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6-Bromo-8-(4-[^{3}H]methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic Acid: A Powerful Tool for Studying Orphan G Protein-Coupled Receptor GPR35

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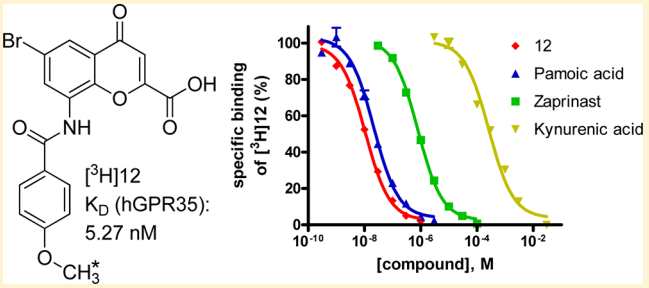
Supporting Information

ABSTRACT: The potent and selective GPR35 agonist 6-bromo-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (**12**) was obtained in tritium-labeled form, designated [^{3}H]PSB-13253, with a specific activity of 36 Ci (1.33 TBq)/mmol. Radiolabeling was achieved by methylation of ethyl 6-bromo-8-(4-((*tert*-butyldimethylsilyl)oxy)-benzamido)-4-oxo-4*H*-chromene-2-carboxylate (**19**) with [^{3}H]methyl tosylate followed by ester hydrolysis. The radioligand was characterized by kinetic, saturation, and competition assays at membrane preparations of Chinese hamster ovary cells recombinantly expressing the human GPR35. [^{3}H]**12** labeled the receptor with high affinity (K_D = 5.27 nM). Binding was saturable (B_{max} = 12.6 pmol/mg of protein) and reversible. Affinities of selected standard ligands and a library of amidochromen-4-one-2-carboxylates were determined. Binding data mostly correlated with potencies determined in β-arrestin assays. On the basis of the test results, several new fluorine-substituted 6-bromo-8-benzamidochromen-4-one-2-carboxylic acids were obtained, which represent the most potent GPR35 agonists known to date. 6-Bromo-8-(2,6-difluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (**83**; K_i = 0.589 nM, EC_{50} = 5.54 nM) showed the highest affinity with a K_i value in the subnanomolar range.



INTRODUCTION

G protein-coupled receptors (GPCRs) constitute one of the most important classes of pharmacotherapeutic targets. However, the (patho)physiological roles of many GPCRs is far from being well understood. GPCRs whose endogenous agonist is yet unknown belong to the most enigmatic members of this receptor superfamily; they are termed "orphan" receptors. GPR35, a rhodopsin-like class A GPCR, is such an orphan receptor.[1] Although several endogenous compounds, including the tryptophan metabolite kynurenic acid (**1**),[2] some tyrosine metabolites,[3] and lysophosphatidic acid species[4] have been shown to activate the human GPR35, their potential role as physiological agonists is still unclear and controversially discussed.[3,5] GPR35 is related to the orphan receptor GPR55 (sequence identity of the human orthologues 34%) and to subtypes of lysophosphatidic acid receptors (e.g., GPR23/LPA_4R, 28%), P2Y nucleotide receptors (e.g., $P2Y_4$, 27%), and hydroxycarboxylic acid receptors (e.g., HCAR3, 25%).[1,6]

Human and rodent GPR35 were found to be expressed by various cells and organs. High mRNA expression levels could be detected in intestine and colon,[1,2,7] spleen, several cells of the immune system,[2,8] and sensory cells of the peripheral nervous system.[9−11] Although the receptor is poorly investigated, there is some evidence for its possible (patho)-physiological relevance: it may be involved in the regulation of blood pressure, since after genetic deletion of GPR35 in mice their blood pressure was significantly increased.[12] In rodent pain models GPR35 agonists exhibited antinociceptive effects indicating that the receptor may play a role in pain perception.[9,13] Moreover, there is growing evidence that GPR35 is involved in the modulation of immune responses: receptor activation was found to induce firm arrest of monocytes to an endothelium-like matrix, a process that initiates recruitment of leukocytes to inflamed tissues.[14] In addition, GPR35 activation was found to affect cytokine production of human monocytes and type 1 natural killer T cells.[2,8] Furthermore, the receptor has been implicated in the pathogenesis of gastric cancer,[7] type 2 diabetes,[15] and cardiovascular diseases.[16] Thus, GPR35 has great potential to become a drug target for the treatment of various pathophysiological conditions.

Several structurally diverse GPR35 agonists have recently been described, indicating promiscuity of the receptor.[2,11,19,21,23,25] Almost all of them have been discovered by high-throughput screening (HTS) of compound libraries. Apart from the physiological compounds mentioned above, synthetic agonists have been identified. The phosphodiesterase inhibitor zaprinast (**2**) was the first synthetic surrogate agonist and became the most important reference ligand for GPR35.[11]

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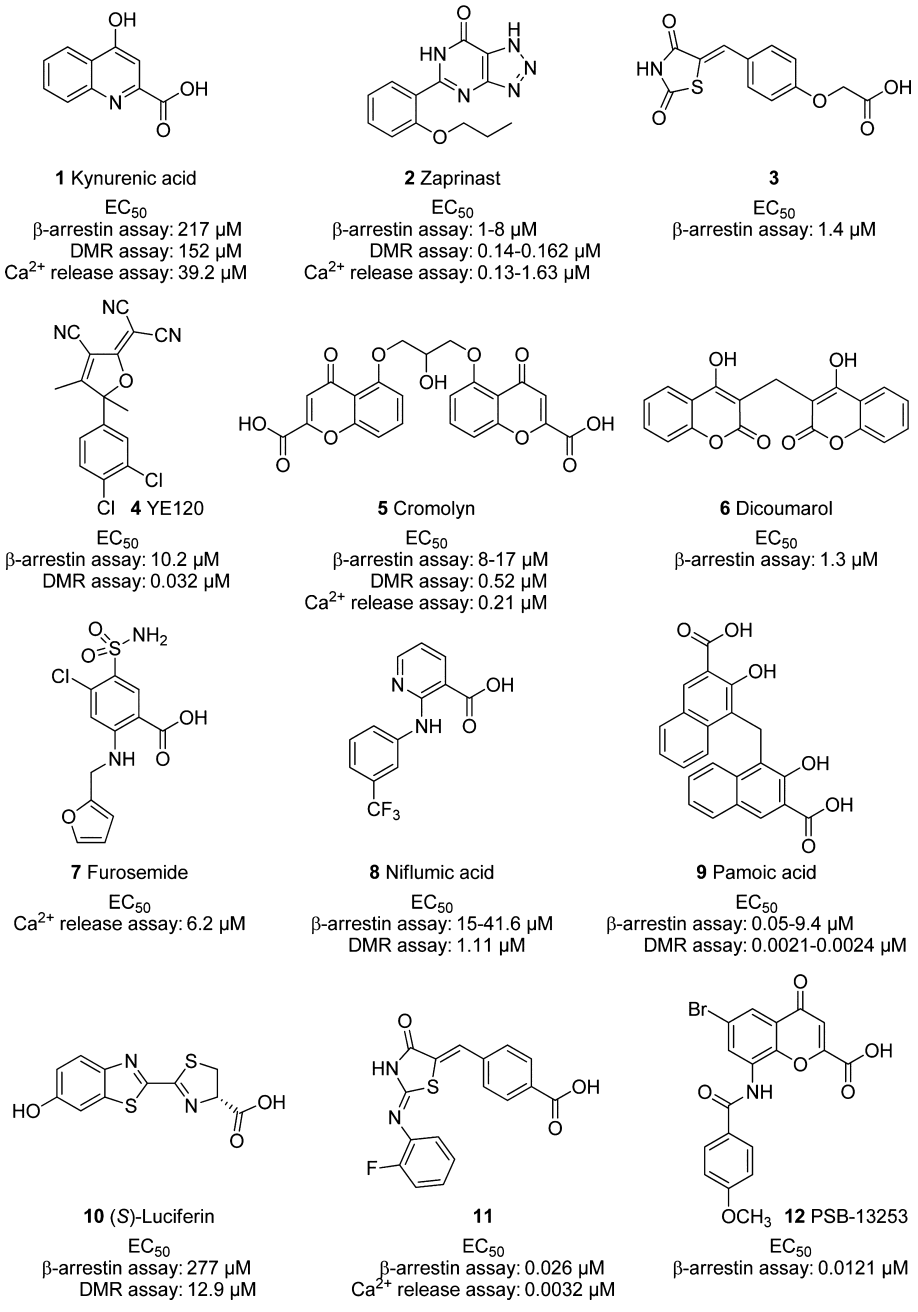

Figure 1. Selected GPR35 agonists. Potencies at the human GPR35 determined in different assay systems are given.[2,3,17−26]

Other synthetic GPR35 agonists include ((dioxothiazolidinylidene)methyl)phenoxyacetic acid (**3**)[27] and the (methylfuranylidene)malononitrile YE120 (**4**).[17] Some approved drugs were also found to activate the human GPR35: the antiallergic drug cromolyn (**5**),[24] the anticoagulant dicoumarol (**6**),[19] loop diuretics such as furosemide (**7**),[23] and the nonsteroidal anti-inflammatory drug niflumic acid (**8**).[19] In addition, pamoic acid (**9**), which is contained in several pharmaceutical products and was previously thought to be pharmacologically inert, was found to act as a partial agonist at the human GPR35.[19,25] Partial agonism was also described for (*S*)-luciferin (**10**), as well as for the above-mentioned therapeutic drugs dicoumarol and niflumic acid.[18,19] Most of the known GPR35 agonists show only moderate potency with EC_{50} values typically in the high micromolar to high nanomolar range. Very few highly potent agonists with EC_{50} values in the low nanomolar range have been described so far: among those are the (thiazolidinylidenemethyl)benzoic acid derivative **11** reported by Graeme Milligan and co-workers[21] and several benzamidochromenonecarboxylic acid derivatives, e.g., **12**, which were recently developed in our group (for structures see Figure 1).[26] Compound **12** represents the most potent agonist for the human GPR35 known to date. In contrast to the large number of agonists, only a few antagonists have been described for GPR35, the most potent ones being CID 2745687 (**13**) and ML 145 (**14**; for structures see Figure 2).[25,28]

GPR35 was found to be coupled to $G_{i/o}$ and G_{13} proteins and to induce *β*-arrestin recruitment upon activation. For the pharmacological characterization of GPR35 ligands *β*-arrestin recruitment assays are most commonly used. In addition, dynamic mass redistribution (DMR) and calcium mobilization

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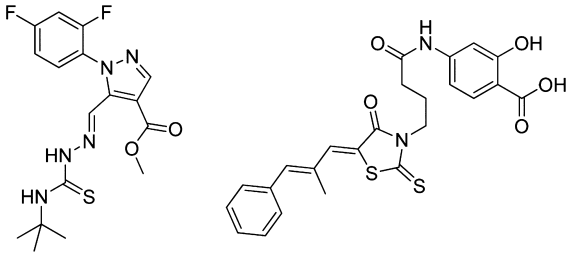


Figure 2. Selected antagonists. IC_{50} values were determined at the human GPR35 versus the agonist zaprinast (4 μM in β-arrestin recruitment assays, 0.5 μM in dynamic mass redistribution assays).[3,20].

