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Structure–Activity Relationship Studies of Coumarin-like Diacid Derivatives as Human G Protein-Coupled Receptor-35 (hGPR35) Agonists and a Consequent New Design Principle

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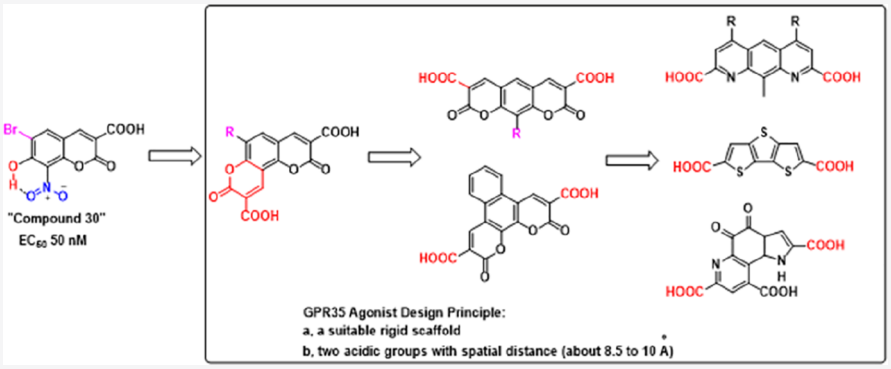

ABSTRACT: A series of coumarin-like diacid derivatives were designed and synthesized as novel agonists of human G-protein-coupled receptor 35 (hGPR35). Active compounds were characterized to possess one acidic group on both sides of a fused tricyclic aromatic scaffold. Most of them functioned as full agonists selective to hGPR35 and exhibited excellent potency at low nanomolar concentrations. Substitution on the middle ring of the scaffold could effectively regulate compound potency. Structure–activity relationship studies and docking simulation indicated that compounds that carried two acidic groups with a proper special distance and attached to a rigid aromatic scaffold would most likely show a potent agonistic activity on hGPR35. Following this principle, we screened a list of known compounds and some were found to be potent GPR35 agonists, and compound **24** even had an EC_{50} of 8 nM. Particularly, a dietary supplement pyrroloquinoline quinone (PQQ) was identified as a potent agonist (EC_{50} = 71.4 nM). To some extent, this principle provides a general strategy to design and recognize GPR35 agonists.

INTRODUCTION

G protein-coupled receptors (GPCRs) represent the largest target of therapeutic agents, mostly because of their substantial involvement in human pathophysiology and their pharmacological tractability.[1] It has been reported that drugs targeting GPCRs account for ∼27% of the global market share of therapeutic drugs, with aggregated sales during 2011–2015 at ∼$890 billion.[2] Given the history of GPCRs successfully being drug targets, there is great passion, as well as a requirement, for discovering lead compounds for orphan GPCRs, natural agonists of which yet remain unidentified.[3] The human orphan G protein-coupled receptor-35 (GPR35) was first discovered in 1998 and expressed in the immune and gastrointestinal systems, dorsal root ganglia, lungs, cerebellum, and brain.[4−6] GPR35 is implicated in a number of diseases such as pain, cancer, coronary artery disease, and hypertension;[5−9] thus, great efforts have been made to identify compounds that can regulate GPR35.

Kynurenic acid (**1**), the tryptophan metabolite, was reported to be the first endogenously expressed GPR35 ligand in 2006.[6] However, its weak potency in a high micromolar range challenged its position as an endogenous ligand. Several other ligands were also postulated to be the natural agonists for GPR35, including 2-acyl lysophosphatidic acid,[10] guanosine-3′,5′-cyclic monophosphate,[11] multiple tyrosine metabolites,[12] and the mucosal chemokine CXCL17,[13] but which is a true natural agonist for GPR35 remains controversial.[14] Therefore, GPR35 has been considered to be an orphan receptor until now. Despite these limitations, more and more synthetic agonists have been identified, including zaprinast that has become the most widely used reference agonist for GPR35.[15]

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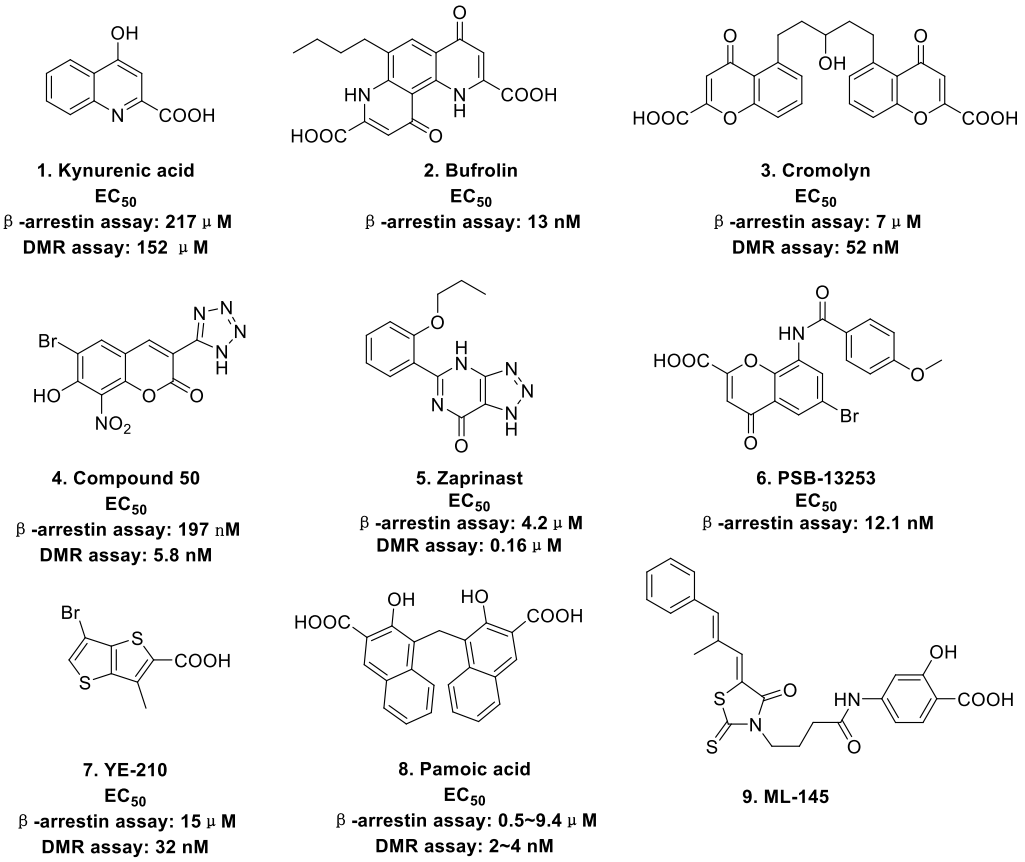

Figure 1. Selected GPR35 agonists with potencies (**1**−**8**) and GPR35 antagonist (**9**).

