc). The product was isolated as a white solid. (299 mg, 78% yield). ^{1}H NMR (600 MHz): δ 1.31 (t, *J* = 7.0 Hz, 3H, CH_3), 4.38 (q, *J* = 7.0 Hz, 2H, CH_2), 7.01 (s, 1H, 3-H), 7.63-7.64 (m, 2H, 5-H, 5'-H), 7.76 (d, *J* = 8.0 Hz, 1H, 6'-H), 7.81 (s, 1H, 3'-H), 8.06 (d, *J* = 8.0 Hz, 1H, 7-H), 10.69 (s, 1H, NH). ^{13}C NMR (151 MHz): δ 14.3 (CH_3), 63.3 (CH_2), 106.9 (d, *J* = 23.1 Hz, C-5), 113.6 (C-3), 118.8 (d, *J* = 29.7 Hz, C-7), 125.7 (d, *J* = 7.7 Hz, C-4a), 128.0 (C-5'), 130.0 (C-3' or C-6'), 130.1 (C-8), 131.4 (C3' or C6'), 132.1 (C-4'), 134.8 (C-1'), 136.1 (C-2'), 146.2 (C-8a), 152.5 (C-2), 159.0 (d, *J* = 245.4 Hz, C-6), 160.2 ($\underline{C}O_2Et$), 164.9 (CO-NH), 176.9 (C-4). LC-MS (*m*/*z*): negative mode 422 $[M-H]^-$, positive mode 424 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 229–230 °C.

Ethyl 6-chloro-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (44). The compound was synthesized using 4-methoxybenzoyl chloride (205 mg, 1.2 mmol) and **19** (241 mg, 0.9 mmol) in a reaction time of 2 days (*work-up A*). The product was isolated as a white powder (318 mg, 88% yield). ^{1}H NMR (343 K): δ 1.30 (t, *J* = 7.1 Hz, 3H, CH_2-$C\underline{H}_3$), 3.86 (s, 3H, OCH_3), 4.38 (q, *J* = 7.1 Hz, 2H, $C\underline{H}_2$-CH_3), 7.00 (s, 1H, 3-H), 7.10 (d, *J* = 8.8 Hz, 2H, 3'-H, 5'-H), 7.80 (d, *J* = 2.5 Hz, 1H, 5-H), 8.00 (d, *J* = 8.5 Hz, 2H, 2'-H, 6'-H), 8.27 (d, *J* = 2.5 Hz, 1H, 7-H), 9.89 (br, 1H, NH). ^{13}C NMR (343 K): δ 13.5 (CH_2-$\underline{C}H_3$), 55.4 (OCH_3), 62.6 ($\underline{C}H_2$-CH_3), 113.4 (C-3), 113.8 (C-3', C-5'), 119.2 (C-7), 124.9 (C-4a), 125.7 (C-1'), 128.3 (C-5), 129.4 (C-2', C-6'), 130.0, 130.3 (C-6, C-8), 147.1 (C-8a), 151.7 (C-2), 159.5 ($\underline{C}O_2Et$), 162.5 (C-4'), 164.7 (CO-NH), 175.8 (C-4). LC-MS (*m*/*z*): negative mode 328 $[M-CO_2H]^-$, 372 $[M-H]^-$. Purity by HPLC-UV (254 nm)-ESI-MS: 98.9%. mp 252–253 °C.

Ethyl 6-bromo-8-(2,4-dichlorobenzamido)-4-oxo-4*H*-chromene-2-carboxylate (48). The compound was synthesized using 2,4-dichlorobenzoyl chloride (251 mg, 1.2 mmol) and **18** (281 mg, 0.9 mmol) in a reaction time of 2 days (*work-up A*: 95:5 DCM/EtOAc). The product was isolated as a white solid (318 mg, 73% yield). ^{1}H NMR: δ 1.33 (t, *J* = 7.1 Hz, 3H, CH_3), 4.40 (q, *J* = 7.1 Hz, 2H, CH_2), 7.00 (s, 1H, 3-H), 7.59 (dd, *J* = 8.2 Hz, *J* = 1.9 Hz, 1H, 5'-H), 7.73 (d, *J* = 8.2 Hz, 1H, 6'-H), 7.76 (d, *J* = 1.6 Hz, 1H, 3'-H), 7.99 (d, *J* = 2.5 Hz, 1H, 5-H), 8.30 (d, *J* = 2.2, 1H, 7-H). ^{13}C NMR: δ 13.6 (CH_3), 62.7 (CH_2), 113.8 (C-3), 117.7 (C-6), 123.6 (C-5), 125.5 (C-4a), 127.3 (C-5'), 129.4 (C-8), 129.4 (C-3' or C-6'), 130.8 (C-3' or C-6'), 131.5 (C-4'), 132.0 (C-7), 134.1 (C-1'), 135.6 (C-2'), 148.0 (C-8a), 152.1 (C-2), 159.5 ($\underline{C}O_2Et$), 164.3 (CO-NH), 175.7 (C-4). LC-MS (*m/z*): negative mode 484 $[M-H]^-$, positive mode 486 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 221–223 °C.