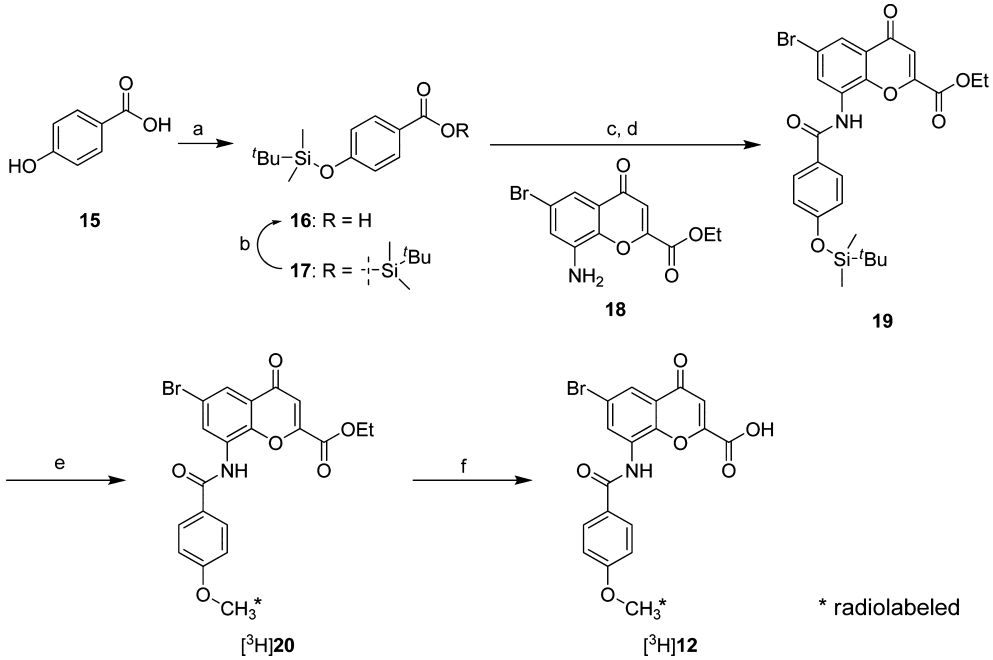
assays (after coexpression with chimeric or promiscuous G proteins) have been described for determining the potency of ligands. It has become evident that considerable differences in the determined EC_{50} values of GPR35 agonists are observed depending on the assay system used. Rank orders of potencies do correlate for the majority of agonists in the different test systems; however, in some cases differences are larger than expected: whereas for many ligands the EC_{50} values determined in DMR or calcium mobilization assays are about 10−30-fold lower than those determined in β-arrestin assays, for some agonists, e.g., pamoic acid, a more than 1000-fold difference in potency was determined (see Figure 1), possibly indicating biased agonism. In general, functional assays have to be carefully analyzed, because the results may be influenced by receptor expression levels, ligand bias, or nonspecific, receptor-independent effects.[29] Therefore, radioligand binding assays provide valuable complementary information, yielding direct affinity data of a test compound at its target receptor. Furthermore, radioligands can be used to quantify receptor expression levels.[30] In addition, radioligand binding data are essential to evaluate the effects of mutations introduced into GPCRs to determine the role of single amino acids in binding or receptor activation.[31,32] Most recently, radioligands were shown to be powerful tools in quality control during experimental steps preceding receptor crystallization,[33] e.g., to characterize stabilizing mutations.[34] Radioligand binding data complement structural data obtained for a given GPCR and thus facilitate discussion of observations from X-ray crystallography.[35] However, up to now, the affinity of ligands at GPR35 could not be directly assessed due to the lack of a high-affinity radioligand.

In the present study, we pursued the development of a suitable radioligand and the establishment of a radioligand binding assay for the human GPR35. A tritiated form of the highly potent GPR35 agonist 6-bromo-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic acid (PSB-13253)[26] was prepared. The radioligand was characterized and applied in competition assays. It thus allowed for the first time the determination of the affinity of a series of ligands in binding studies at the human GPR35. Furthermore, we synthesized a set of new amidochromenone derivatives to further explore their structure−activity relationships (SARs) and to obtain new GPR35 agonists with improved potency.

RESULTS AND DISCUSSION

Chemistry. The synthesis of [^{3}H]**12** was performed as depicted in Scheme 1. A suitable precursor (**19**) was prepared for radiolabeling, which would allow for the introduction of tritium atoms by a one-step conversion of the *tert*-butyldimethylsilyl ether **19** to the corresponding [^{3}H]methyl ether **20**.[36] Thus, *p*-hydroxybenzoic acid (**15**) was reacted with

Scheme 1. Preparation of the GPR35 Radioligand [^{3}H]12[a]



[a]Reagents and conditions: (a) *tert*-butyldimethylsilyl chloride, DIPEA, DMF, rt, overnight; (b) 5% aq NaOH solution, THF, rt, 5 min, overall yield 70%; (c) $SOCl_2$, cat. DMF, CH_2Cl_2, argon, rt, 30 min; (d) **18**, DIPEA, DCM, THF, argon, rt, 2 d, yield 85% (for both steps); (e) [^{3}H]methyl tosylate, potassium fluoride (anhydrous), DMF, rt, overnight; (f) K_2CO_3, THF, EtOH, H_2O, rt, 4 h, diluted aq HCl (1 N).

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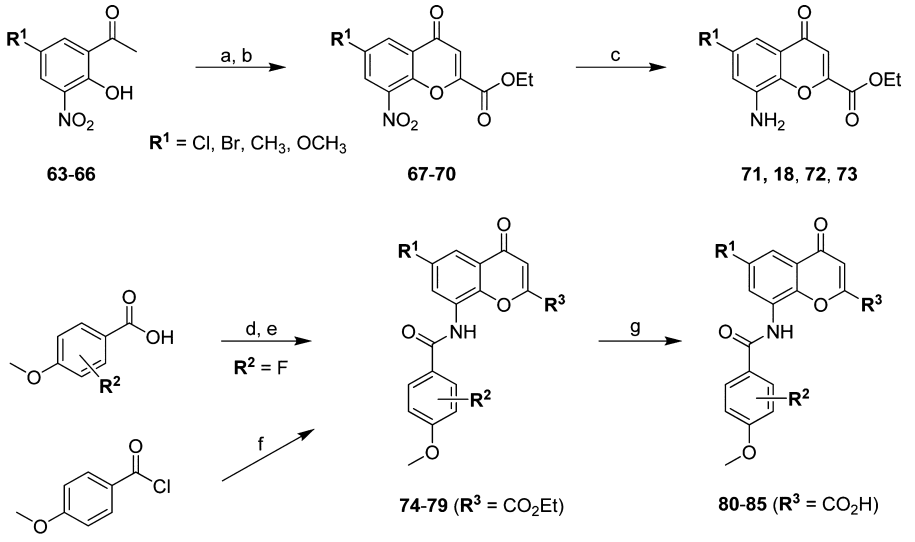
Scheme 2. Synthesis of New 6-Substituted 8-Benzamidochromen-4-one-2-carboxylic Acid Derivatives[a]



		compd	yield (%)	compd	yield (%)
R^1	R^2	R^3 = CO_2Et		R^3 = CO_2H	
Cl	2-F	**74**	82	**80**	80
Br	2-F	**75**	64	**81**	95
Br	3-F	**76**	74	**82**	75
Br	2,6-*di*-F	**77**	51	**83**	91
Me	H	**78**	81	**84**	86
OMe	H	**79**	85	**85**	93

[a]Reagents and conditions: (a) $(COOEt)_2$, KO^{t}Bu, DMF, argon, 0−5 °C, 2−3 h; concd aq HCl, ice−water; (b) concd aq HCl, EtOH, reflux, overnight, yield 79−82%; (c) $SnCl_2 \cdot 2H_2O$, aq HCl (2 N), EtOH, 65−70 °C, 20 min, yield 80−95%; (d) $SOCl_2$, cat. DMF, CH_2Cl_2, argon, rt, 30 min; (e) **18** or **71**, DIPEA, DCM, THF, argon, rt, 2 d, yield 51−82%; (f) **72** or **73**, DIPEA, DCM, THF, argon, rt, 2 d, yield 81−85%; (g) K_2CO_3, THF, EtOH, H_2O, rt, overnight, aq HCl (2 N), H_2O, yield 75−95%.

tert-butyldimethylsilyl chloride in the presence of *N*,*N*-diisopropylethylamine (DIPEA) as a base in *N*,*N*-dimethylformamide (DMF) at room temperature (rt) overnight,[37] yielding the *tert*-butyldimethylsilyl ether **16**[38] and its *tert*-butyldimethylsilyl ester derivative **17**.[39] The undesired ester **17** was transformed into **16** by alkaline hydrolysis,[40] treating **17** with aqueous NaOH solution in tetrahydrofuran (THF) at rt for 5 min. A good overall yield of 70% was obtained. Reaction of **16** with thionyl chloride in the presence of DMF as a catalyst in dichloromethane (DCM) at rt for 30 min afforded the corresponding acid chloride, which was subsequently coupled with aminochromenone **18**[26,41] in the presence of DIPEA as a base in a 6:1 mixture of DCM and THF at rt for 2 d, providing amide **19** in a very good yield of 85%. The precursor molecule **19** was custom-labeled by Quotient Bioresearch, Cardiff, U.K., using [^{3}H]methyl tosylate (84 Ci/mmol) in the presence of anhydrous potassium fluoride as a base in DMF at rt. The obtained product [^{3}H]**20** was finally hydrolyzed by treatment with potassium carbonate in a 4:3:1 mixture of THF, water, and ethanol at rt for 4 h, affording the radioligand [^{3}H]**12**, which was purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The overall yield from 100 mCi of [^{3}H]methyl tosylate was 7.8 mCi. The identity of [^{3}H]**12** was confirmed by mass spectrometric analysis. [^{3}H]**12** displayed a specific activity of 36 Ci/mmol (1.33 TBq/mmol) and a radiochemical purity of >99.9% as determined by HPLC−UV (254 nm).

The preparation of compounds **21**−**62** has previously been described.[26,42,43] The amidochromene derivatives **80**−**85** were obtained by a recently developed method as depicted in Scheme 2.[26] Treatment of 5′-substituted 2′-hydroxy-3′-nitroacetophenones **63**−**66** with diethyl oxalate and potassium *tert*-butoxide as a base in DMF at 0−5 °C for 2−3 h yielded the appropriate diketone intermediates, which were subsequently reacted with hydrochloric acid in ethanol under reflux to their chromen-4-one derivatives **67**,[26] **68**,[41] **69**,[44] and **70** in good yields (79−82%). Acetophenone **66** was prepared by nitration of 1-(2-hydroxy-5-methoxyphenyl)ethanone with anhydrous nitric acid in glacial acetic acid at rt for 2 h.[45] A moderate yield of 62% was obtained. The nitro groups of intermediates **67**−**70** were reduced[46] using tin(II) chloride in 2 N aqueous hydrochloric acid and ethanol, yielding the corresponding amino derivatives **18**,[41] **71**,[26] **72**,[44] and **73** in very good to excellent yields (80−95%). Treatment of 4-methoxybenzoic acids containing fluorine substitution at different positions with thionyl chloride in the presence of DMF as a catalyst in DCM at rt for 30 min yielded the corresponding acid chlorides. The acid chlorides were coupled with the amines **18** and **71** in the presence of DIPEA as a base in a 6:1 mixture of DCM and THF at rt, affording the amides **74**−**77** in moderate to good yields. For further variation of the 6-position of the chromen-4-one scaffold, 4-methoxybenzoyl chloride was reacted with the aniline derivatives **72** (R^1 = CH_3) and **73** (R^1 = OCH_3) as described above, yielding the amides **78** and **79**, respectively, in very good yields (81−85%). In the last step, the ethyl ester

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groups of **74**−**79** were hydrolyzed under mild basic conditions.[26] Very good to excellent yields of 75−95% were obtained for the final products **80**−**85**.