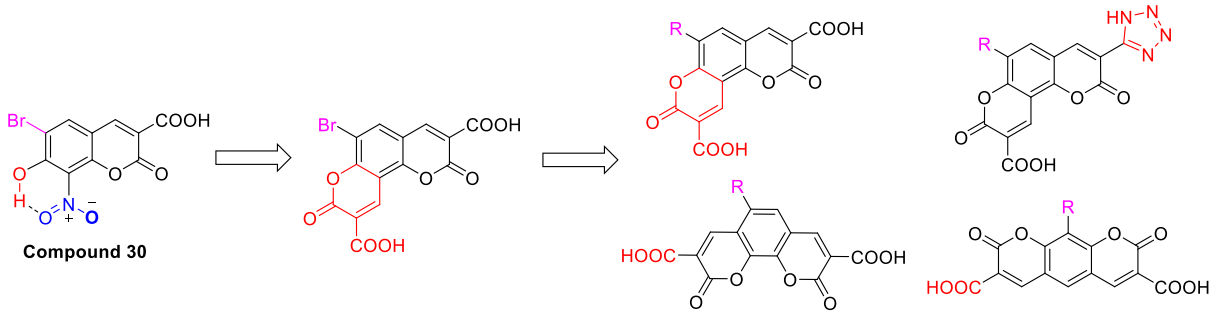

Figure 2. Conceptual development of the novel GPR35 agonists starting from "compound **30**" via 6-bromo-8-formyl-7-hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid to evolve to the general structure of the novel coumarin-like dicarboxylic derivatives reported herein.

The GPR35 sequence contains numerous positively charged amino acids that face the binding pocket.[16] This is the possible reason for what most GPR35 agonists comprise one or two acidic groups (Figure 1), for example, YE-210 (**7**) and PSB-13253 (**6**) individually having one carboxyl group;[17,18] "compound **50**" (**4**) with one tetrazole group;[19] bufrolin (**2**), cromolyn (**3**), and pamoic acid (**8**) individually containing two carboxyl groups.[20,21] As in Figure 1, ML145 (**9**), a known potent GPR35 antagonist, has a carboxyl group also.[22] Several docking models also confirmed a significant interaction between the acidic group and some amino acid residues on GPR35.[20]

Although a number of compounds have been screened to look for GPR35 agonists and thus a few detailed structure−activity relationship (SAR) studies were generated, these works and related drug designs were relatively random.[17−19] When analyzing structure characterization of active compounds, we noticed an interesting phenomenon: bearing two carboxyl groups on aromatic moieties, bufrolin, cromolyn, and pamoic acid exhibited much better agonistic activity than their partial analogues kynurenic acid, chromocarb, and 3-hydroxy-2-naphthoic acid (Figure S1), respectively. Although such a phenomenon is less reported, it inspired us to look into more diacid compounds to explore whether the diacid structure is more advantageous to GPR35 agonists. We previously reported a series of coumarin derivatives that were able to act as powerful GPR35 agonists. The coumarin scaffold easily derivatives and is usually seen in drug designs.[23,24] Thus, we designed and synthesized a series of novel diacid compounds based on coumarin-like scaffolds and studied their SAR and binding interaction with GPR35 through docking simulation, which lead to an empirical principle for a GPR35 agonist design. More significantly, we found, for the first time, that PQQ (Pyrroloquinoline quinone) is a potent GPR35 agonist based on this principle.

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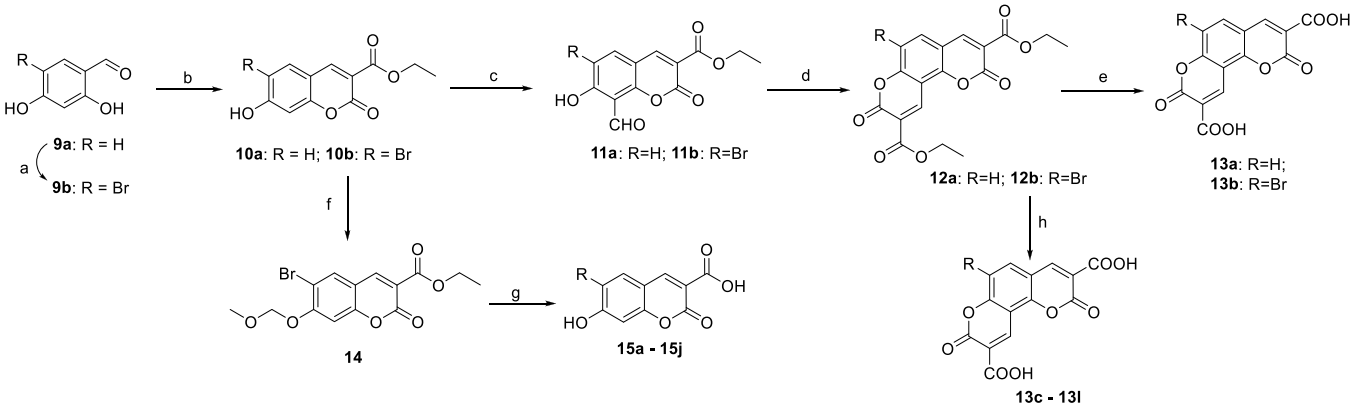
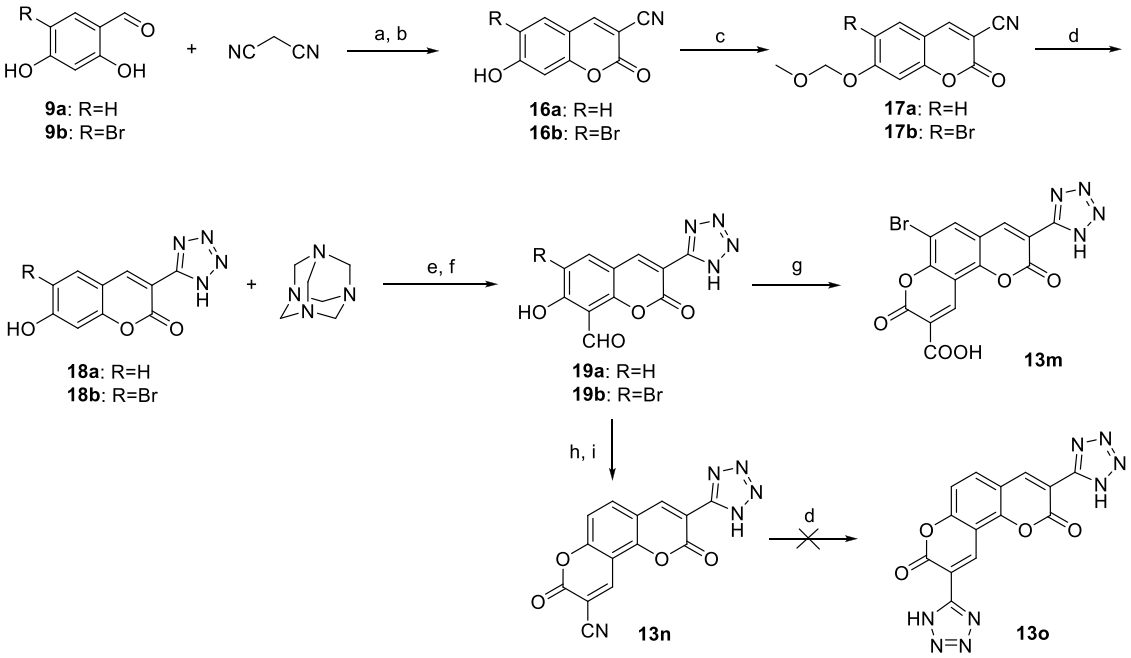
Scheme 1. Syntheses of Coumarin 3,9-Dicarboxylic Acid Derivatives 13a−13l and Coumarin 3-Carboxylic Acid Derivatives 15a−15j[a]



[a]Reagents and conditions: (a) Br_2, AcOH, rt, 1 h. (b) Diethyl malonate, Piperidine, 80 °C, 6 h. (c) i: HMTA, TFA, 80 °C, overnight; ii: HCl (2 M), 80 °C, 1 h. (d) Diethyl malonate, Piperidine, 80 °C, 6 h. (e) NaOH, 1 h, rt. (g, h) i: arylboronic acids, $PdCl_2(PPh)_2$, K_2CO_3, dioxane/H_2O = 5:1, 80 °C, overnight; ii: HCl (2 M), 80 °C, 2 h.