Ethyl 6-bromo-8-(3,4-dichlorobenzamido)-4-oxo-4*H*-chromene-2-carboxylate (49). The compound was synthesized using 3,4-dichlorobenzoyl chloride (251 mg, 1.2 mmol) and **18** (281 mg, 0.9 mmol) in a reaction time of 2 days (*work-up A*). The product was isolated as a white powder (259 mg, 71% yield). ^{1}H NMR: δ 1.23 (t, *J* = 7.1 Hz, 3H, CH_3), 4.33 (q, *J* = 7.1 Hz, 2H, CH_2), 7.02 (s, 1H, 3-H), 7.87 (d, *J* = 8.2 Hz, 1H, 5'-H), 7.97 (dd, *J* = 8.3 Hz, *J* = 2.0 Hz, 1H, 6'-H), 8.00 (d, *J* = 2.5 Hz, 1H, 5-H), 8.23-8.24 (m, 2H, 7-H, 2'-H), 10.64 (br, 1H, NH). ^{13}C NMR: δ 13.8 (CH_3), 62.9 (CH_2), 114.0 (C-3), 117.9 (C-6), 124.1 (C-5), 125.7 (C-4a), 128.2 (C-6'), 129.8 (C-2'), 129.9 (C-8), 131.2 (C-5'), 131.7 (C-3'), 133.4 (C-7), 134.1 (C-1'), 135.2 (C-4'), 148.7 (C-8a), 152.0 (C-2), 159.7 ($\underline{C}O_2Et$), 163.5 (CO-NH), 176.1 (C-4). LC-MS (*m/z*): negative mode 484 $[M-H]^-$, positive mode 486 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 206–207 °C.

Ethyl 6-bromo-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (51). The compound was synthesized using 4-methoxybenzoyl chloride (205 mg, 1.2 mmol) and **18** (281 mg, 0.9 mmol) in a reaction time of 2 days (*work-up A*: 98:2 DCM/EtOAc). The product was isolated as a white powder (354 mg, 89% yield). ^{1}H NMR: δ 1.30 (t, *J* = 7.1 Hz, 3H, CH_3), 3.87 (s, 3H, OCH_3), 4.38 (q, *J* = 7.1 Hz,

2H, CH_2), 7.00 (s, 1H, 3-H), 7.10 (d, *J* = 8.8 Hz, 2H, 3'-H, 5'-H), 7.94 (d, *J* = 2.2 Hz, 1H, 5-H), 8.00 (d, *J* = 8.8 Hz, 2H, 2'-H, 6'-H), 8.38 (d, *J* = 2.5 Hz, 1H, 7-H), 9.88 (br, 1H, NH). ^{13}C NMR: δ 13.5 (CH_3), 55.4 (OCH_3), 62.6 (CH_2), 113.5 (C-3), 113.8 (C-3', C-5'), 117.7 (C-6), 122.4 (C-5), 125.2, 125.7 (C-10, C-1'), 129.4 (C-2', C-6'), 130.4 (C-8), 131.0 (C-7), 147.5 (C-8a), 151.8 (C-2), 159.4 ($\underline{C}O_2Et$), 162.5 (C-4'), 164.7 (CO-NH), 175.7 (C-4). LC-MS (*m*/*z*): negative mode 444 $[M-H]^-$, positive mode 448 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 248–249 °C.