The structures of all final products were confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy and by HPLC analysis coupled to electrospray ionization mass spectrometry (LC−ESI-MS), which was also used to determine the compounds' purity.

Pharmacological Characterization of the New Radioligand. We could previously show that unlabeled **12** is a highly potent, fully efficacious agonist at human GPR35 as determined in β-arrestin assays.[26] Therefore, we selected this compound for tritium-labeling to obtain the first radioligand for the human GPR35 (see Scheme 1). In preliminary studies [^{3}H]**12** was found to bind with high affinity to membrane preparations of CHO cells recombinantly expressing the human GPR35. Furthermore, we found that addition of magnesium ions to the incubation buffer led to a significant increase in specific binding of the radioligand (see below). Similar observations with agonist radioligands were reported for several other GPCRs.[47−50] Therefore, as applied in many agonist radioligand binding assays,[49,51−54] we supplemented the incubation buffer with $MgCl_2$ (10 mM). The influence of ions on radioligand binding was studied in more detail after comprehensive pharmacological characterization of the radioligand (see below). Saturation experiments were performed using a wide range of radioligand concentrations, 10 μg of protein per sample, and a buffer consisting of 50 mM Tris, 10 mM $MgCl_2$, pH 7.4 (Figure 3). The results of the initial experiments suggested an incubation time of 150 min to be sufficient for reaching stable equilibrium binding. In three separate experiments, each performed in duplicate, a K_D value of 5.27 ± 0.18 nM was determined, and a high B_{max} value of 12.6 ± 1.2 pmol/mg of protein was calculated for membrane preparations of recombinant, GPR35-expressing CHO cells. In experiments assessing association and dissociation kinetics, 5 nM radioligand and 10 μg of protein were employed. In association experiments the radioligand and receptor protein were incubated for up to 200 min. Dissociation was induced by the addition of a high concentration of pamoic acid (10 μM) after 100 min of preincubation. Equilibrium binding was reached after 60 min and was found to be stable for at least 200 min at room temperature (Figure 4).

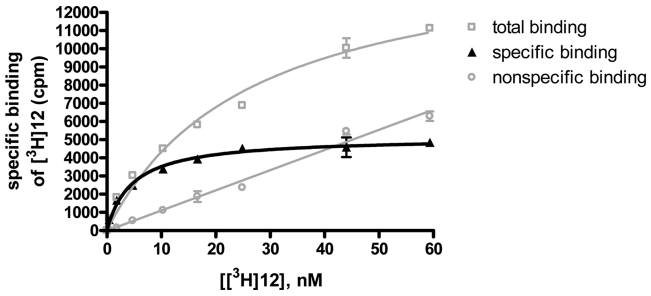

Figure 3. Saturation binding of [^{3}H]**12** to human GPR35 recombinantly expressed in CHO cells (10 μg of protein per sample). The data represent the means from a typical experiment performed in duplicate.

All subsequent radioligand binding studies were performed using a radioligand concentration of 5 nM, 10 μg of protein per sample, and an incubation time of 100 min in 50 mM Tris buffer, supplemented with 10 mM $MgCl_2$, pH 7.4 (unless otherwise indicated).

In experiments using different protein amounts, we could show that radioligand binding was strictly protein-dependent and that specific radioligand binding to membrane preparations of untransfected CHO cells was negligible (Supporting Information Figure 3), although a hypothetical GPR35-like receptor was predicted to be expressed in Chinese hamsters (accession no. XP_003499301). We previously showed that **12** is selective for human GPR35 versus its orthologues in rodents (mice and rats).[26] Therefore, one would expect **12** to have only a low affinity at the putative GPR35-like receptor in Chinese hamsters, which would preclude the receptor to be labeled by the radioligand. However, pamoic acid was used to determine nonspecific binding in these experiments. Since this compound was shown to be inactive at murine receptors,[20] one might expect it to also be inactive at the GPR35-like receptor expressed in Chinese hamsters. To verify that specific binding at membrane preparations of nontransfected cells was not underestimated, zaprinast (an agonist with high potency at rodent GPR35) was used in some experiments to determine nonspecific binding. We could not observe any significant difference for the determined nonspecific binding no matter whether pamoic acid or zaprinast was used. Thus, the hypothetical Chinese hamster GPR35-like receptor either is not labeled by the radioligand or is not expressed in CHO cells, or it does not exist. Furthermore, nonspecific binding barely rose with increasing protein concentrations, and it was nearly the same for samples without protein as for samples to which

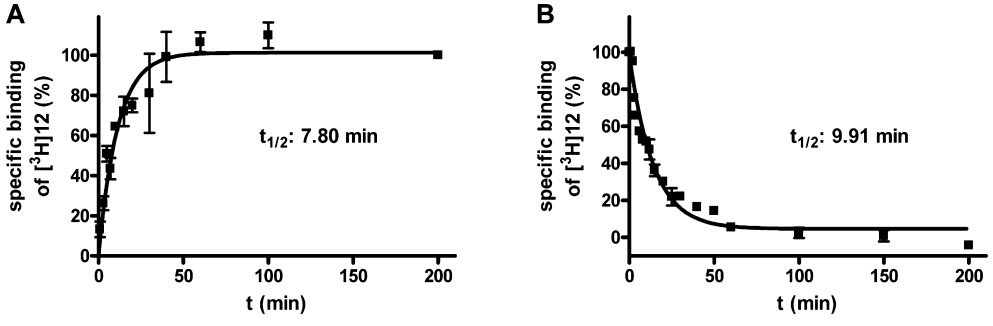

Figure 4. Kinetics of [^{3}H]**12** binding to human GPR35 recombinantly expressed in CHO cells (membrane preparations): (A) association and (B) dissociation curves (k_{on} = 3 640 000 M^{-1} min^{-1}, k_{off} = 0.0707 min^{-1}). Dissociation was induced by the addition of pamoic acid (10 μM) after 100 min of preincubation. Radioligand binding experiments were performed using a final concentration of 5 nM [^{3}H]**12**. The data represent the means of two independent experiments performed in duplicate.

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membrane preparations (of transfected or untransfected CHO cells) had been added. Thus, we can conclude that the radioligand has the advantage of very low to negligible nonspecific binding to CHO cell membranes. In addition, the radioligand did not appear to label members of receptor families that are closely related to GPR35 and are endogenously expressed in CHO cells (e.g., LPA and P2Y receptors).[55−57] In preliminary experiments, we also tested the radioligand at membrane preparations of CHO cells stably expressing mouse or rat GPR35 using a higher concentration of 15 nM [^{3}H]**12**, 100 μg of protein per sample, and an incubation time of 2 h. Nonspecific binding was determined in the presence of 250 μM zaprinast. Whereas specific binding of the radioligand at the human receptor amounted to about 90% of the total binding under similar conditions, it was very low at both rodent receptors with approximately 20% at the rat and only 8% at the mouse GPR35. As we could previously show **12** to have only moderate to weak potencies at the murine receptor orthologues in β-arrestin recruitment assays,[26] we can assume that the radioligand binds with only low affinity to the mouse and rat GPR35. Thus, [^{3}H]**12** is not a suitable radioligand for the labeling of murine GPR35 receptors.

Effects of Cations on Radioligand Binding. Next we investigated the effects of cations on radioligand binding. For that purpose we performed homologous competition experiments in which we omitted $MgCl_2$ addition to the incubation buffer or exchanged it for 10 mM $CaCl_2$ or 100 mM NaCl using 15 μg of protein per sample (see Figure 5A). When 50 mM Tris buffer, pH 7.4, without addition of any salts was used, specific binding amounted to approximately 65% of the total binding under the applied conditions. After addition of 10 mM $MgCl_2$ or 10 mM $CaCl_2$, specific binding increased to 90% and 85%, respectively, of the total binding, while the different cations barely affected nonspecific binding. Moreover, we found a 20-fold increase in the affinity of **12** induced by divalent cations (K_D values determined by homologous competition: without additional ions, 115 ± 32 nM; in the presence of 10 mM $MgCl_2$, 5.65 ± 0.28 nM; in the presence of 10 mM $CaCl_2$, 5.92 ± 0.18 nM; Figure 5B). Although in particular magnesium ions had previously been found to increase the affinity and the number of detected binding sites for agonists at several GPCRs,[47,50] the underlying molecular mechanism for this phenomenon is still unknown. However, magnesium ions are believed to shift the GPCR conformation toward an active state.[58] Therefore, a larger proportion of receptors would be in a high-affinity state for agonists and could thus be labeled by agonist radioligands. It would be interesting to address the question of whether divalent cations only modulate the receptor allosterically or whether they are additional mediators of ligand binding in some cases, e.g., by forming a coordination complex with the respective ligand and the receptor. Interestingly, while magnesium ions had been found to cause an increase in agonist affinity at many GPCRs, the effects of calcium ions appear to be strongly receptor-dependent, as they had been reported to increase[47] or decrease[59] the binding affinity or to not affect the affinity at all.[60]

In contrast to the effects observed for divalent ions, addition of 100 mM NaCl caused a loss of specific radioligand binding. This is in agreement with the results found for agonists at other GPCRs.[48,61−64] Recently, in a high-resolution crystal structure of the human A_{2A} adenosine receptor, a water cluster containing a sodium ion was found to bind to highly conserved amino acid residues, e.g., Asn1.50, Asp2.50, Ser3.39, Trp6.48,

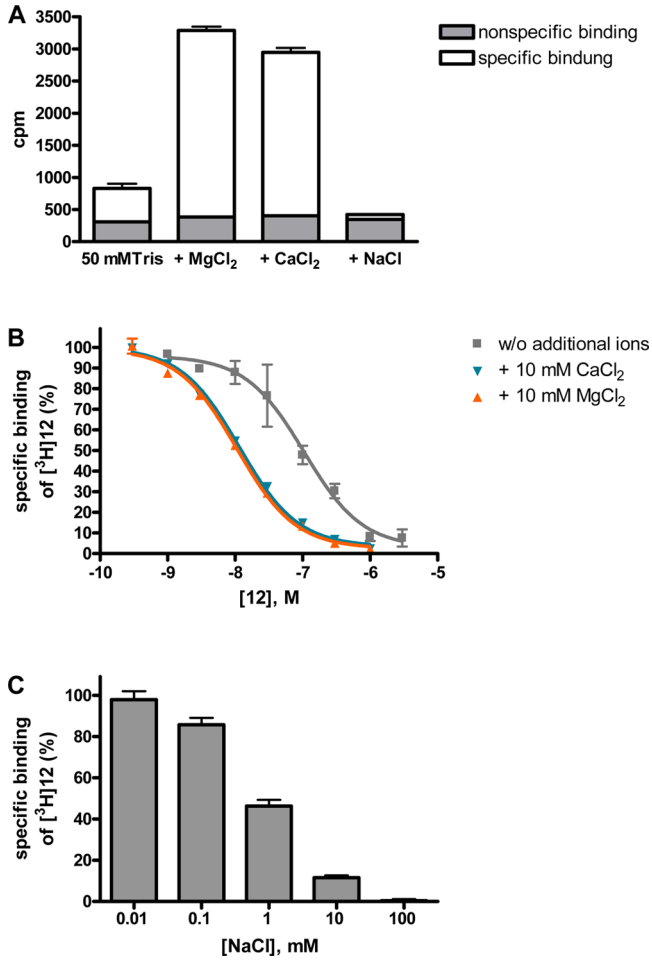

Figure 5. Effects of cations on binding of [^{3}H]**12**: (A) effect on binding capacity, (B) effect on affinity, (C) influence of sodium ions on specific binding of [^{3}H]**12** in an incubation buffer supplemented with 10 mM $MgCl_2$. Radioligand binding experiments were performed using 5 nM [^{3}H]**12** and membrane preparations of CHO cells recombinantly expressing human GPR35. The data represent the means of three independent experiments performed in duplicate.