Scheme 2. Syntheses of Coumarin 3-Tetrazyl-9-carboxylic Derivatives 13m and 13n[a]



[a]Reagents and conditions: (a) CH_3COONH_4, H_2O, rt, 6 h. (b) HCl, H_2O, 75 °C, 2 h. (c) MOMCl, Et_3N, DCM, rt, 3 h. (d) NaN_3, $AlCl_3$, THF, 80 °C, overnight. (e) TFA, 80 °C, overnight. (f) HCl (2 M), 80 °C, 1 h. (g) Meldrum's acid, CH_3COONH_4, EtOH, rt, 2 h. (h) malononitrile, CH_3COONH_4, rt, 4 h. (i) HCl (2 M), H_2O, 75 °C, 2 h.

RESULTS AND DISCUSSION

Structure Consideration. Our previous work indicated that the acidic group on the 3-position of coumarin derivatives is crucial for compounds to maintain their GPR35 activity. In addition, the 7-hydroxy and 8-nitro groups also played important roles in the high agonistic activity for several compounds, such as "compound **30**", which is possibly related to the cyclic structure formed by an intramolecular hydrogen bond between the hydroxyl and nitro. Due to this consideration, we designed a series of coumarin-like tricyclic derivatives and introduced an acidic group on the additional ring. Figure 2 presents some representative structures.

Chemistry. A series of coumarin-like 3,9-dicarboxylic acids with various aromatic substituents at the 6-position were first synthesized (Scheme 1). The coumarin skeletons **10a** and **10b** were created using Knoevenagel condensation of diethyl malonate and **9a** and **9b**, respectively. An 8-aldehyde group was introduced to construct compounds **11a** and **11b** through Duff reaction by refluxing **10** with urotropin in trifluoroacetic acid. Another Knoevenagel condensation of **11** and diethyl malonate was carried out with piperidine as a base to produce **12a** and **12b**. By a hydrolysis reaction, dicarboxylic products **13a** and **13b** were obtained with excellent yields. Further derivatives **13c−13l** could be prepared by Suzuki−Miyaura cross-coupling of **12b** with suitable aromatic boronic acids. As coumarin-like mono-acid control compounds, **15a−15k** could be synthesized by similar cross-coupling reactions using **14** as a common intermediate.

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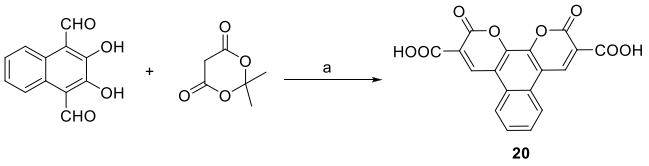
Considering that the tetrazyl group was often used as an isostere of carboxyl, we also tried to conduct such a replacement on compounds **13a** and **13b**. As shown in Scheme 2, 3-cyanocoumarin **16** could be prepared in a one-pot method as reported in literature.[25] Via a [3+2] cycloaddition reaction of nitriles with sodium azide catalyzed by $AlCl_3$ in refluxing THF, intermediate **17** directly became the 3-(1*H*-tetrazol-5-yl)-2*H*-chromen-2-one derivative **18** along with losing an MOM protecting group. We also performed this reaction with a naked phenolic hydroxyl group but obtained no product. Then, the aldehyde group was introduced at the 8-position by Duff reaction to create compound **19**. Subsequent products **13m** and **13n** could be obtained through Knoevenagel condensation with Meldrum's acid and malononitrile, respectively.[26] However, preparation of compound **13o** was beyond the conventional [3+2] cycloaddition reaction of nitriles with sodium azide.

Coumarin 3,10-dicarboxylic acid derivative **20** could be readily prepared in an excellent yield by Knoevenagel condensation of 2,3-dihydroxynaphthalene-1,4-dicarbaldehyde with Meldrum's acid in water with a catalytic amount of ammonium acetate (Scheme 3).

Scheme 3. Synthesis of Coumarin 3,10-Dicarboxylic Acid Derivative 20[a]



[a]Reagents and conditions: (a) CH_3COONH_4, H_2O, rt, 6 h.

Besides the U-shaped molecular structure presented in compounds **13** and **20**, linear coumarin 3,7-dicarboxylic derivatives **23a−23f** were also designed and synthesized. As shown in Scheme 4, salicylaldehyde derivatives **21a−21c** were treated with piperidine as a base in diethyl malonate at 80 °C to construct intermediates **22a−22c**. Target compounds **23a−23c** bearing two symmetric carboxyl groups were yielded by hydrolysis of the ethyl esters with NaOH at room temperature. Biphenyl analogues **23d−23f** could be prepared from **22a** by Suzuki−Miyaura cross-coupling with suitable aromatic boronic acids as mentioned above.

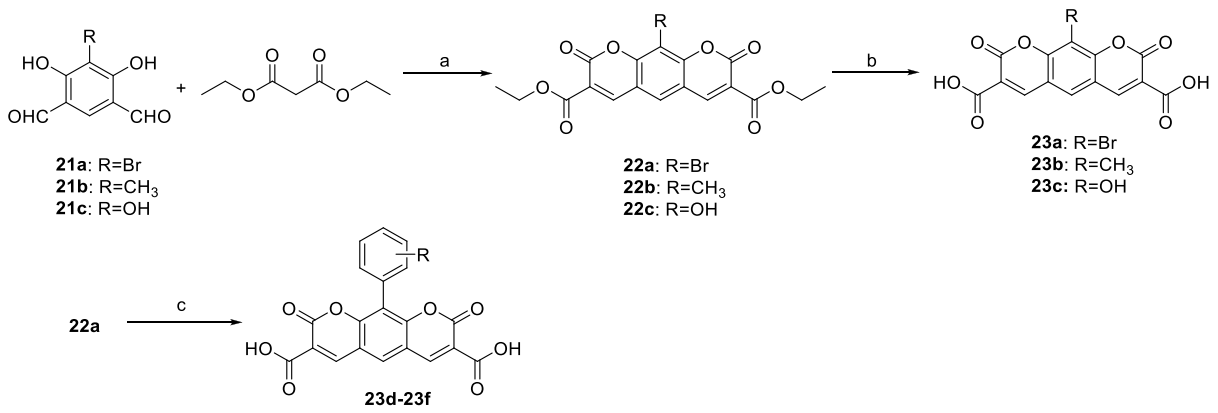
Pharmacological Evaluation. Dynamic mass redistribution (DMR) detected by a label-free resonant waveguide grating biosensor was applied to profile compound activity at GPR35 endogenously expressed in human colorectal adenocarcinoma cell line HT-29.[27] In this work, zaprinast was used as a full GPR35 agonist and tool molecule in DMR activation and desensitization experiments. It triggered a robust DMR signal in HT-29 cells with an EC_{50} of 0.34 ± 0.03 μM. All coumarin-like diacid derivatives not only gave rise to concentration-dependent DMR signals in HT-29 but also, after 1 h incubation with the cells, desensitized DMR responses induced by 1 μM zaprinast that was added later (Tables 1−3). For all derivatives, the potency to trigger DMR was found to be almost equivalent to that to desensitize the zaprinast response, suggesting that these compounds acted as GPR35 agonists.