Ethyl 6-bromo-8-(2-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (53). The compound was synthesized using 2-methoxybenzoyl chloride (205 mg, 1.2 mmol) and **18** (281 mg, 0.9 mmol) in a reaction time of 2 days (*work-up B*). Column chromatography (95:5 DCM/EtOAc) afforded the product as a white solid (340 mg, 85% yield). ^{1}H NMR (343 K): δ 1.36 (t, *J* = 7.1 Hz, 3H, CH_3), 4.16 (s, 3H, OCH_3), 4.45 (q, *J* = 7.1 Hz, 2H, CH_2), 7.03 (s, 1H, 3-H), 7.16-7.20 (m, 1H, 4'-H), 7.33 (d, *J* = 8.5 Hz, 1H, 3'-H), 7.62 (ddd, *J* = 7.3 Hz, *J* = 1.7 Hz, 1H, 5'-H), 7.84 (d, *J* = 2.5 Hz, 1H, 5-H), 8.10 (dd, *J* = 7.8 Hz, *J* = 1.9 Hz, 1H, 6'-H), 9.03 (d, *J* = 2.5 Hz, 1H, 7-H), 10.65 (s, 1H, NH). ^{13}C NMR (343 K): δ 13.9 (CH_3), 57.1 (OCH_3), 63.1 (CH_2), 113.1 (C-3'), 114.2 (C-3), 118.4 (C-6), 120.6 (C-1'), 121.0 (C-5'), 121.5 (C-5), 125.3 (C-4a), 127.1 (C-6'), 130.5 (C-8), 131.8 (C-7), 134.5 (C-4'), 145.2 (C-8a), 152.0 (C-2), 157.8 (C-2'), 159.7 ($\underline{C}O_2Et$), 163.6 (CO-NH), 176.0 (C-4). LC-MS (*m*/*z*): negative mode 444 $[M-H]^-$, positive mode 448 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.8%. mp 196–197 °C.

Ethyl 6-bromo-8-(3,4-dimethoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (54). The compound was synthesized using 3,4-dimethoxybenzoyl chloride (241 mg, 1.2 mmol) and **18** in a reaction time of 2 days (281 mg, 0.9 mmol) (*work-up A*: 98:2 DCM/EtOAc). The product was isolated as a white powder (327 mg, 76% yield). ^{1}H NMR: δ 1.25 (t, *J* = 7.1 Hz, 3H, CH_2-$C\underline{H}_3$), 3.85, 3.86 (s, 6H, OCH_3), 4.34 (q, *J* = 7.1 Hz, 2H, $C\underline{H}_2$-CH_3), 7.02 (s, 1H, 3-H), 7.12 (d, *J* = 8.5 Hz, 1H, 5'-H), 7.59 (d, *J* = 2.2 Hz, 1H, 2'-H), 7.66 (dd, *J* = 8.4 Hz, *J* = 2.1 Hz, 1H, 6'-H), 7.94 (d, *J* = 2.5 Hz, 1H, 5-H), 8.32 (d, *J* = 2.5 Hz, 1H, 7-H), 10.14 (br, 1H, NH). ^{13}C NMR: δ 13.8 (CH_2-$\underline{C}H_3$), 55.8, 55.9 (OCH_3),

62.9 ($\underline{C}H_2$-CH_3), 111.3 (C-2', C-5'), 113.9 (C-3), 118.0 (C-6), 121.4 (C-6'), 123.0 (C-5), 125.5, 125.7 (C-4a, C-1'), 130.6 (C-8), 132.1 (C-7), 148.1 (C-3'), 148.6 (C-8a), 152.0 (C-4'), 152.4 (C-2), 159.7 ($\underline{C}O_2Et$), 164.9 (CO-NH), 176.2 (C-4). LC-MS (*m*/*z*): negative mode 474 $[M-H]^-$, positive mode 476 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.7%. mp 221–223 °C.