Asn7.45, Asn7.49, and Tyr7.53.[62] Mutagenesis studies at different GPCRs had shown that among these amino acid residues mainly Asp2.50, which is conserved in GPR35, was mediating effects induced by sodium ions.[62] By comparing different crystal structures, the sodium binding pocket was found to collapse upon conformational change during receptor activation.[62] Thus, sodium ions are negative allosteric modulators affecting agonist binding at many GPCRs. We showed that sodium ions act in a similar fashion at GPR35. To avoid misinterpretation of competition binding data determined with sodium salts of test compounds, e.g., cromolyn, we wanted to explore the effect of sodium ions in more detail. We found that increasing concentrations of sodium chloride added to the standard incubation buffer (supplemented with 10 mM $MgCl_2$) gradually reduced specific binding of [^{3}H]**12** starting at a concentration of approximately 100 μM. At 100 mM sodium chloride specific binding was found to be completely abolished (Figure 5C). Since the receptor is fully activated in the presence of a high extracellular sodium chloride concentration,[26] the sodium binding site is likely not accessible from the extracellular space.

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Affinity of Known GPR35 Ligands. The affinity and potency of a selection of known GPR35 ligands was determined in radioligand binding and β-arrestin recruitment assays, respectively (Table 1, Figure 1). Radioligand binding assays were perfomed as described above. β-Arrestin recruitment assays were conducted using CHO cells expressing human GPR35 C-terminally tagged with an enzyme fragment as well as a fusion protein of β-arrestin and a deletion mutant of β-galactosidase (Pathhunter assay, DiscoverX, Fremont, CA). Upon receptor activation, the receptor recruits β-arrestin molecules and enzyme complementation occurs. Thus, measured enzyme activity correlates with receptor activation. The compounds were initially tested at a concentration of 10 μM. Inhibition curves and concentration−response curves were determined for compounds that caused more than 50% inhibition of radioligand binding and a 50% effect in β-arrestin recruitment assays (compared to the effect of 30 μM zaprinast), respectively.

Table 1. Potency and Affinity of Selected Standard GPR35 Ligands

	radioligand binding assays[a]	β-arrestin recruitment assays	
compd	K_i ± SEM (μM)	EC_{50} or IC_{50}[b] ± SEM (μM) (% effect[c] ± SEM)	E_{max}[c]
	Agonists		
1, kynurenic acid	137 ± 6[d]	(25 ± 5)[e]	
2, zaprinast	0.401 ± 0.015	1.96[f]	100
3	0.0508 ± 0.0023	0.349 ± 0.052	107
4	0.655 ± 0.035	(1 ± 4)	
5, cromolyn	2.34 ± 0.04[d]	1.26[f]	119
6, dicoumarol	0.0390 ± 0.0038	9.78 ± 1.73	107
7, furosemide	3.27 ± 0.28	11.2 ± 0.9	96
8, niflumic acid	3.61 ± 0.07	30.8 ± 3.1	97
9, pamoic acid	0.0115 ± 0.0010	1.20 ± 0.13	95
10, (*S*)-luciferin	9.86 ± 1.41	(11 ± 1)	
12	0.00518 ± 0.00033	0.0121[f]	121
	Antagonists		
13	0.0422 ± 0.0029	0.574 ± 0.050	
14	0.00876 ± 0.00043	0.522 ± 0.066	

[a]Affinities were determined in competition binding experiments using 5 nM [^{3}H]**12** and membrane preparations of CHO cells recombinantly expressing human GPR35. [b]IC_{50} values were determined versus 5 μM zaprinast. [c]Effects were normalized to the signal induced by 30 μM zaprinast, corresponding to a maximal response at the human GPR35. Screenings were performed at a concentration of 10 μM unless otherwise noted. [d]The inhibition curve was extrapolated. [e]Screened at 100 μM concentration. [f]Results were previously published.[26]

All known GPR35 ligands investigated in the present study inhibited binding of the radioligand [^{3}H]**12**, and full inhibition curves could be determined. Thus, all compounds appeared to share the same binding site with **12** or at least overlapping binding sites. It became evident that for most agonists the determined K_i values were about 2−9-fold lower than their EC_{50} values determined in β-arrestin assays. Surprisingly, the same was true for the two investigated antagonists, K_i values being much lower than the previously published IC_{50} values.

Especially for the dimeric compounds **6** and **9**, potency and affinity differed by more than 100-fold. Interestingly, both compounds have previously been found to be partial agonists, although in the applied β-arrestin assay only the efficacy of pamoic acid appeared to be somewhat reduced. Partial agonists have long been thought to induce less desensitization of GPCRs than full agonists. However, this concept has been challenged, and partial agonists may be able to cause a degree of desensitization similar to that of full agonists.[65] Interestingly, except for compound **8**, all ligands which had been found to be partial agonists (namely, **6**, **9**, and **10**) showed a disproportionately higher affinity in binding studies than one would have expected on the basis of functional data. The much lower potency in β-arrestin recruitment assays might, however, be due to biased signaling like had already been shown for **9** in a comparison of β-arrestin (lower potency) versus DMR assays (much higher potency, Figure 1). Thus, certain partial agonists might be biased, inducing β-arrestin recruitment with lower potency than other signaling pathways.

Compound **9** had for a long time been thought to be pharmacologically inert and is therefore used as an adjuvant in several pharmaceutical products. Although its agonistic activity had previously been described, its high affinity discovered in radioligand binding studies was surprising. This leads to the question of whether **9** causes adverse effects or could even be pharmacologically beneficial, e.g., in the antihelmintic treatment using the pamoate of pyrantel or pyrvinium. Pamoates are known as salts with low solubility; thus, beneficial effects of drugs applied as pamoates were thought to be dependent on their physicochemical properties rather than pharmacological effects of the anion. However, in the intestine and especially in cells of the intestinal endothelium, there is a very high expression of GPR35. As pamoic acid was shown to bind with very high affinity to GPR35, the receptor is likely to be activated, thereby possibly influencing the therapeutic outcome of the treatment. GPR35 is also known to be expressed on several cells of the immune system and was shown to be a functional modulator. Pamoic acid might also act on these cells. However, to our knowledge, no comprehensive study has been performed to determine the level of absorption of pamoic acid. In future studies it would be interesting to address the question of whether and how GPR35 signaling may be involved in effects by drugs administered as salts of pamoic acid. Among the investigated standard GPR35 ligands, **12** exhibited the highest affinity for the human GPR35.

Structure−Activity Relationships. As previously described, several chromen-4-one-2-carboxylic acids were identified and optimized as GPR35 agonists in our group.[26] Using the new radioligand [^{3}H]**12**, we have now determined the affinity of a series of 42 chromenone derivatives. On the basis of an SAR analysis performed with the obtained binding data, we designed, synthesized, and tested a set of six novel derivatives to obtain GPR35 agonists with improved affinity (see Tables 2−4). In general, affinity and potency determined in radioligand binding assays and β-arrestin recruitment assays, respectively, correlated well for the set of tested compounds (r^2 = 0.943 determined in a two-tailed Pearson test; for the scatter plot see Figure 6). Accordingly, SARs discussed for data obtained in functional assays could essentially be confirmed by the data determined in radioligand binding assays; however, we could also find some differences. For high potency and affinity, a hydrogen bond acceptor in the 8-position of the chromenone core structure appeared to be beneficial (compare **21**, K_i = 13.6,

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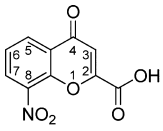
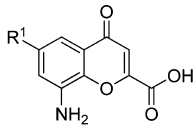
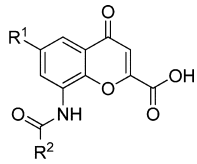
Table 2. Affinity of 8-Substituted Chromen-4-one-2-carboxylic Acid Derivatives at Human GPR35



21



22, 23



24-31

compd	R^1	R^2	radioligand binding assays[a] $K_i \pm$ SEM (μM) (% inhibition ± SEM)
21			13.6 ± 0.7
22	H		(7 ± 2)
23	Br		3.01 ± 0.30
24	H	methyl	18.5 ± 0.5
25	H	ethyl	6.77 ± 0.80
26	H	2-methoxy-2-oxoethyl	18.6 ± 1.0
27	H	cyclohexyl	3.58 ± 0.26
28	H	phenyl	3.29 ± 0.19
29	H	benzyl	2.72 ± 0.07
30	H	2-naphthyl	0.714 ± 0.041
31	H	2-quinolinyl	0.691 ± 0.056

[a]Affinities were determined in competition binding experiments using 5 nM [^{3}H]**12** and membrane preparations of CHO cells recombinantly expressing hGPR35. Screenings were performed at a concentration of 10 μM.

and **22**, which could barely displace the radioligand at a concentration of 10 μM). Introduction of nonpolar, bulky substituents via an amide linker in the 8-position of the core structure gradually increased the affinity of the compounds: methyl in **24** (K_i = 18 500 nM), ethyl in **25** (K_i = 6770 nM), cyclohexyl in **27** (K_i = 3580 nM), and phenyl in **28** (K_i = 3290 nM). Additionally, the introduction of bulky bicyclic substituents, e.g., 2-naphthyl (**30**, K_i = 714 nM, EC_{50} = 4670 nM) and 2-quinolinyl (**31**, K_i = 691 nM, EC_{50} = 1340 nM), increased affinity and, for the latter, also increased potency. Introduction of an even bulkier, nonpolar group could possibly further increase affinity. Since the efficacy of **31** is decreased, it might be a suitable scaffold to develop potent partial agonists at the GPR35.

Halogen atoms in the 6-position of the chromenone core greatly increased the potency and affinity of the compounds (compare **36**, 6-H, K_i = 221 nM, EC_{50} = 346 nM, **41**, 6-F, K_i = 25.4 nM, EC_{50} = 112 nM, **46**, 6-Cl, K_i = 5.50 nM, EC_{50} = 16.8 nM, and **12**, 6-Br, K_i = 5.18 nM, EC_{50} = 12.1 nM). The order of affinity and potency was as follows: 6-Br > 6-Cl > 6-F > 6-H. Interestingly, a fluorine atom in that position had a stronger impact on affinity than on potency. We previously hypothesized that a 6-substituent with a large van der Waals radius was beneficial to fill a hydrophobic pocket in the receptor protein.[26] To distinguish between the substituent's effect caused by spatial extension and that caused by negative inductive effects on the adjacent aromatic ring system, we synthesized the two novel compounds **84** and **85**. Addition of a methyl group (which has a radius comparable to that of a bromine atom) in the 6-position (**84**) led to a markedly increased affinity and potency (K_i = 42.8 nM, EC_{50} = 37.7 nM) compared to those of **36** (6-H, K_i = 221 nM, EC_{50} = 346 nM); however, the methyl group had a larger effect on potency than on affinity. Taken together with the observation of the effects of a fluorine substituent in that position (which has a radius comparable to that of a hydrogen atom), one can conclude that the negative inductive effect of the 6-substituent is mainly important for high-affinity binding of the ligand. Further extension as found in **85** (6-methoxy) was well-tolerated (K_i = 20.5 nM, EC_{50} = 25.5 nM), and synthesis of additional compounds with different 6-substituents appears to be promising for future studies. Up to now a bromine atom in the 6-position has been most beneficial for high affinity and potency.