A DMR antagonist assay using the known GPR35 antagonist compound **9** (ML-145) confirmed that compound **9** concentration dependently and completely blocked the DMR responses generated by the diacid derivatives that were added at concentrations equal to individual EC_{80}−EC_{100}. Together with the desensitization assay results, it suggested that the diacid compounds generated DMR responses in the HT-29 cells specifically through the activation of GPR35. Therefore, these compounds were GPR35 agonists.

Structure−Activity Relationship Analysis. Based on the molecular scaffold (Figure S6), the coumarin-like diacid compounds were roughly classified into a U-shaped type (Table 1) and a linear type (Table 3), and the coumarin mono-acid compounds **15a−15j** (Table 2) were used as control compounds.

Compound **13a**, the simplest diacid derivative, showed a moderate agonist property (EC_{50} 0.31 μM). When a 6-position bromine was introduced, the resulting compound **13b** showed a 7-fold increase in potency (EC_{50} 0.045 μM). This result was in accord with an early report that such an introduction to 3-carboxylic-coumarin could improve the agonistic activity.[19] To expand molecular diversity, the bromine was replaced with various substituted phenyl groups. The 6-phenyl derivative **13c** (EC_{50} 0.048 μM) showed almost the same activity to **13b**, but the potency of **13j** with a bigger substituent 6-(*para*-isopropyl)-phenyl increased 3.5-fold (EC_{50} 0.013 μM) compared to that of **13b**, suggesting a possible lipophilic pocket opposite to the 6-position of the coumarin scaffold.[18] The 6-aryl derivatives with a

Scheme 4. Syntheses of [3,2-*g*]Chromene Derivatives 23a−23f[a]



[a]Reagents and conditions: (a) Piperidine, 80 °C, 6 h. (b) NaOH, H_2O, 1 h, rt. (c) i: arylboronic acids, $PdCl_2(PPh)_2$, K_2CO_3, dioxane/H_2O = 5:1, 80 °C, overnight; ii: HCl (2 M), 80 °C, 2 h.

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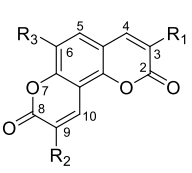
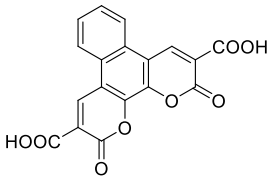
Table 1. Potencies of the U-Shaped Coumarin-like Tricyclic Derivatives 13a−13n and 20 in DMR Assays on HT-29



13a-13n



20

Compd	R_1	R_2	R_3	EC_{50}[a] (μM)	Desensitization IC_{50}[b] (μM)	Antagonist IC_{50}[c] (μM)
zaprinast				0.34 ± 0.03		
13a	COOH	COOH	H	0.31 ± 0.02	0.30 ± 0.02	0.43 ± 0.05
13b	COOH	COOH	Br	0.045 ± 0.006	0.026 ± 0.003	0.54 ± 0.15
13c	COOH	COOH		0.048 ± 0.006	0.016 ± 0.002	0.20 ± 0.02
13d	COOH	COOH		0.083 ± 0.006	0.022 ± 0.002	0.48 ± 0.05
13e	COOH	COOH		0.015 ± 0.002	0.003 ± 0.001	0.17 ± 0.03
13f	COOH	COOH		0.020 ± 0.002	0.007 ± 0.002	0.47 ± 0.05
13g	COOH	COOH		0.022 ± 0.003	0.006 ± 0.001	0.44 ± 0.06
13h	COOH	COOH		0.017 ± 0.002	0.006 ± 0.001	0.47 ± 0.06
13i	COOH	COOH		0.033 ± 0.003	.0 ± 0.001	1.03 ± 0.07
13j	COOH	COOH		0.013 ± 0.001	0.008±0.002	0.30 ± 0.24
13k	COOH	COOH		0.037 ± 0.006	0.015 ± 0.005	0.21 ± 0.12
13l	COOH	COOH		0.092 ± 0.011	0.027 ± 0.003	0.35 ± 0.18
13m		COOH	Br	0.018 ± 0.002	0.018 ± 0.003	0.83 ± 0.13
13n		CN	H	2.92 ± 0.52	0.73 ± 0.13	2.53 ± 0.30
20				0.017±0.001	0.010±0.001	0.46±0.24

[a]EC_{50} to trigger DMR. [b]IC_{50} to desensitize cells upon repeated stimulation with 1 μM zaprinast. [c]IC_{50} of the known GPR35 antagonist **9** to block the agonist-induced DMR.

variety of substituents at the *ortho*-, *para*-, or *meta*-position of phenyl were further investigated. Most compounds among **13d−13l** exhibited a considerable increase in potency compared to the unsubstituted **13c**, which might be due to a better filling of the lipophilic pocket by the substituted phenyls. The only exception was **13d**, and the introduction of *ortho*-methoxyl halved its potency (EC_{50} 0.083 μM). Because the *para*-methoxy and *ortho*-fluoro substitutions appeared more favorable (**13f**, EC_{50} 0.020 μM; **13g**, EC_{50} 0.022 μM), we would like to investigate whether a combination of the two substitutions could further improve potency. However, the resulting compound **13l** bearing *o*-fluoro and *p*-methoxy showed a significantly reduced potency (EC_{50} 0.092 μM) compared with its monosubstituted counterparts. More substituents led to an undesired effect probably due to the reason that those groups were too big to properly fit in the lipophilic pocket of GPR35. The replacement of phenyl (**13c**) with thiophene (**13k**) brought in no apparent effect to the potency (**13k**, EC_{50} 0.037 μM). Noticeably, with a different scaffold arrangement, analogue **20** also showed a competitive GPR35 agonist potency (EC_{50} 0.017 μM).

We previously demonstrated that tetrazolyl was a more effective active group than carboxyl in similar molecules in terms of GPR35 potency,[19] based on which compound **13m** with a tetrazolyl on the 3-position of scaffold **13** was designed. With the replacement of the carboxyl with tetrazolyl, compound **13m** showed a 2-fold increase in potency (EC_{50} 0.018 μM) compared to compound **13b**. Compound **13n** with a cyano unit in the 9-position showed a sharp reduction (EC_{50} 2.9 μM). It indicated that the presence of two acidic groups in the molecule was extremely important to maintain a high activity.