Ethyl 6-bromo-8-(2-chloro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (56). The compound was synthesized using 2-chloro-4-methoxybenzoic acid (224 mg, 1.2 mmol) and **18** (281 mg, 0.9 mmol). The precipitate was filtered off, washed three times with 1 mL of DCM each and dried in vacuum at 50 °C. The product was isolated as a white powder (350 mg, 81% yield). ^{1}H NMR (343 K): δ 1.34 (t, *J* = 7.1 Hz, 3H, CH_2-$C\underline{H}_3$), 3.87 (s, 3H, OCH_3), 4.40 (q, *J* = 7.1 Hz, 2H, $C\underline{H}_2$-CH_3), 7.00 (s, 1H, 3-H), 7.07 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H, 5'-H), 7.15 (d, *J* = 2.2 Hz, 1H, 3'-H), 7.77 (d, *J* = 8.5 Hz, 1H, 6'-H), 7.95 (d, *J* = 2.5 Hz, 1H, 5-H), 8.41 (d, *J* = 2.5 Hz, 1H, 7-H), 10.07 (br, 1H, NH). ^{13}C NMR (343 K): δ 13.0 (CH_2-$\underline{C}H_3$), 55.2 (OCH_3), 62.0 ($\underline{C}H_2$-CH_3), 112.5 (C-5'), 113.1 (C-3), 114.8 (C-3'), 117.1 (C-6), 122.1 (C-5), 124.7 (C-4a), 126.1 (C-1'), 129.1 (C-8), 130.1 (C-6'), 130.7 (C-7), 131.0 (C-2'), 146.7 (C-8a), 151.3 (C-2), 158.8 ($\underline{C}O_2Et$), 160.9 (C-4'), 163.8 (CO-NH), 175.0 (C-4). LC-MS (*m*/*z*): negative mode 480 $[M-H]^-$, positive mode 482 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 223–224 °C.

8-(4-Methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (69). The compound was synthesized using **35** (161 mg, 0.44 mmol). Recrystallization from 1:1 acetone/ethanol afforded the product as a white powder (127 mg, 85% yield). ^{1}H NMR: δ 3.85 (s, 3H, OCH_3), 6.94 (s, 1H, 3-H), 7.09 (d, *J* = 8.8 Hz, 2H, 3'-H, 5'-H), 7.54 (dd, *J* = 7.9 Hz, 1H, 6-H), 7.89 (dd, *J* = 8.0 Hz, *J* = 1.4 Hz, 1H, 5-H), 8.01 (d, *J* = 8.8 Hz, 2H, 2'-H, 6'-H), 8.09 (dd, *J* = 7.6 Hz, *J* = 1.3 Hz, 1H, 7-H), 10.04 (s, 1H, NH). ^{13}C NMR: δ 55.6 (CH, OCH_3), 113.51 (C-3), 114.0 (C-3', C-5'), 121.3 (C-5), 124.5 (C-4a), 125.7 (C-7), 126.2 (C-1'), 128.6 (C-8), 129.8 (C-2', C-6'), 130.8 (C-6), 149.4 (C-8a), 153.1 (C-2), 161.4 (CO_2H),

162.4 (C-4'), 165.0 (CO-NH), 177.6 (C-4). LC-MS (*m*/*z*): negative mode 294 $[M-CO_2H]^-$, 338 $[M-H]^-$, positive mode 340 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.8%. mp 277-279 °C (decomp.).

8-(2,4-Dichlorobenzamido)-6-fluoro-4-oxo-4*H*-chromene-2-carboxylic acid (72). The compound was synthesized using **38** (170 mg, 0.40 mmol) and was isolated as a white powder (154 mg, 97% yield). ^{1}H NMR: δ 6.95 (s, 1H, 3-H), 7.59-7.61 (m, 2H, 5-H, 5'-H), 7.76-7.80 (m, 2H, 3'-H, 6'-H), 8.13-8.15 (m, 1H, 7-H), 10.66 (s, 1H, NH). ^{13}C NMR: δ 106.2 (d, *J* = 23.8 Hz, C-5), 113.0 (C-3), 117.6 (d, *J* = 29.3 Hz, C-7), 125.3 (d, *J* = 9.2 Hz, C-4a), 127.6 (C-5'), 129.6 (C-3' or C-6'), 129.7 (d, *J* = 11.0 Hz, C-8), 131.1 (C3' or C6'), 131.7 (C-4'), 134.5 (C-1'), 135.8 (C-2'), 145.4 (C-8a), 153.3 (C-2), 158.4 (d, *J* = 245.6 Hz, C-6), 161.1 (CO_2H), 164.6 (CO-NH), 176.8 (C-4). LC-MS (*m*/*z*): negative mode 350 $[M-CO_2H]^-$, 394 $[M-H]^-$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 282-283 °C (decomp.).