We next tested a series of **28** derivatives with varying substituents at the distal phenyl moiety and different halogen substituents in the 6-position of the core structure. SARs deduced from radioligand binding data were mostly comparable to those previously discussed for β-arrestin recruitment assays: substituents were tolerated in the *para*-position, where a methoxy substituent had the greatest impact on affinity and potency (compare **28**, K_i = 3290 nM, EC_{50} = 4910 nM, with **36**, K_i = 221 nM, EC_{50} = 346 nM, or **47**, K_i = 81.6 nM, EC_{50} = 303 nM, with **12**, K_i = 5.18 nM, EC_{50} = 12.1 nM), followed by a chlorine substituent (compare **47**, K_i = 81.6 nM, EC_{50} = 303 nM, with **49**, K_i = 4.79 nM, EC_{50} = 25.1 nM). Most substituents in the *ortho*- and *meta*-positions led to a decrease in affinity and potency, except for *o*- and *m*-chlorine substitution (compare **49**, *p*-Cl, K_i = 4.79 nM, EC_{50} = 25.1 nM, with **50**, *o*,*p*-di-Cl, K_i = 0.938 nM, EC_{50} = 16.4 nM, and **51**, *m*,*p*-di-Cl, K_i = 4.79 nM, EC_{50} = 25.1 nM). Interestingly, a chlorine substituent in the *ortho*-position led to an 87-fold increase in affinity, but only to an 18-fold increase in potency (compare **49** with **50**). A similar effect for an *o*-chlorine substituent was found when **12** (K_i = 5.18 nM, EC_{50} = 12.1 nM) was compared to **58** (K_i = 1.12 nM, EC_{50} = 11.1 nM): only the affinity, but not the potency, of the compound was positively affected by that substitution. Thus, a substituent in the *ortho*-position causing a negative inductive effect was beneficial for ligand binding, but somewhat less so for potency determined in β-arrestin assays. On the basis of these results, we synthesized the novel fluoro-substituted compounds **80** (6-Cl, *o*-F, K_i = 1.92 nM, EC_{50} = 6.06 nM), **81** (6-Br, *o*-F, K_i = 1.37 nM, EC_{50} = 4.45 nM), **82** (6-Br, *m*-F, K_i = 1.64 nM, EC_{50} = 4.37 nM), and **83** (6-Br, *o*,*o*′-di-F, K_i = 0.589 nM, EC_{50} = 5.54 nM). The three compounds were found to be very potent agonists, showing high affinity in radioligand binding and high potency in β-arrestin recruitment assays. Compound **83** was the agonist with the highest affinity for the human GPR35, exhibiting a subnanomolar K_i value. In β-arrestin recruitment assays **81**, **82**, and **83** were about equally potent, showing EC_{50} values in the one-digit nanomolar range. These new compounds represent the most potent agonists at the human GPR35 known to date. At the same time, we were able to improve the druglikeness[66] of our series of compounds by introduction of fluoro substituents at the benzoyl residue. We calculated the ligand efficiency (LE) and ligand-lipophilicity efficiency (LLE)[67,68] for the most potent chromenone acids, **81**, **82**, and **83**. The compounds **81** and **82**, containing one fluoro substituent, showed, besides a high potency at the human GPR35 receptor, an ideal LE of 0.31 and a high LLE of 5.21 and 5.22, respectively. For **83**, containing two fluoro substituents, a slightly decreased LE of 0.29 and LLE of 4.97 was determined. Thus, the new, fluorinated, highly potent chromenone acids may be useful pharmacological tools due to their druglike properties.

Furthermore, we tested **42**, an ethyl ester of compound **41**, to address the question of whether the negative charge of the

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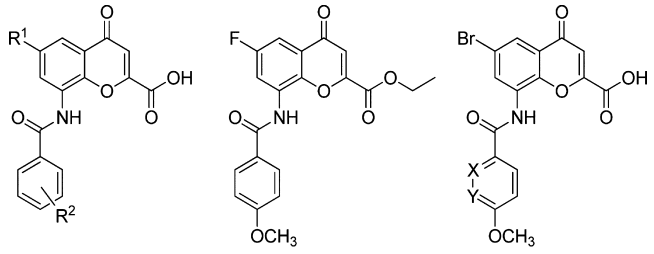
Table 3. Affinity of 8-Benzamidochromen-4-one-2-carboxylic Acid Derivatives at Human GPR35



12, **32-41**, **43-60** **42** **61** (X = N, Y = CH) **62** (X = CH, Y= N)

		R^2			radioligand binding assays[a]
compd	R^1	*ortho*	*meta*	*para*	K_i ± SEM (μM)
32	H	H	CH_3	H	4.78 ± 0.36
33	H	H	NO_2	H	4.39 ± 0.17
34	H	H	H	CH_3	0.697 ± 0.033
35	H	H	H	CF_3	10.3 ± 0.8
36	H	H	H	OCH_3	0.221 ± 0.020
37	H	H	H	Br	0.233 ± 0.014
38	F	Cl	Cl	H	0.119 ± 0.004
39	F	Cl	H	Cl	0.00412 ± 0.00009
40	F	H	Cl	Cl	0.0115 ± 0.0005
41	F	H	H	OCH_3	0.0254 ± 0.0023
42					0.198 ± 0.019
43	Cl	H	H	H	0.138 ± 0.013
44	Cl	H	CH_3	H	0.250 ± 0.012
45	Cl	H	H	NO_2	0.183 ± 0.006
46	Cl	H	H	OCH_3	0.00550 ± 0.00012
47	Br	H	H	H	0.0816 ± 0.0056
48	Br	H	CH_3	H	0.128 ± 0.001
49	Br	H	H	Cl	0.00479 ± 0.00044
50	Br	Cl	H	Cl	0.000938 ± 0.000097
51	Br	H	Cl	Cl	0.00218 ± 0.00005
52	Br	H	H	CN	0.148 ± 0.014
12	Br	H	H	OCH_3	0.00518 ± 0.00033
54	Br	H	H	ethoxy	0.0984 ± 0.0017
55	Br	OCH_3	H	H	0.331 ± 0.021
56	Br	H	OCH_3	OCH_3	0.228 ± 0.014
57	Br	H		OCH_2O	0.0150 ± 0.0015
58	Br	Cl	H	OCH_3	0.00112 ± 0.00009
59	H	H	H	cyclopropylmethoxy	3.71 ± 0.43
60	H	H	H	propoxy	8.06 ± 0.22
61					0.0568 ± 0.0056
62					0.0257 ± 0.0022

[a]Affinities were determined in competition binding experiments using 5 nM [^{3}H]**12** and membrane preparations of CHO cells recombinantly expressing human GPR35.

compounds was essential for activation of the receptor. We had previously found that the potency was the same for both compounds, free acid and ester (EC_{50} = 112 nM). We previously hypothesized that the ester of **42** might have been cleaved by esterases present in the cell-based β-arrestin assay. In radioligand binding assays we found the affinity of ester **42** (K_i = 198 nM) to be significantly lower compared to that of **41** (K_i = 25.4 nM). As we used purified membrane preparations for radioligand binding assays, there were no soluble esterases present in the assay. Nevertheless, membrane-bound esterases might have been present, potentially leading to partial hydrolysis of **42**. Our results indicate that a negatively charged carboxylate may be important for high-affinity binding.

CONCLUSIONS

We developed the first agonist radioligand, [^{3}H]**12**, for the human GPR35, which allows the determination of ligand affinity at the receptor. [^{3}H]**12** was found to exhibit high affinity for the human GPR35 along with a very low degree of nonspecific binding. Its binding was discovered to be modulated by ions, magnesium and calcium chloride increasing its affinity, while sodium chloride inhibited binding. For full agonists from different structural classes, the determined affinities correlated in most cases quite well with EC_{50} values obtained in functional β-arrestin assays. On the basis of SAR analyses, we were able to design and obtain optimized fluorine-substituted 8-benzamidochromen-4-one-2-carboxylic acid de-

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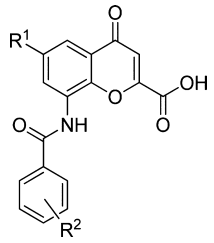
Table 4. Affinity and Potency of New 8-Benzamidochromen-4-one-2-carboxylic Acid Derivatives at Human GPR35



		R^2			radioligand binding assays[a]	β-arrestin recruitment assays	
compd	R^1	*ortho*	*meta*	*para*	K_i ± SEM (μM)	EC_{50} ± SEM (μM)	E_{max}[b]
80	Cl	F	H	OCH_3	0.00192 ± 0.00012	0.00606 ± 0.00088	111
81	Br	F	H	OCH_3	0.00137 ± 0.00011	0.00445 ± 0.00030	120
82	Br	H	F	OCH_3	0.00164 ± 0.00018	0.00437 ± 0.00048	120
83	Br	di-F	H	OCH_3	0.000589 ± 0.000076	0.00554 ± 0.00029	119
84	CH_3	H	H	OCH_3	0.0428 ± 0.0032	0.0377 ± 0.0036	118
85	OCH_3	H	H	OCH_3	0.0205 ± 0.0022	0.0255 ± 0.0027	124

[a]Affinities were determined in competition binding experiments using 5 nM [^{3}H]**12** and membrane preparations of CHO cells recombinantly expressing human GPR35. [b]Effects were normalized to the signal induced by 30 μM zaprinast, corresponding to a maximal response at the human GPR35.

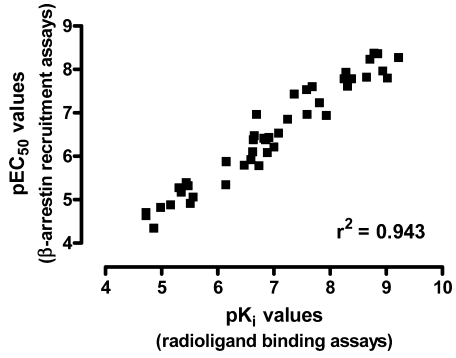


Figure 6. Scatter plot. pEC_{50} values of 8-substituted chromen-4-one-2-carboxylic acid derivatives were plotted against their respective pK_i values to test for correlation. r^2 was determined in a two-tailed Pearson test.

rivatives. Compounds **81**, **82**, and **83** showed EC_{50} and K_i values in the very low nanomolar to subnanomolar range. Thus, these compounds represent the most potent GPR35 agonists known to date. Both the radioligand and the newly developed highly potent agonists represent powerful pharmacological tools to further explore pharmacology and (patho)physiology of the yet poorly characterized orphan GPR35.

EXPERIMENTAL SECTION

General Procedures. All commercially available reagents were used as purchased (Acros, Alfa Aesar, Sigma-Aldrich, or Fluorochem). Solvents were employed 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. 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 either on a Bruker Avance 500 MHz NMR spectrometer at 500 MHz (^{1}H) or 126 MHz (^{13}C) or on a Bruker Ascend 600 MHz NMR spectrometer at 600 MHz (^{1}H) or 151 MHz (^{13}C). DMSO-d_6 was employed as a solvent at 303 K, unless otherwise noted. Chemical shifts are reported in parts per million relative to the deuterated solvent, that is, DMSO, δ(^{1}H) 2.49 ppm, δ(^{13}C) 39.7 ppm. In some cases, $CDCl_3$ was used as a solvent (δ(^{1}H) 7.26 ppm, δ(^{13}C) 77.0 ppm) at 298 K. Coupling constants J are given in hertz, and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and 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 LC−MS instrument (Applied Biosystems API 2000 LC−MS/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 was injected into an HPLC column (Macherey-Nagel Nucleodur 3 μm C18, 50 × 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 equal to or greater than 95%.