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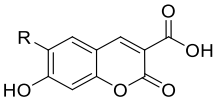
Table 2. Potency of the Coumarin Mono-Acid Derivatives 15a−15j in DMR Assays on HT-29



15a-15j

Compd	R	EC_{50}[a] (μM)	Desensitization IC_{50}[b] (μM)	Antagonist IC_{50}[c] (μM)
15a	Br	4.34 ± 0.36	3.76 ± 0.41	0.13 ± 0.04
15b		2.74 ± 0.42	2.03 ± 0.15	0.33 ± 0.18
15c		23.93 ± 7.71	17.58 ± 3.17	0.12 ± 0.04
15d		1.60 ± 0.30	1.38 ± 0.15	2.26 ± 0.68
15e		2.33 ± 0.37	1.11 ± 0.10	0.15 ± 0.03
15f		1.84 ± 0.28	1.32 ± 0.21	0.80 ± 0.23
15g		2.18 ± 0.40	1.02 ± 0.14	0.71 ± 0.16
15h		8.63 ± 2.39	5.41 ± 2.09	1.06 ± 0.69
15i		2.07 ± 0.66	1.80 ± 0.44	0.47 ± 0.10
15j		2.88 ± 0.44	1.45 ± 0.42	0.16 ± 0.08

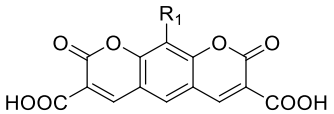
[a]EC_{50} to trigger DMR. [b]IC_{50} to desensitize cells upon repeated stimulation with 1 μM zaprinast. [c]IC_{50} of the known GPR35 antagonist **9** to block the agonist-induced DMR.

7-Hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid derivatives **15a−15j** were used as the mono-acid control compounds to further examine the effect of two acidic substituents on the scaffold **13**. It was found that all derivatives presented a relatively poor potency (EC_{50} > 1 μM, Table 2), about 100-fold less active than the corresponding compound **13**. But when comparing results in Tables 1 and 2, we noticed an intriguing phenomenon that the results seemed to have the same trend of activity among the variations with substituents on the 6-position. For instance, for methoxy substituents, the activity pattern of the compounds **13d−13f** and **15c−15e** (Figure 3A) was the same: *meta* ≈ *para* > *ortho*. So was the activity pattern of the fluoro compounds **13g−13i** and **15f−15h** (Figure S3B). The above results were noteworthy and unambiguously indicated that the two acidic substituents on the coumarin-like scaffold played an important role in improving GPR35 agonist potency.

Furthermore, a series of linear tricyclic compounds **23a−23f** were investigated. 3,7-Dicarboxyl derivative **23** could be considered as an isomer of the 3,9-dicarboxyl scaffold **13**. Results in Table 3 showed that **23a−23f** also had GPR35 agonist activities at a submicromolar level. For compounds **23a−23c**, the bromine substitution (**23a**, EC_{50} 0.025 μM) maintained much better potency than methyl (**23b**, EC_{50} 0.140 μM) and hydroxyl (**23c**, EC_{50} 0.390 μM) on the 10-position of the scaffold (Figure 4A). The introduction of *ortho*-, *meta*-, and *para*-methoxyl substituents to phenyls also endowed the corresponding compounds with excellent agonistic potency, especially the *para*-methoxyl. The rank order of potency was as follows: **23f** (EC_{50} 0.017 μM) > **23d** (EC_{50} 0.065 μM) > **23e** (EC_{50} 0.092 μM) (Figure 4B). These results indicated that the 10-position on scaffold **23** had a good tolerance to larger lipophilic substituents.

For the three types of diacid compounds **13**, **20**, and **23**, we believed that the spatial distance between two acidic groups should be a key structural parameter as it was related to whether

Table 3. Potency of the Linear Coumarin-like Tricyclic Derivatives 23a−23f in DMR Assays on HT-29



23a-23f

Compd	R_1	EC_{50}[a] (μM)	Desensitization IC_{50}[b] (μM)	Antagonist IC_{50}[c] (μM)
23a	Br	0.025±0.002	0.015 ± 0.004	0.29 ± 0.08
23b	CH_3	0.14±0.02	0.042 ± 0.006	0.35 ± 0.05
23c	OH	0.39±0.06	0.25 ± 0.02	0.59 ± 0.21
23d		0.065±0.005	0.027 ± 0.005	0.48 ± 0.05
23e		0.092±0.008	0.026 ± 0.006	0.62 ± 0.11
23f		0.017±0.001	0.002 ± 0.001	0.18 ± 0.17

[a]EC_{50} to trigger DMR. [b]IC_{50} to desensitize cells upon repeated stimulation with 1 μM zaprinast. [c]IC_{50} of the known GPR35 antagonist **9** to block the agonist-induced DMR.

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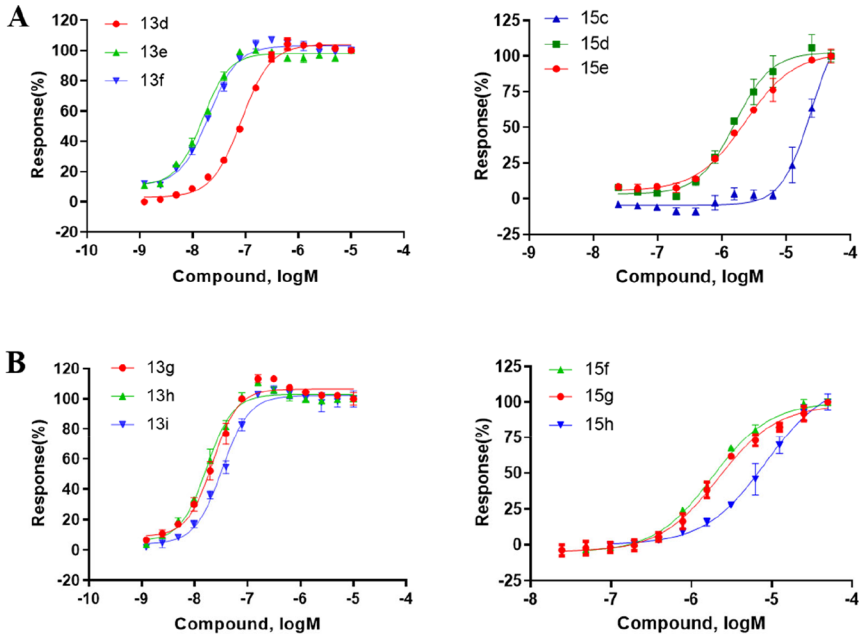

Figure 3. (A, B) Amplitude comparisons of the DMR induced by coumarin-like 3,9-dicarboxyl derivatives **13d−13i** and 3-carboxylic acid coumarin derivatives **15c−15h** as a function of concentrations. The data represented mean ± sd from two independent measurements, each with four replicates (n = 8).