6-Chloro-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (78). The compound was synthesized using **44** (177 mg, 0.44 mmol) and was isolated as a white powder (161 mg, 98% yield). ^{1}H NMR: δ 3.86 (s, 3H, OCH_3), 6.96 (s, 1H, 3-H), 7.10 (d, *J* = 8.8 Hz, 2H, 3'-H, 5'-H), 7.80 (d, *J* = 2.5 Hz, 1H, 5-H), 8.00 (d, *J* = 8.8 Hz, 2H, 2'-H, 6'-H), 8.22 (d, *J* = 2.5 Hz, 1H, 7-H), 10.13 (s, 1H, NH). ^{13}C NMR: δ 55.7 (OCH_3), 113.5 (C-3), 114.1 (C-3', C-5'), 119.8 (C-7), 125.2 (C-4a), 125.8 (C-1'), 129.3 (C-5), 129.9 (C-2', C-6'), 130.0, 130.5 (C-6, C-8), 147.7 (C-8a), 153.4 (C-2), 161.1 (CO_2H), 162.6 (C-4'), 165.0 (CO-NH), 176.5 (C-4). LC-MS (*m*/*z*): negative mode 328 $[M-CO_2H]^-$, 372 $[M-H]^-$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.8%. mp 278-280 °C (decomp.).

6-Bromo-8-(2,4-dichlorobenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (82). The compound was synthesized using **48** (146 mg, 0.30 mmol) and was isolated as a white solid (124 mg, 91% yield). ^{1}H NMR: δ 6.97 (s, 1H, 3-H), 7.60 (dd, *J* = 8.2 Hz, *J* = 1.9 Hz, 1H, 5'-H), 7.76 (d, *J* = 8.2 Hz, 1H, 6'-H), 7.78 (d, *J* = 1.6 Hz, 1H, 3'-H), 7.98 (d, *J* = 2.5 Hz, 1H, 5-H), 8.36 (br, 1H, 7-H), 10.66 (s, 1H, NH). ^{13}C NMR: δ 113.8 (C-3), 117.8 (C-6), 123.8 (C-5), 125.7 (C-4a), 127.6 (C-5'), 129.4 (C-8), 129.6 (C-3'

or C-6'), 131.1 (C-3' or C-6'), 131.7 (C-4'), 132.1 (C-7), 134.5 (C-1'), 135.7 (C-2'), 148.1 (C-8a), 153.4 (C-2), 161.1 (CO_2H), 164.7 (CO-NH), 176.2 (C-4). LC-MS (*m*/*z*): negative mode 412 $[M\text{-}CO_2H]^-$, 456 $[M\text{-}H]^-$, positive mode 458 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 284-286 °C (decomp.).

6-Bromo-8-(3,4-dichlorobenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (83). The compound was synthesized using **49** (194 mg, 0.40 mmol) and was isolated as a white powder (180 mg, 98% yield). ^{1}H NMR: δ 6.94 (s, 1H, 3-H), 7.83 (d, *J* = 8.2 Hz, 1H, 5'-H), 7.92 (dd, *J* = 8.4 Hz, *J* = 2.1 Hz, 1H, 6'-H), 7.96 (d, *J* = 2.5 Hz, 1H, 5-H), 8.20-8.21 (m, 2H, 7-H, 2'-H), 10.58 (s, 1H, NH). ^{13}C NMR: δ 113.7 (C-3), 117.8 (C-6), 124.1 (C-5), 125.7 (C-4a), 128.2 (C-6'), 129.7 (C-8), 129.1 (C-2'), 131.2 (C-5'), 131.7 (C-3'), 133.3 (C-7), 134.1 (C-1'), 135.2 (C-4'), 148.7 (C-8a), 153.3 (C-2), 161.1 (CO_2H), 163.7 (CO-NH), 176.3 (C-4). LC-MS (*m*/*z*): negative mode 412 $[M\text{-}CO_2H]^-$, 456 $[M\text{-}H]^-$, positive mode 458 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 281-282 °C (decomp.).

6-Bromo-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (85). The compound was synthesized using **51** (196 mg, 0.44 mmol) and was isolated as a white powder (174 mg, 95% yield). ^{1}H NMR: δ 3.86 (s, 3H, OCH_3), 6.97 (s, 1H, 3-H), 7.09 (d, *J* = 8.8 Hz, 2H, 3'-H, 5'-H), 7.93 (d, *J* = 2.5 Hz, 1H, 5-H), 7.99 (d, *J* = 8.8 Hz, 2H, 2'-H, 6'-H), 8.33 (d, *J* = 2.2 Hz, 1H, 7-H), 10.11 (s, 1H, NH). ^{13}C NMR: δ 55.7 (OCH_3), 113.6 (C-3), 114.0 (C-3', C-5'), 117.8 (C-6), 122.9 (C-5), 125.5, 125.8 (C-10, C-1'), 129.9 (C-2', C-6'), 130.5 (C-8), 132.0 (C-7), 148.1 (C-8a), 153.3 (C-2), 161.1 (CO_2H), 162.6 (C-4'), 165.0 (CO-NH), 176.4 (C-4). LC-MS (*m*/*z*): negative mode 372 $[M\text{-}CO_2H]^-$, 416 $[M\text{-}H]^-$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.6%. mp 278-279 °C (decomp.).