Compounds **18**, **21**−**62**, **67**, **68**, and **71** were synthesized as described.[26] Compounds **16**,[38] **69**,[44] and **72**[44] had previously been described but were now obtained by new methodologies, and additional structural characterization data are provided in the present study (for detailed synthetic and experimental data see the Supporting Information).

Ethyl 6-Bromo-8-(4-((*tert*-butyldimethylsilyl)oxy)-benzamido)-4-oxo-4*H*-chromene-2-carboxylate (19). 4-((*tert*-Butyldimethylsilyl)oxy)benzoic acid (**16**; 303 mg, 1.2 mmol) and three drops of anhydrous DMF were dissolved in 5 mL of DCM. 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 thionyl chloride were distilled off under reduced pressure using a glass filter pump. Cooling in an ice-bath induced crystallization of the desired acid chloride. In the meantime, ethyl 8-amino-6-bromo-4-oxo-4*H*-chromene-2-carboxylate (**18**; 281 mg, 0.9 mmol) and DIPEA (0.20 mL, 1.2 mmol) were dissolved in 3 mL of DCM and 1 mL of anhydrous THF. 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 for 2 days under an argon atmosphere. Column chromatography (DCM/EtOAc, 98:2) afforded the product as a white solid (419 mg, 85% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 0.24 (s, 6H, $SiCH_3$), 0.97 (s, 9H, $C(CH_3)_3$), 1.23 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 4.32 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 7.00−7.03 (m, 3H, 3-H, 3′-H, 5′-H), 7.94−7.97 (m, 3H, 5-H, 2′-H, 6′-

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H), 8.30 (d, J = 2.5 Hz, 1H, 7-H), 10.15 (br, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ −4.42 ($SiCH_3$), 13.7 ($CH_2\underline{C}H_3$), 18.1 ($\underline{C}(CH_3)_3$), 25.6 ($C(\underline{C}H_3)_3$), 62.9 ($\underline{C}H_2CH_3$), 113.8 (C-3), 118.0 (C-6), 120.0 (C-3′, C-5′), 123.1 (C-5), 125.5 (C-4a), 126.7 (C-1′), 130.0 (C-2′, C-6′), 130.5 (C-8), 132.0 (C-7), 148.1 (C-8a), 152.0 (C-2), 158.9 (C-4′), 159.7 ($\underline{C}O_2Et$), 164.9 (CONH), 176.1 (C-4). LC−MS (m/z): negative mode 544 $[M - H]^-$, positive mode 546 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 99.8%. Mp: 167−168 °C.

6-Bromo-8-(4-[^{3}H]methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic Acid ([^{3}H]12). The radioligand was obtained by custom-labeling (Quotient Bioresearch, Cardiff, U.K.) using the following procedure. [^{3}H]Methyl tosylate (100 mCi) with a specific activity of 84 Ci/mmol (3.11 TBq/mmol) was dissolved in DMF (1 mL), and anhydrous potassium fluoride (0.1 mg, 1.7 μmol) was added, followed by **19** (1.0 mg, 1.8 μmol). The mixture was stirred at 25 °C for 5 h and then left to stand at rt overnight. The reaction mixture was partially purified by application to a 5 g silica column eluting with DCM/EtOAc (98:2) to yield [^{3}H]**20**. The ester was subsequently dissolved in THF (6 mL) and ethanol (1.5 mL), and a solution of potassium carbonate (79 mg, 0.57 mmol) in water (4.5 mL) was slowly added. The reaction mixture was stirred at 25 °C for 4 h. Water was added (4 mL), and the mixture was acidified with diluted aq HCl (1 N) until pH < 2 was reached. The reaction mixture was purified by preparative HPLC (see below). After HPLC separation, the eluent was removed under reduced pressure, and the compound was dissolved in ethanol and stored in solution at −20 °C. [^{3}H]**12** was obtained with a radiochemical purity of >99.9% and a specific activity of 36 Ci/mmol (1.33 TBq/mmol). The overall yield from 100 mCi of [^{3}H]methyl tosylate was 7.8 mCi. Preparative HPLC was performed using an Ultrasphere ODS column (250 × 10 mm), applying a gradient of water/acetonitrile from 100:0 to 0:100 in the presence of 0.1% trifluoroacetic acid within 60 min with a flow rate of 3 mL/min. Analysis of [^{3}H]**12** was performed using an Atlantis T3 column (150 × 4.6 mm, particle size 5 μm), applying a gradient of water/acetonitrile from 80:20 to 0:100 in the presence of 0.1% trifluoroacetic acid at 22 °C within 15 min and subsequently for 10 min with a flow rate of 1.0 mL/min, and by mass spectrometry. Radiochemical purity was detected at 254 nm.

General Procedure for the Synthesis of Compounds 69 and 70. The appropriate 5′-substituted 2′-hydroxy-3′-nitroacetophenone (4.0 mmol) and diethyl oxalate (1.3 mL, 9.8 mmol) were dissolved in 27 mL of anhydrous DMF under an argon atmosphere and were cooled to 0−5 °C. Potassium *tert*-butoxide (1.79 g, 15.6 mmol) was added, and the reaction mixture was stirred under an argon atmosphere for 2−3 h at 0−5 °C. Then a cooled solution of concd (12 M) aq HCl solution (2.6 mL, 31.2 mmol) in 27 mL of water was added, and the mixture was extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in 20 mL of EtOH, and concd (12 M) aq HCl (1.3 mL, 15.6 mmol) was subsequently added. The reaction mixture was refluxed overnight under an 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 72 and 73. The appropriate nitro derivative (2.5 mmol) was suspended in a mixture of EtOH (7 mL) and diluted aq HCl (2 N, 7 mL). Tin(II) chloride dihydrate (2.26 g, 10.0 mmol) was subsequently added, and the reaction mixture was heated at 65 °C until the solid was completely dissolved. The mixture was stirred for an additional 20 min at the same temperature. The EtOH was removed under reduced pressure, and the resulting suspension was added to 50 mL of EtOAc containing sodium carbonate (7 g). The precipitated tin complex was filtered off and washed with 30 mL of EtOAc. The filtrate was washed with brine (30 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 74−77. 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 30−40 min. The DCM and excess 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). 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 d under an argon atmosphere. For workup conditions, see the information for individual compounds.

General Procedure for the Synthesis of Compounds 78 and 79. 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 (3.5 mL) and anhydrous THF (0.9 mL). Then a solution of the 4-methoxybenzoyl chloride (205 mg, 1.2 mmol) in DCM (3 mL) was added, and the reaction mixture was stirred at rt for 2 d under an argon atmosphere. The precipitate was filtered off, washed two 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.

General Procedure for the Synthesis of Compounds 80−85. 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 pH ≤ 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 the information for individual compounds).

1-(2-Hydroxy-5-methoxy-3-nitrophenyl)ethanone (66). A mixture of anhydrous nitric acid (0.46 mL, 11.1 mmol) and glacial acetic acid (0.85 mL, 13.5 mmol) was added dropwise to a solution of 1-(2-hydroxy-5-methoxyphenyl)ethanone (1.66 g, 10.0 mmol) in glacial acetic acid (6.7 mL) over a period of 1 h. The reaction mixture was stirred at room temperature for 2 h and subsequently stirred to 60 mL of ice−water. The precipitate was filtered off and washed twice with 2 mL of water each. Recrystallization from 1:1 ethanol/water afforded the product as an orange-colored solid (1.31 g, 62% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 2.70 (s, 3H, $COCH_3$), 3.84 (s, 3H, OCH_3), 7.74 (d, J = 3.1 Hz, 1H, 6-H), 7.79 (d, J = 3.2 Hz, 1H, 4-H), 12.20 (br, 1H, OH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 28.5 ($CO\underline{C}H_3$), 56.6 (OCH_3), 115.8 (C-4), 122.5 (C-6), 124.1 (C-1), 138.3 (C-3), 147.9 (C-2), 150.5 (C-4), 204.0 ($\underline{C}OCH_3$). LC−MS (m/z): negative mode 209 $[M - H]^-$, positive mode 211 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 98.8%. Mp: 112−114 °C.

Ethyl 8-Nitro-6-methoxy-4-oxo-4*H*-chromene-2-carboxylate (70). The compound was synthesized using **66** (845 mg, 4.0 mmol) and was isolated as a white solid (917 mg, 78% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 1.34 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 3.95 (s, 3H, OCH_3), 4.39 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 7.02 (s, 1H, 3-H), 7.72 (d, J = 3.2 Hz, 1H, 5-H), 8.16 (d, J = 3.2 Hz, 1H, 7-H). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 14.0 ($CH_2\underline{C}H_3$), 56.8 (OCH_3), 63.0 ($\underline{C}H_2CH_3$), 111.5 (C-5), 113.5 (C-3), 119.2 (C-7), 126.3 (C-4a), 140.0 (C-8), 141.9 (C-8a), 152.2 (C-2), 155.8 (C-6), 159.5 ($\underline{C}O_2Et$), 175.7 (C-4). LC−MS (m/z): positive mode 294 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 96.8%. Mp: 175 °C.

Ethyl 8-Amino-6-methoxy-4-oxo-4*H*-chromene-2-carboxylate (73). The compound was synthesized using **70** (733 mg, 2.5 mmol) and was isolated as an orange-colored solid (623 mg, 95% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 1.35 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 3.76 (s, 3H, OCH_3), 4.38 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 5.56 (br, 2H, NH_2), 6.61 (d, J = 2.9 Hz, 1H, 7-H), 6.70 (d, J = 3.0 Hz, 1H, 5-H), 6.84 (s, 1H, 3-H). ^{13}C NMR (126 MHz, DMSO-

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d_6, 303 K): δ 14.0 ($CH_2\underline{C}H_3$), 55.5 (OCH_3), 62.7 ($\underline{C}H_2CH_3$), 91.6 (C-5), 105.8 (C-7), 112.5 (C-3), 125.1 (C-4a), 139.8 (C-8 or C-8a), 140.2 (C-8 or C-8a), 151.3 (C-2), 157.7 (C-6), 160.2 ($\underline{C}O_2Et$), 177.5 (C-4). LC−MS (m/z): negative mode 262 $[M - H]^-$, positive mode 264 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 98.7%. Mp: 144−145 °C.

Ethyl 6-Chloro-8-(2-fluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (74). The compound was synthesized using 2-fluoro-4-methoxybenzoic acid (204 mg, 1.2 mmol) and ethyl 8-amino-6-chloro-4-oxo-4*H*-chromene-2-carboxylate (**71**; 241 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 filtrate was concentrated under reduced pressure and separated by column chromatography on a column of silica gel (95:5 DCM/EtOAc), yielding further product. The amide was isolated as a white powder (310 mg, 82% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ 1.37 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 3.89 (s, 3H, OCH_3), 4.43 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 6.97−7.02 (m, 3H, 3-H, 3′-H, 5′-H), 7.74 (d, J = 2.5 Hz, 1H, 5-H), 7.95 (dd, J = 9.1 Hz, 1H, 6′-H), 8.57 (d, J = 2.5 Hz, 1H, 7-H), 9.64 (d, J = 11.7 Hz, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 343 K): δ 13.5 ($CH_2\underline{C}H_3$), 56.1 (OCH_3), 62.7 ($\underline{C}H_2CH_3$), 101.9 (d, J = 28.4 Hz, C-3′), 111.3 (C-5′), 113.0 (d, J = 11.9 Hz, C-1′), 113.6 (C-3), 118.5 (C-7), 124.7 (C-4a), 125.6 (C-5), 129.8 (C-6 or C-8), 130.2 (C-6 or C-8), 132.3 (d, J = 3.7 Hz, C-6′), 145.4 (C-8a), 151.7 (C-2), 159.4 ($\underline{C}O_2Et$), 161.1 (d, J = 249.3 Hz, C-2′), 161.3 (d, J = 3.7 Hz, C-4′), 164.1 (d, J = 12.8 Hz, CONH), 175.7 (C-4). LC−MS (m/z): negative mode 418 $[M - H]^-$, positive mode 420 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 100.0%. Mp: 212−213 °C.