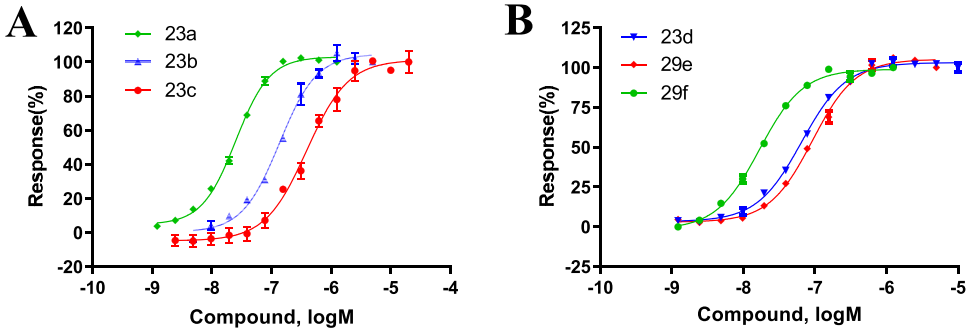

Figure 4. (A, B) Amplitudes of the DMR induced by coumarin-like 3,7-dicarboxyl derivatives **23a−23f** as a function of concentrations. The data represented mean ± sd from two independent measurements, each with four replicates (n = 8).

the two acidic groups could synergistically interact with cationic residues of the receptor. The spatial distance between the two carboxyl carbons of **13b** was 8.568 Å, **20** was 9.539 Å, and **23a** was 9.382 Å according to ChemBio 3D measurement. However, the distance difference did not seem to to result in a distinct division in potency among the compounds. We speculated an appropriate distance ranging from 8.5 to 10 Å.

The above results indicated that the introduction of two acidic groups in coumarin-like tricyclic scaffolds generally resulted in good GPR35 agonist activity. Therefore, we speculated a general phenomenon with caution that, carrying two acidic groups (such as carboxyl or tetrazolyl) on a rigid aromatic scaffold, compound would produce a GPR35 agonistic activity. The coumarin-like tricyclic structure provided a rigid scaffold to maintain a relatively favorable spatial distance between the acidic groups. Other rigid scaffolds may also work for the GPR35 activity. Although the electronic effect of different scaffolds might also have influence on compound activity, the scaffold geometry should be more fundamental. For instance, bufrolin is a potent GPR35 agonist and has a phenanthroline-like tricyclic structure, geometrically similar to compound **13**. Following these empirical principles, we tried to search and prepare more compounds with those structural characters and test their GPR35 activity.

10-Methyl-4,6-dioxo-1,4,6,9-tetrahydropyrido[3,2-*g*]-quinoline-2,8-dicarboxylic acid **24** and its analogue **25** were reported as potential antiallergy agents in the 1970s.[28] Their synthetic procedures are the same as described in literature (Scheme 5).[28,34] Compounds **24** and **25** have a quinoline-like tricyclic scaffold, and the spatial distance between two carboxyl groups is about 9.356 Å, and thus, they meet the above principle. Indeed, the DMR assays showed that both were GPR35 agonists (Table 4), especially compound **24** that exhibited rather outstanding agonistic activity with an EC_{50} of 0.008 μM. Compound **25** is the methylation product of **24**. Its GPR35 agonistic potency decreased about 12-fold, but it was still desirable (EC_{50} 0.1 μM) (Figure S2). Compound **26** with 4,6-dichloro substituents is a novel derivative of compound **24** and exhibited an increased GPR35 agonistic activity (EC_{50} 0.054 μM) compared to 25. It indicated that hydrogen bond receptors at 4- and 6-positions of the quinoline-like tricyclic scaffold may be more favorable to activity.

Dithieno[3,2-b:2′,3′-*d*]thiophene-2,6-dicarboxylic acid **27** was another diacid compound bearing a much different fused

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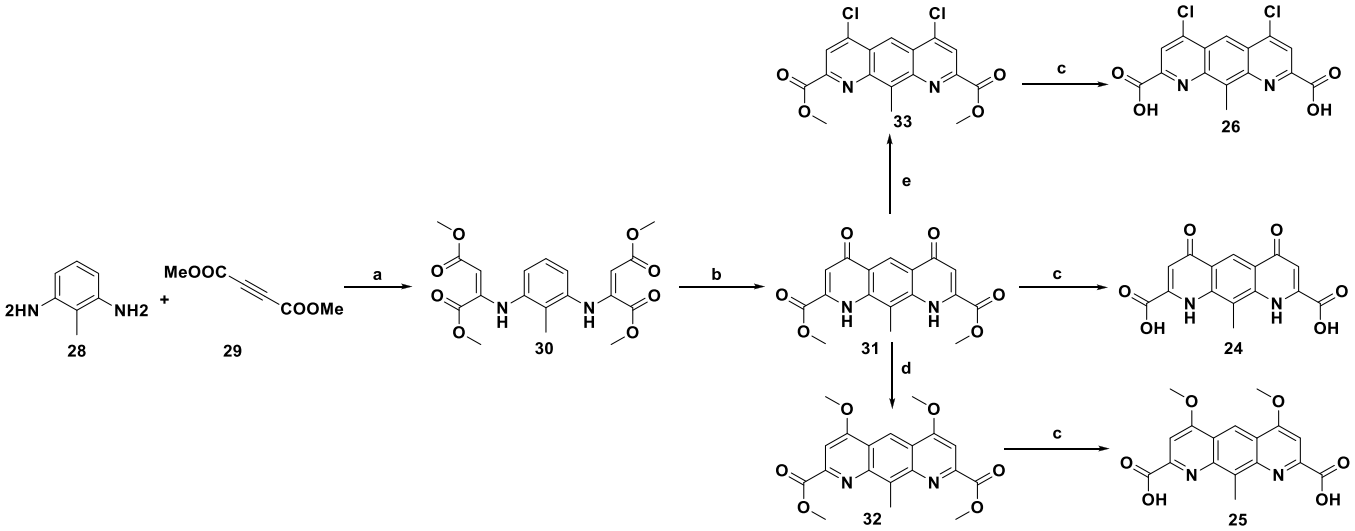
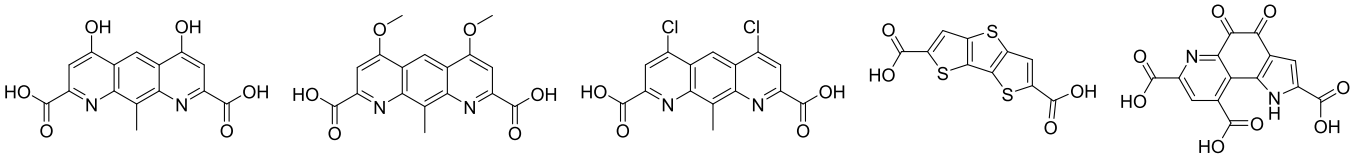
Scheme 5. Syntheses of 24−26[a]



[a]Reagents and conditions: (a) MeOH, 0 °C to rt, overnight. (b) Diphenyl ether, 260 °C, 2 h. (c) NaOH (2 M), MeOH, rt, 1 h. (d) MeI, K_2CO_3, DMF, 60 °C, 12 h. (e) $POCl_3$, 90 °C.