6-Bromo-8-(2-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (87). The compound was synthesized using **53** (196 mg, 0.44 mmol). Recrystallization from 1:1 acetone/ethanol (10 mL) afforded the product as a pale yellow powder (158 mg, 86% yield). ^{1}H NMR: δ 4.13 (s, 3H, OCH_3), 6.96 (s, 1H,

3-H), 7.15 (dd, *J* = 7.4 Hz, 1H, 4'-H), 7.29 (d, *J* = 8.2 Hz, 1H, 3'-H), 7.62 (ddd, *J* = 7.7 Hz, *J* = 1.7 Hz, 1H, 5'-H), 7.75 (d, *J* = 2.5 Hz, 1H, 5-H), 8.08 (dd, *J* = 7.7 Hz, *J* = 1.7 Hz, 1H, 6'-H), 9.06 (d, *J* = 2.2 Hz, 1H, 7-H), 10.68 (s, 1H, NH). ^{13}C NMR: δ 56.9 (OCH_3), 112.7 (C-3'), 113.8 (C-3), 118.4 (C-6), 119.9 (C-1'), 120.6 (C-5'), 121.4 (C-5), 125.1 (C-4a), 126.3 (C-6'), 130.4 (C-8), 131.9 (C-7), 134.7 (C-4'), 144.7 (C-8a), 153.0 (C-2), 157.7 (C-2'), 161.2 (CO_2H), 163.4 (CO-NH), 176.2 (C-4). LC-MS (*m*/*z*): negative mode 374 $[M-CO_2H]^-$, 416 $[M-H]^-$, positive mode 420 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 278-279 °C (decomp.).

6-Bromo-8-(3,4-dimethoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (88). The compound was synthesized using **54** (210 mg, 0.44 mmol) and was isolated as a white powder (192 mg, 97% yield). ^{1}H NMR: δ 3.85, 3.86 (s, 6H, OCH_3), 6.97 (s, 1H, 3-H), 7.12 (d, *J* = 8.5 Hz, 1H, 5'-H), 7.57 (d, *J* = 1.9 Hz, 1H, 2'-H), 7.64 (dd, *J* = 8.4 Hz, *J* = 2.0 Hz, 1H, 6'-H), 7.92 (d, *J* = 2.5 Hz, 1H, 5-H), 8.38 (d, *J* = 2.5 Hz, 1H, 7-H), 10.10 (s, 1H, NH). ^{13}C NMR: δ 55.8, 55.9 (OCH_3), 111.0, 111.4 (C-2', C-5'), 113.6 (C-3), 117.9 (C-6), 121.5 (C-6'), 122.7 (C-5), 125.5, 125.8 (C-4a, C-1'), 130.4 (C-8), 131.4 (C-7), 147.8 (C-3'), 148.6 (C-8a), 152.3 (C-4'), 153.3 (C-2), 161.2 (CO_2H), 165.0 (CO-NH), 176.4 (C-4). LC-MS (*m*/*z*): negative mode 402 $[M-CO_2H]^-$, 448 $[M-H]^-$, positive mode 450 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp 264-265 °C (decomp.).

6-Bromo-8-(2-chloro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (90). The compound was synthesized using **56** (212 mg, 0.44 mmol) and was isolated as a white powder (190 mg, 95% yield). ^{1}H NMR: δ 3.85 (s, 3H, OCH_3), 6.97 (s, 1H, 3-H), 7.06 (dd, *J* = 8.7 Hz, *J* = 2.4 Hz, 1H, 5'-H), 7.16 (d, *J* = 2.5 Hz, 1H, 3'-H), 7.75 (d, *J* = 8.8 Hz, 1H, 6'-H), 7.93 (d, *J* = 2.5 Hz, 1H, 5-H), 8.44 (br, 1H, 7-H), 10.34 (s, 1H, NH). ^{13}C NMR: δ 56.1 (OCH_3), 113.4 (C-5'), 113.7 (C-3), 115.5 (C-3'), 117.9 (C-6), 123.0 (C-5), 125.6 (C-4a), 127.1 (C-1'), 129.9 (C-8), 131.0 (C-6'), 131.6 (C-7), 131.9 (C-2'), 147.5 (C-8a), 153.4 (C-2), 161.1 (CO_2H), 161.6 (C-4'), 164.9 (CO-NH), 176.3 (C-4). LC-MS (*m*/*z*):

negative mode 408 $[M\text{-}CO_2H]^-$, 452 $[M\text{-}H]^-$, positive mode 454 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.7%. mp 281-282 °C (decomp.).