Ethyl 6-Bromo-8-(2-fluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (75). The compound was synthesized using 2-fluoro-4-methoxybenzoic acid (204 mg, 1.2 mmol) and ethyl 8-amino-6-bromo-4-oxo-4*H*-chromene-2-carboxylate (**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 solid (267 mg, 64% yield). ^{1}H NMR (600 MHz, DMSO-d_6, 303 K): δ 1.35 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 3.88 (s, 3H, OCH_3), 4.41 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 7.00 (dd, J = 8.9 Hz, J = 2.4 Hz, 1H, 5′-H), 7.04 (s, 1H, 3-H), 7.07 (dd, J = 13.8 Hz, J = 2.3 Hz, 1H, 3′-H), 7.89 (d, J = 2.4 Hz, 1H, 5-H), 7.94 (dd, J = 8.9 Hz, 1H, 6′-H), 8.68 (d, J = 2.4 Hz, 1H, 7-H), 9.82 (s, 1H, NH). ^{13}C NMR (151 MHz, DMSO-d_6, 303 K): δ 14.2 ($CH_2\underline{C}H_3$), 56.7 (OCH_3), 63.4 ($\underline{C}H_2CH_3$), 102.5 (d, J = 28.6 Hz, C-3′), 111.9 (C-5′), 113.6 (d, J = 13.2 Hz, C-1′), 114.4 (C-3), 118.6 (C-6), 122.3 (C-5), 125.7 (C-4a), 129.3 (C-7), 130.5 (C-8), 133.0 (d, J = 3.3 Hz, C-6′), 146.6 (C-8a), 152.3 (C-2), 160.0 ($\underline{C}O_2Et$), 161.7 (d, J = 248.7 Hz, C-2′), 162.0 (C-4′), 164.1 (d, J = 12.1 Hz, CONH), 176.4 (C4). LC−MS (m/z): negative mode 464 $[M - H]^-$, positive mode 464 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 100.0%. Mp: 216 °C.

Ethyl 6-Bromo-8-(3-fluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (76). The compound was synthesized using 3-fluoro-4-methoxybenzoic acid (204 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 filtrate was concentrated under reduced pressure and separated by column chromatography on a column of silica gel (98:2 DCM/EtOAc), yielding further product. The amide was isolated as a white solid (309 mg, 74% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ 1.29 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 3.96 (s, 3H, OCH_3), 4.37 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 7.00 (s, 1H, 3-H), 7.34 (dd, J = 8.5 Hz, 1H, 5′-H), 7.83−7.89 (m, 2H, 2′-H, 6′-H), 7.97 (d, J = 2.4 Hz, 1H, 5-H), 8.32 (d, J = 2.4 Hz, 1H, 7-H), 10.05 (s, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 343 K): δ 13.4 ($CH_2\underline{C}H_3$), 56.4 (OCH_3), 62.6 ($\underline{C}H_2CH_3$), 113.6 (C-3 or C-6′), 113.7 (C-3 or C-6′), 115.1 (d, J = 19.2 Hz, C-2′), 117.6 (C-6), 123.0 (C-5), 124.8 (d, J = 2.7 Hz, C-5′), 125.3 (C-4a), 126.0 (d, J = 5.5 Hz, C-1′), 130.0 (C-8), 131.8 (C-7), 147.9 (C-8a), 150.5 (d, J = 10.1 Hz, C-4′), 151.0 (d, J = 245.6 Hz, C-3′), 151.8 (C-2), 159.4 ($\underline{C}O_2Et$), 163.7 (CONH), 175.7 (C4). LC−MS (m/z): negative mode 461 $[M - H]^-$, positive mode 466 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 97.2%. Mp: 251 °C.

Ethyl 6-Bromo-8-(2,6-difluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (77). The compound was synthesized using 2,6-difluoro-4-methoxybenzoic acid (226 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 filtrate was concentrated under reduced pressure and separated by column chromatography on a column of silica gel (95:5 DCM/EtOAc), yielding further product. The amide was isolated as a white powder (221 mg, 51% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ 1.34 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 3.87 (s, 3H, OCH_3), 4.39 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 6.85 (d, J = 10.0 Hz, 2H, 3′-H, 5′-H), 7.00 (s, 1H, 3-H), 7.97 (d, J = 2.4 Hz, 1H, 5-H), 8.33 (d, J = 2.4 Hz, 1H, 7-H), 10.27 (s, 1H, NH). ^{13}C NMR (151 MHz, DMSO-d_6, 303 K): δ 14.2 ($CH_2\underline{C}H_3$), 57.0 (OCH_3), 63.3 ($\underline{C}H_2CH_3$), 99.1 (d, J = 28.6 Hz, C-3′, C-5′), 106.8−107.1 (m, C-1′), 114.4 (C-3), 118.3 (C-6), 124.0 (C-5), 126.1 (C-4a), 129.7 (C-8), 132.1 (C-7), 148.1 (C-8a), 152.6 (C-2), 159.3 (C-4′), 160.1 ($\underline{C}O_2Et$), 160.0−161.7 (m, C-2′, C-6′), 162.6 (t, J = 14.3 Hz, CONH), 176.4 (C4). LC−MS (m/z): negative mode 482 $[M - H]^-$, positive mode 484 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 98.6%. Mp: 263 °C.

Ethyl 8-(4-Methoxybenzamido)-6-methyl-4-oxo-4*H*-chromene-2-carboxylate (78). The compound was synthesized using ethyl 8-amino-6-methyl-4-oxo-4*H*-chromene-2-carboxylate (**72**; 226 mg, 0.9 mmol). The product was isolated as a white powder (278 mg, 81% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ 1.27 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 2.46 (s, 3H, $ArCH_3$), 3.87 (s, 3H, OCH_3), 4.35 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 6.93 (s, 1H, 3-H), 7.07−7.10 (m, 2H, 3′-H, 5′-H), 7.68−7.69 (m, 1H, 5-H), 7.98 (d, J = 2.1 Hz, 1H, 7-H), 8.00−8.03 (m, 2H, 2′-H, 6′-H), 9.85 (s, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 343 K): δ 13.5 ($CH_2\underline{C}H_3$), 20.5 ($ArCH_3$), 55.4 (OCH_3), 62.4 ($\underline{C}H_2CH_3$), 113.3 (C-3), 113.7 (C-3′, C-5′), 120.2 (C-5), 123.9 (C-4a), 126.1 (C-1′), 128.2 (C-8), 129.4 (C-2′, C-6′), 130.9 (C-7), 135.3 (C-6), 147.2 (C-8a), 151.5 (C-2), 159.7 ($\underline{C}O_2Et$), 162.1 (C-4′), 164.7 (CONH), 176.9 (C4). LC−MS (m/z): negative mode 380 $[M - H]^-$, positive mode 382 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 98.2%. Mp: 219 °C.

Ethyl 6-Methoxy-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylate (79). The compound was synthesized using **73** (237 mg, 0.9 mmol). The product was isolated as a white solid (304 mg, 85% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ 1.30 (t, J = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 3.87 (s, 3H, 6-OCH_3 or 4′-OCH_3), 3.90 (s, 3H, 6-OCH_3 or 4′-OCH_3), 4.36 (q, J = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 6.93 (s, 1H, 3-H), 7.09 (d, J = 8.8 Hz, 2H, 3′-H, 5′-H), 7.27 (d, J = 3.1 Hz, 1H, 5-H), 7.85 (d, J = 3.0 Hz, 1H, 7-H), 8.00 (d, J = 9.1 Hz, 2H, 2′-H, 6′-H), 9.76 (s, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 343 K): δ 13.5 ($CH_2\underline{C}H_3$), 55.4 (6-OCH_3 or 4′-OCH_3), 55.8 (6-OCH_3 or 4′-OCH_3), 62.4 ($\underline{C}H_2CH_3$), 101.1 (C-5), 112.5 (C-3), 113.8 (C-3′, C-5′), 118.0 (C-7), 124.7 (C-4a), 126.0 (C-1′), 129.3 (C-2′, C-6′), 129.7 (C-8), 143.5 (C-8a), 151.3 (C-2), 156.6 (C-6), 159.7 ($\underline{C}O_2Et$), 162.3 (C-4′), 164.6 (CONH), 176.5 (C4). LC−MS (m/z): negative mode 396 $[M - H]^-$, positive mode 398 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 98.9%. Mp: 217−218 °C.

6-Chloro-8-(2-fluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic Acid (80). The compound was synthesized using **74** (168 mg, 0.40 mmol). Recrystallization from 1:1 acetone/ethanol afforded the product as a white powder (126 mg, 80% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 3.87 (s, 3H, OCH_3), 6.96−7.03 (m, 3H, 3-H, 3′-H, 5′-H), 7.72 (d, J = 2.5 Hz, 1H, 5-H), 7.92 (dd, J = 9.0 Hz, 1H, 6′-H), 8.56 (d, J = 2.5 Hz, 1H, 7-H), 9.77 (d, J = 11.7 Hz, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 56.3 (OCH_3), 102.1 (d, J = 27.5 Hz, C-3′), 111.6 (C-5′), 113.3 (d, J = 11.9 Hz, C-1′), 113.6 (C-3), 118.7 (C-7), 124.9 (C-4a), 125.8 (C-5), 130.0 (C-6 or C-8), 130.2 (C-6 or C-8), 132.6 (d, J = 3.7 Hz, C-6′), 145.7 (C-8a), 153.1 (C-2), 160.3 (CO_2H), 161.3 (d, J = 249.3 Hz, C-2′), 161.7 (d, J = 3.7 Hz, C-4′), 164.2 (d, J = 11.9 Hz, CONH), 176.4 (C-4). LC−MS (m/z): negative mode 346 $[M - CO_2H]^-$, 390 $[M - H]^-$, positive mode 392 $[M + H]^+$. Purity by HPLC−UV (254 nm)−ESI-MS: 99.3%. Mp: 279−280 °C dec.

6-Bromo-8-(2-fluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic Acid (81). The compound was synthesized

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using **75** (204 mg, 0.44 mmol). Recrystallization from 1:1 acetone/ethanol afforded the product as a white solid (170 mg, 95% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 3.87 (s, 3H, OCH_3), 6.96–7.04 (m, 3H, 3-H, 5′-H, 3′-H), 7.88 (d, J = 2.4 Hz, 1H, 5-H), 7.92 (dd, J = 8.9 Hz, 1H, 6′-H), 8.68 (d, J = 2.4 Hz, 1H, 7-H), 9.81 (d, J = 10.2 Hz, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 56.4 (OCH_3), 102.1 (d, J = 27.5 Hz, C-3′), 111.6 (C-5′), 113.4 (d, J = 11.9 Hz, C-1′), 113.6 (C-3), 118.1 (C-6), 121.9 (C-5), 125.3 (C-4a), 128.7 (C-7), 130.1 (C-8), 132.5 (d, J = 2.8 Hz, C-6′), 146.2 (C-8a), 153.3 (C-2), 161.0 (CO_2H), 161.2 (d, J = 249.3 Hz, C-2′), 161.7 (d, J = 3.7 Hz, C-4′), 164.1 (d, J = 11.9 Hz, CONH), 176.3 (C4). LC–MS (m/z): negative mode 390 $[M - CO_2H]^-$, 434 $[M - H]^-$, positive mode 436 $[M + H]^+$. Purity by HPLC–UV (254 nm)–ESI-MS: 100.0%. Mp: 289–290 °C dec.