Table 4. Potency of the Predicted Compounds 24−27 and PQQ in DMR assays on HT-29



compd	EC_{50} (μM)[a]	desensitization IC_{50} (μM)[b]	antagonist IC_{50} (μM)[c]
24	0.008 ± 0.001	0.002 ± 0.002	0.789 ± 0.123
25	0.100 ± 0.015	0.065 ± 0.007	0.282 ± 0.033
26	0.054 ± 0.006	0.015 ± 0.002	2.529 ± 0.847
27	0.043 ± 0.003	0.020 ± 0.002	0.494 ± 0.062
PQQ	0.071 ± 0.010	0.030 ± 0.009	0.582 ± 0.087

[a]EC_{50} to trigger DMR. [b]IC_{50} to desensitize cells upon repeated stimulation with 1 μM zaprinast. [c]IC_{50} of the known GPR35 antagonist **9** to block the agonist-induced DMR.

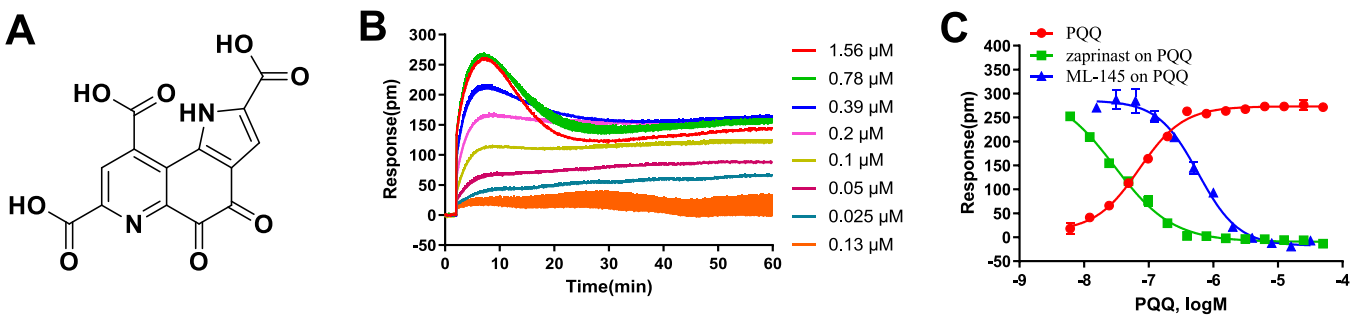


Figure 5. (A) Structure of PQQ. (B) Real-time kinetic responses of PQQ at different concentrations in HT-29 cells. (C) DMR amplitudes of PQQ, the concentration-dependent desensitization of 400 nM zaprinast DMR by PQQ and the concentration-dependent inhibition of the DMR of 1 μM PQQ by ML145. The data represented mean ± sd from two independent measurements, each with four replicates (n = 8).

tricyclic scaffold and a spatial distance of 8.679 Å between two carboxyl groups. It was initially reported as an intermediate during the preparation of optoelectronic materials.[29] Based on our principle, compound **27** effectively activated the GPR35 receptor with an EC_{50} of 0.043 μM (Figure S2).

By searching natural compound libraries, we found another example meeting the principle. Pyrroloquinoline quinone (PQQ), an oxidoreductase co-factor widely present in methylotrophic bacteria,[30] is a natural product of great interest. It not only takes part in the redox reaction *in vivo* but also has some special biological activities and physiological functions, such as regulation of the nervous system, improvement of immune function, and inhibition of tumor growth and metastasis.[31] PQQ has been used as a dietary supplement to reduce the ischemia−reperfusion injury caused by heart attack or stroke.[32,33] PQQ has a rigid pyrrolo[2,3-*f*]quinoline scaffold

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and three carboxyl groups, two of which are located at the ends of the scaffold with a distance of 8.959 Å, and the third one is at the middle. As expected, the DMR assays showed that PQQ had a nanomolar GPR35 agonistic activity (EC_{50} 0.071 μM) and could be antagonized by the specific antagonist ML-145 (Figure 5). To our knowledge, this should be the first time that PQQ was discovered to activate GPR35; the current pharmacological investigations of PQQ are controversial, and we hope our finding of its agonistic activity on GPR35 may shed lights on them.

Receptor Selectivity. To assess the receptor selectivity of these novel GPR35 agonists, some compounds were selected for further DMR assays. In CHO-K1 host cells, the selected compounds showed no signals at all (Figure S3). However, on CHO-K1-hGPR35 cells, all selected compounds exhibited outstanding agonist activity, especially compounds **20** and **24** with EC_{50} values of 0.312 and 1.08 nM, respectively (Table 5).

Table 5. Potency of the Selected Compounds on CHO-K1-hGPR35 Cells in DMR Assays

compd	EC_{50} (nM)[a]	desensitization IC_{50} (nM)[b]
zaprinast	62.83 ± 1.81	48.15 ± 1.62
13f	6.93 ± 0.29	2.45 ± 0.12
13m	25.92 ± 1.11	15.90 ± 0.71
20	0.31 ± 0.02	0.47 ± 0.05
23a	12.72 ± 0.49	9.31 ± 0.25
23f	62.83 ± 1.81	48.15 ± 1.62
24	1.08 ± 0.16	0.74 ± 0.06
25	8.13 ± 0.70	6.11 ± 0.54
26	5.08 ± 0.44	3.52 ± 0.30

[a]EC_{50} to trigger DMR. [b]IC_{50} to desensitize cells upon repeated stimulation with 400 nM zaprinast.

In other cell lines separately expressing M_3, D_2, μ-opioid, FFA4, and H_1 receptors, no apparent signals were observed when compounds were added at a concentration of 1 μM (Figure S4), suggesting no activity on these receptors. Therefore, these selected fused tricyclic diacid compounds had a selective agonistic activity on GPR35.

Molecular Docking Studies. To further investigate the binding mode of the diacid agonists with suggested structural characters on the GPR35 protein, preliminary molecular docking studies had been attempted using a homology model and several representative agonists, shown in Figure S5. The results showed that, for each agonist, there were strong electrostatic and hydrogen binding interactions between its acidic groups and cationic Arg residues in the binding pocket. We noticed that Arg100/151/164 seemed to be more inclined to such interactions, possibly because they were located in a spatial range that was right approachable to both acidic groups (about 8.5−10 Å between the two acidic groups). It has also been reported that the GPR35 binding pocket contains abundant cationic residues,[16] and thus, it provided favorable conditions for the diacid compounds to have dual interactions with the receptor. The simulation results also indicated that, apart from providing a suitable molecular geometry and spatial distance between two acidic groups, the aromatic scaffold played an important supporting role in the formation of hydrophobic interactions with surrounding residues, such as Leu237/258 and Pro176, and such interactions were seen in almost all docking models.

In this work, we included acid groups, carboxyl and tetrazyl, and the fused tricyclic aromatic scaffolds in the compound design. Despite the limitation of experiments and simulations, we still could propose that a combination of two acidic groups and a suitable rigid scaffold would allow compounds to activate GPR35, and this proposal might serve as a principle for future GPR35 agonist designs.