Ethyl 6-bromo-8-(6-methoxynicotinamido)-4-oxo-4*H*-chromene-2-carboxylate (98). The compound was synthesized using 6-methoxynicotinic acid (184 mg, 1.2 mmol) and **18** (281 mg, 0.9 mmol). The precipitate was filtered off, washed three times with 1 mL of DCM each and dried in vacuum at 50 °C. The product was isolated as a white powder (318 mg, 79% yield). ^{1}H NMR (343 K): δ 1.29 (t, *J* = 7.1 Hz, 3H, $CH_2\text{-}C\underline{H}_3$), 3.98 (s, 3H, OCH_3), 4.37 (q, *J* = 7.1 Hz, 2H, $C\underline{H}_2\text{-}CH_3$), 6.97 (d, *J* = 8.8 Hz, 1H, 5'-H), 7.00 (s, 1H, 3-H), 7.97 (d, *J* = 2.2 Hz, 1H, 5-H), 8.26 (dd, *J* = 8.7 Hz, *J* = 2.4 Hz, 1H, 4'-H), 8.31 (d, *J* = 2.8 Hz, 1H, 2'-H), 8.86 (d, *J* = 2.5 Hz, 1H, 7-H), 10.19 (br, 1H, NH). ^{13}C NMR (343 K): δ 13.4 ($CH_2\text{-}\underline{C}H_3$), 53.6 ($OCH_3$), 62.6 ($\underline{C}H_2\text{-}CH_3$), 110.2 (C-5'), 113.6 (C-3), 117.6 (C-6), 123.0 (C-3'), 123.1 (C-5), 125.3 (C-4a), 130.0 (C-8), 132.0 (C-7), 138.3 (C-4'), 147.5 (C-2'), 147.5 (C-8a), 151.8 (C-2), 159.3 ($\underline{C}O_2Et$), 163.7 (C-6'), 165.8 (CO-NH), 175.7 (C-4). LC-MS (*m*/*z*): negative mode 447 $[M\text{-}H]^-$, positive mode 449 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 100.0%. mp: 252–253 °C.

6-Bromo-8-(6-methoxynicotinamido)-4-oxo-4*H*-chromene-2-carboxylic acid (102). The compound was synthesized using **98** (197 mg, 0.44 mmol) and was isolated as a white powder (180 mg, 98% yield). ^{1}H NMR (343 K): δ 3.98 (s, 3H, OCH_3), 6.95-6.97 (m, 2H, 3-H, 5'-H), 7.96 (d, *J* = 1.6 Hz, 1H, 5-H), 8.24 (dd, *J* = 8.5 Hz, *J* = 1.9 Hz, 1H, 4'-H), 8.34 (d, *J* = 1.6 Hz, 1H, 2'-H), 8.84 (d, *J* = 1.3 Hz, 1H, 7-H), 10.15 (s, 1H, NH). ^{13}C NMR (343 K): δ 53.6 (OCH_3), 110.2 (C-5'), 113.3 (C-3), 117.5 (C-6), 123.0 (C-5), 123.1 (C-3'), 125.3 (C-4a), 129.9 (C-8), 131.7 (C-7), 138.4 (C-4'), 147.5 (C-2'), 147.8 (C-8a), 153.2 (C-2), 160.6 (CO_2H), 163.8 (C-6'), 165.8 (CO-NH), 175.9 (C-4). LC-MS (*m*/*z*): negative mode 375 $[M\text{-}CO_2H]^-$, 419 $[M\text{-}H]^-$, positive mode 419 $[M+H]^+$. Purity by HPLC-UV (254 nm)-ESI-MS: 99.7%. mp 273 °C (slow decomp.).