6-Bromo-8-(3-fluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic Acid (82). The compound was synthesized using **76** (204 mg, 0.44 mmol). Recrystallization from 1:1 acetone/ethanol afforded the product as a white solid (144 mg, 75% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 3.94 (s, 3H, OCH_3), 6.97 (s, 1H, 3-H), 7.36 (dd, J = 8.5 Hz, 1H, 5′-H), 7.84–7.89 (m, 2H, 2′-H, 6′-H), 7.96 (d, J = 2.4 Hz, 1H, 5-H), 8.28 (d, J = 2.4 Hz, 1H, 7-H), 10.27 (s, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 56.5 (OCH_3), 113.6 (C-3 or C-6′), 113.7 (C-3 or C-6′), 115.5 (d, J = 20.1 Hz, C-2′), 117.8 (C-6), 123.4 (C-5), 125.3 (d, J = 2.8 Hz, C-5′), 125.6 (C-4a), 126.0 (d, J = 6.4 Hz, C-1′), 130.2 (C-8), 132.6 (C-7), 148.4 (C-8a), 150.6 (d, J = 9.2 Hz, C-4′), 151.0 (d, J = 244.7 Hz, C-3′), 153.3 (C-2), 161.1 (CO_2H), 164.1 (CONH), 176.4 (C4). LC–MS (m/z): negative mode 390 $[M - CO_2H]^-$, 434 $[M - H]^-$, positive mode 438 $[M + H]^+$. Purity by HPLC–UV (254 nm)–ESI-MS: 99.8%. Mp: 296–297 °C dec.

6-Bromo-8-(2,6-difluoro-4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic Acid (83). The compound was synthesized using **77** (97 mg, 0.20 mmol). Recrystallization from 1:1 acetone/ethanol afforded the product as a white solid (83 mg, 91% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 3.85 (s, 3H, OCH_3), 6.89 (d, J = 10.0 Hz, 2H, 3′-H, 5′-H), 6.97 (s, 1H, 3-H), 7.95 (d, J = 2.4 Hz, 1H, 5-H), 8.40 (s 1H, 7-H), 10.62 (s, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 56.7 (OCH_3), 98.9 (d, J = 27.5 Hz, C-3′, C-5′), 106.6–106.9 (m, C-1′), 113.8 (C-3), 117.9 (C-6), 123.3 (C-5), 125.7 (C-4a), 129.4 (C-8), 130.9 (C-7), 147.3 (C-8a), 153.4 (C-2), 159.1 (C-4′), 160.4 (dd, J = 249.3 Hz, J = 10.1 Hz, C-2′, C-6′), 161.0 (CO_2H), 162.6 (t, J = 13.7 Hz, CONH), 176.2 (C4). LC–MS (m/z): negative mode 408 $[M - CO_2H]^-$, 452 $[M - H]^-$, positive mode 456 $[M + H]^+$. Purity by HPLC–UV (254 nm)–ESI-MS: 100.0%. Mp: 296–297 °C dec.

8-(4-Methoxybenzamido)-6-methyl-4-oxo-4*H*-chromene-2-carboxylic Acid (84). The compound was synthesized using **78** (168 mg, 0.44 mmol). Recrystallization from 1:1 acetone/ethanol afforded the product as a white solid (134 mg, 86% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 2.45 (s, 3H, $ArCH_3$), 3.85 (s, 3H, OCH_3), 6.91 (s, 1H, 3-H), 7.09 (d, J = 9.1 Hz, 2H, 3′-H, 5′-H), 7.68–7.69 (m, 1H, 5-H), 7.92 (d, J = 2.2 Hz, 1H, 7-H), 8.00 (d, J = 8.8 Hz, 2H, 2′-H, 6′-H), 10.00 (s, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 20.8 ($ArCH_3$), 55.6 (OCH_3), 113.4 (C-3), 114.0 (C-3′, C-5′), 120.7 (C-5), 124.1 (C-4a), 126.2 (C-1′), 128.3 (C-8), 129.7 (C-2′, C-6′), 131.9 (C-7), 135.5 (C-6), 147.7 (C-8a), 152.9 (C-2), 161.4 (CO_2H), 162.4 (C-4′), 165.0 (CONH), 177.5 (C4). LC–MS (m/z): negative mode 308 $[M - CO_2H]^-$, 352 $[M - H]^-$, positive mode 354 $[M + H]^+$. Purity by HPLC–UV (254 nm)–ESI-MS: 99.7%. Mp: 283–284 °C dec.

6-Methoxy-8-(4-methoxybenzamido)-4-oxo-4*H*-chromene-2-carboxylic Acid (85). The compound was synthesized using **79** (175 mg, 0.44 mmol). Recrystallization from 1:1 acetone/ethanol afforded the product as a white solid (151 mg, 93% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 303 K): δ 3.85 (s, 3H, 6-OCH_3 or 4′-OCH_3), 3.88 (s, 3H, 6-OCH_3 or 4′-OCH_3), 6.91 (s, 1H, 3-H), 7.09 (d, J = 8.9 Hz, 2H, 3′-H, 5′-H), 7.27 (d, J = 3.1 Hz, 1H, 5-H), 7.77 (d, J = 3.1 Hz, 1H, 7-H), 8.00 (d, J = 8.9 Hz, 2H, 2′-H, 6′-H), 10.01 (s, 1H, NH). ^{13}C NMR (126 MHz, DMSO-d_6, 303 K): δ 55.7 (6-OCH_3 or 4′-OCH_3), 56.1 (6-OCH_3 or 4′-OCH_3), 101.2 (C-5), 112.7 (C-3), 114.0 (C-3′, C-5′), 119.1 (C-7), 124.9 (C-4a), 126.1 (C-1′), 129.8 (C-2′, C-6′), 129.9 (C-8), 144.2 (C-8a), 152.7 (C-2), 156.6 (C-6), 161.4 (CO_2H), 162.5 (C-4′), 165.0 (CONH), 177.2 (C4). LC–MS (m/z): negative mode 324 $[M - CO_2H]^-$, 368 $[M - H]^-$, positive mode 370 $[M + H]^+$. Purity by HPLC–UV (254 nm)–ESI-MS: 100.0%. Mp: 276–277 °C dec.

Membrane Preparations for Radioligand Binding Assays. CHO cells were transfected with human GPR35 using a retroviral transfection system as previously described.[69] In brief, GP$^+$envAM12 packaging cells were transfected with the plasmid pLXSN carrying the human GPR35 cDNA. After production of pseudotyped retroviruses, the virus-containing supernatant of the packaging cells was sterile-filtered and added to CHO cells. Successfully transfected CHO cells were selected for a week using DMEM/F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.8 mg/mL G418. Afterward the cells were maintained in the same medium containing a reduced concentration of G418 (0.2 mg/mL) and plated on cell culture dishes. Membrane preparations were produced as previously described:[31] After removal of the culture medium, the cells were washed with phosphate-buffered saline and immediately frozen at −20 °C. After thawing, the cells were scraped off in a sterile buffer containing 5 mM Tris–HCl and 2 mM EDTA (pH 7.4). The cells were mechanically lysed and homogenized using an Ultra-Turrax (11000 rpm for 15 s). The homogenate was centrifuged for 10 min at 1000g and 4 °C. The obtained supernatant was subsequently spun down at 48000g and 4 °C for 1 h. The pellets were resuspended in sterile 50 mM Tris–HCl buffer (pH 7.4). After homogenization the membrane preparation was aliquoted and stored at −80 °C until usage. The membrane preparation's protein concentration was determined using the method described by Lowry.[70]

Radioligand Binding Assays. Radioligand binding assays were performed using [^{3}H]**12** in a final volume of 400 μL containing 10 μL of DMSO or test compound dissolved in 100% DMSO, 190 μL of buffer (sterile 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4), 100 μL of radioligand solution (final concentration 5 nM, except for saturation experiments), and 100 μL of membrane preparation (10 μg of protein per sample, except for binding assays to determine the effects of cations, for which 15 μg was used). The membrane preparation and radioligand were diluted in sterile buffer containing 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4. Saturation experiments were performed over a concentration range of 0.4–60 nM [^{3}H]**12** and were incubated for 150 min. Nonspecific binding was determined using 250 μM zaprinast. Three independent assays were performed in duplicate. For all other assays nonspecific binding was determined in the presence of 10 μM pamoic acid. Association experiments were performed over a time range of 200 min. Dissociation was initiated by the addition of 10 μM pamoic acid after 100 min of preincubation. Two independent experiments were performed in duplicate. For competition experiments the assay mixture was incubated for 100 min at rt and subsequently filtered through GF/B glass fiber filters using a Brandel harvester (Brandel, Gaithersburg, MD). The filters were washed three times with 2–3 mL of ice-cold 50 mM Tris–HCl buffer, pH 7.4. Remaining radioactivity was determined by liquid scintillation counting using Tri-Carb 1810 TR (Perkin-Elmer, Waltham, MA; counter efficiency approximately 52%). Three to four independent experiments were performed in duplicate.

***β*-Arrestin Recruitment Assay.** The Pathhunter β-arrestin recruitment assay cell line was purchased from DiscoverX (Fremont, CA). That CHO cell line stably expressed β-arrestin fused to an N-terminal deletion mutant of β-galactosidase (β-arrestin–EA) and the human GPR35, which was C-terminally tagged with an enzyme-fragment. The cells were seeded into 96-well plates (Thermo Scientific, Waltham, MA) at a density of 20 000 cells per well in 90 μL of cell plating medium 2 (DiscoverX) approximately 24 h before the assay. The compounds were diluted in DMSO followed by a second dilution step in cell plating medium 2 at a ratio of 1:10. Compound dilutions (10 μL per well) were added. The final DMSO concentration did not exceed 1%. After 90 min of incubation, 50 μL of detection reagent (DiscoverX) per well was added. After 60 min of

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incubation an NXT plate reader (Perkin-Elmer, Meriden, CT) was used to measure luminescence. Three to four independent experiments were performed, each in duplicate. All pharmacological 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 **16**, **69**, and **72**, high-performance liquid chromatogram and mass spectrum of [^{3}H]**12**, protein dependence of radioligand binding experiments, complete set of affinity and potency data for 8-substituted chromen-4-one-2-carboxylic acid derivatives, and calculation of physicochemical properties of selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Ar, aryl; compd, compound; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMR, dynamic mass redistribution; GPCR, G protein-coupled receptor; HCAR, hydroxycarboxylic acid receptor; LE, ligand efficiency; LLE, ligand-lipophilicity efficiency; LPAR, lysophosphatidic acid receptor; PA, pamoic acid; PSB, Pharmaceutical Sciences Bonn; RP-HPLC, reversed-phase high-performance liquid chromatography; rt, room temperature; SARs, structure−activity relationships; THF, tetrahydrofuran

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