Pharmacological assays

For β-arrestin assays, CHO cell lines were used that stably expressed β-arrestin fused to an N-terminal deletion mutant of β-galactosidase (β-arrestin-EA) and receptors C-terminally tagged with an enzyme-fragment. Cell lines transfected with human GPR35 and GPR55 were purchased, while cell lines expressing human GPR35b, mouse and rat GPR35 were created by transfecting a parental CHO β-arrestin-EA cell line with the respective plasmids (Pathhunter® β-arrestin assays, DiscoverX, Fremont, CA). On the day before the assay, cells were seeded into 96-well plates (Thermo Scientific, Waltham, MA) at a density of 20000 cells per well in 90 µL of cell plating medium 2 (DiscoverX, Fremont, CA) or Optimem medium supplemented with 2% FCS, 100 units/mL penicillin G, 100 µg/mL streptomycin, 300 µg/mL hygromycin B and 1% ultraglutamin (Invitrogen, Carlsbad, CA or Sigma-Aldrich, St. Louis, MO). Compound dilutions were prepared in DMSO and further diluted in cell plating medium 2 or phosphate buffered saline in a ratio of 1:10. In antagonist assays 5 µL of compound dilutions were added to each well. After 30 min of incubation, 5 µL of standard agonist were added (final concentration: 5 µM zaprinast for human GPR35, 1 µM LPI for human GPR55). In agonist assays 10 µL of compound dilutions per well were added. Final DMSO concentration did not exceed 1%. After 90 min of incubation, 50 µL of detection reagent (DiscoverX, Fremont, CA) per well were added. Luminescence was determined after 60 min using a Topcount NXT plate reader (Perkin Elmer, Meriden, CT). Three to four independent experiments were performed, each in duplicate.[56] All data were analyzed using GraphPad Prism, Version 4.02 (GraphPad Inc., La Jolla, CA).

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and ^{1}H and ^{13}C NMR spectral data for compounds **12**, **13**, **16–18**, **21–54, 36**, **37**, **39–43**, **45–47**, **50**, **52**, **55**, **57–68**, **70**, **71**, **73–77**, **79–81**, **84**, **86**, **89**, **91**, **95–97**, **99–101** and **103**, and experimental procedures for investigating the hydrolytic stability of **85**. This material is available free of charge in the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

AHR, aryl hydrocarbon acid receptor; AMPAR, α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor; Ar, aryl; compd, compound; clogP, calculated partition coefficient; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DNA pol., DNA polymerase; GABA, γ-aminobutyric acid; GPCR, G protein-coupled receptor; LE, ligand efficiency; LLE (LipE), ligand-lipophilicity efficiency; LPI, lysophospatidylinositol; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartic acid; PDE, phosphodiesterase; PSA, polar surface area; rt, room temperature; SARs, structure-activity relationships; THF, tetrahydrofuran.

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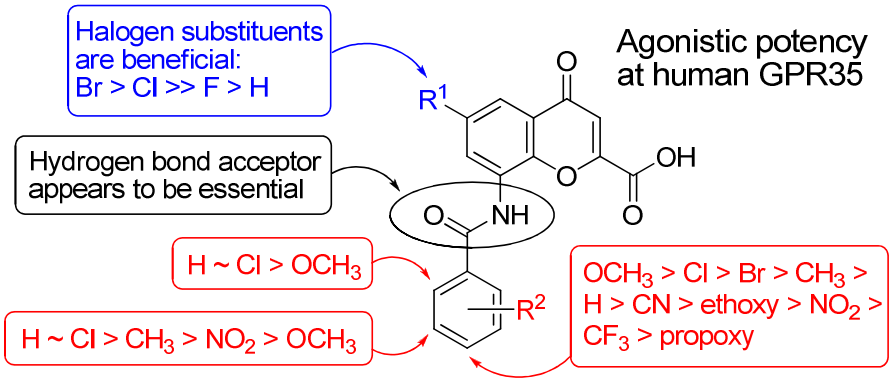
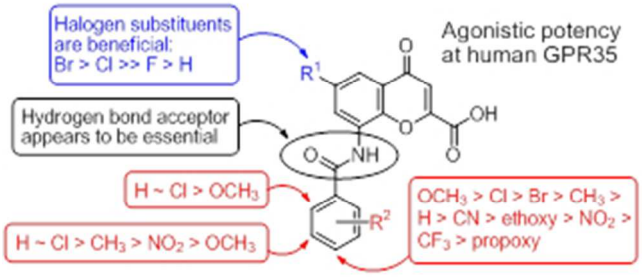
Table of Contents Graphic





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