CONCLUSIONS

In conclusion, a series of coumarin-like diacid derivatives with U-shaped and linear fused tricyclic structures were synthesized and evaluated to be agonists of an orphan receptor GPR35. Most compounds behaved as selective and full GPR35 agonists and exhibited excellent potency at low nanomolar concentrations. Compounds carrying two acid groups, such as carboxyl or tetrazyl, had significantly increased potency on GPR35 compared to mono acid analogues. Combining information of some known agonists bearing different scaffolds, we believe that such an increase is associated with the fact that the bonding pocket of GPR35 is rich in cation residues to create a favorable condition to form multiple electrostatic interactions between the acid groups of ligand and the receptor. Based on the structural features, more known compounds, such as **24**−**27** and PQQ, were identified as potent GPR35 agonists. Particularly the natural product PQQ, it is commercially used as a dietary supplement to reduce the ischemia−reperfusion injury caused by heart attack or stroke, and here, it was found to have powerful agonistic activity on GPR35 for the first time, which would provide more theoretical basis for its controversial pharmacological research. The docking simulation of some representative agonists further rationalized the advantages of those diacid compounds bearing a tricyclic aromatic scaffold. These results promoted us to propose that suitable combination of two acidic groups and a rigid scaffold would possibly endow the compound with a potent agonistic activity on GPR35. We believe, to some extent, this principle will provide a general method for designing and recognize GPR35 agonists.

EXPERIMENTAL SECTION

General. All experimental reagents and solvents were obtained from various providers and used without any additional purification or drying except for tetrahydrofuran, which was distilled over calcium. The purity of all coumarin-like compounds was ≥95%. The reactions were monitored by thin-layer chromatography (TLC). If necessary, the products were purified with column chromatography. NMR data were collected on a Bruker Ascend 600 MHz NMR spectrometer at 600 MHz (^{1}H) or 151 MHz (^{13}C). The chemical shifts were reported in parts per million (ppm) relative to the deuterated solvent DMSO-d_6; that is, δ ^{1}H, 2.49 ppm; ^{13}C, 39.7 ppm. High-resolution mass spectral (HRMS) analyses were performed on an Agilent 1290 Infinity LC instrument (Agilent, USA) coupled to an Agilent 6540 series QTOF-MS (Agilent, USA) equipped with an ESI source, a diode-array detector (DAD), an automatic sample injector, a degasser, and a column thermostat.

The purity of all final compounds analyzed by high-pressure liquid chromatography (HPLC) was >95%. The determination of purity was conducted on a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) with an ACQUITY UPLC HSS T3 column (2.1 × 100 mm,1.8 μm). Elution was performed with a gradient of water/acetonitrile (containing 0.1% formic acid) from 95/5 to 5/95 for 15 min and maintained 5/95 for another 15 min. The flow rate was 200 μL/min. Peaks were detected at 290 or 254 nm.

The synthesis and structural characterization data of the active compounds and their intermediates are described as below. For the

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detailed synthetic and structural characterization data of other compounds see the Supporting Information.

Screening for Pan-Assay Interference Compounds (PAINS). Screening of all target compounds for PAINS via the public tool http://zinc15.docking.org/patterns/home74 yielded no hits.

*General Procedure of Method A for the Syntheses of Compounds **15a–15j**.* A mixture of the **14** (1.0 equiv) and arylboronic acids (1.5 equiv) in 30 mL degassed (by sonication followed by stream of nitrogen) dioxane/H_2O (5:1) was evacuated and flushed with nitrogen. K_2CO_3 (2.2 equiv) and $PdCl_2(PPh_3)_2$ (0.1 equiv) were added, and the reaction mixture was stirred under a nitrogen atmosphere overnight. Hydrochloric acid solution (2 M) was added to adjust pH to 4–5, and the resulting solution was stirred for 2 h more. Dioxane was evaporated under reduced pressure. The mixture was diluted with water (30 mL) and extracted three times with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was washed with a modicum of methyl alcohol (MeOH) (3 × 2 mL) and dried under vacuum at 50 °C.

*General Procedure of Method B for the Syntheses of Compounds **13a, 13b, and 23a–23c**.* The appropriate ethyl carboxylate derivatives (1.0 equiv) were added into a solution of NaOH (2.5 equiv) in water (10 mL). The reaction mixture was stirred at rt until a clear solution was obtained. The mixture was acidified to pH 4–5 with hydrochloric acid solution (2 M). The obtained precipitate was filtered off, washed with a modicum of MeOH (3 × 2 mL), and dried under vacuum at 50 °C.

*General Procedure of Method C for the Syntheses of Compounds **13c–13l and 23d–23f**.* A mixture of the **12b** or **22a** (1.0 equiv) and aryboronic acids (1.5 equiv) in 30 mL degassed (by sonication followed by stream of nitrogen) dioxane/H_2O (5:1) was evacuated and flushed with nitrogen. K_2CO_3 (2.2 equiv) and $PdCl_2(PPh_3)_2$ (0.1 equiv) were added, and the reaction mixture was stirred under a nitrogen atmosphere overnight. Hydrochloric acid solution (2 M) was added to adjust pH to 4–5, and the resulting solution was stirred for 2 h more. Dioxane was evaporated under reduced pressure. The mixture was diluted with water (30 mL) and extracted three times with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated under reduced pressure. The crude product was purified by column chromatography (9:1 DCM/MeOH).

*2,8-Dioxo-2,8-dihydropyrano[2,3-f]chromene-3,9-dicarboxylic Acid (**13a**).* As described in method B, compound **13a** was obtained from **12a** with 81% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.83 (s, 1H), 8.74 (s, 1H), 8.20 (d, J = 8.8 Hz, 1H), 7.45 (d, J = 8.8 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.16, 163.83, 158.22, 155.77, 155.74, 152.61, 148.87, 140.88, 135.52, 119.09, 117.49, 114.44, 113.59, 107.30. Purity: 97.74%. HRMS for $C_{14}H_6O_8$: calcd, $[M + H]^+$ 303.0135, $[M + H_2O + H]^+$ 321.0241, found 303.0135, 321.0245.

*6-Bromo-2,8-dioxo-2,8-dihydropyrano[2,3-f]chromene-3,9-dicarboxylic Acid (**13b**).* As described in method B, compound **13b** was obtained from **12b** with 83% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.77 (s, 1H), 8.71 (s, 1H), 8.56 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.95, 163.54, 155.34, 154.99, 154.36, 151.82, 147.83, 140.77, 137.18, 119.54, 118.32, 115.51, 108.66, 104.77. Purity: 97.87%. HRMS for $C_{14}O_8H_5Br$: calcd, $[M - H]^-$ 334.9197 and 336.9176, found 334.9115 and 336.9100.

*2,8-Dioxo-6-phenyl-2,8-dihydropyrano[2,3-f]chromene-3,9-dicarboxylic Acid (**13c**).* As described in method C, compound **13c** was obtained from **12b** and phenylboronic acid with 42% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.91 (s, 1H), 8.86 (s, 1H), 8.35 (s, 1H), 7.70 (d, J = 7.2 Hz, 2H), 7.62 (t, J = 7.6 Hz, 2H), 7.55 (t, J = 7.4 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.19, 163.87, 155.80, 155.54, 155.10, 151.84, 148.77, 141.05, 135.45, 134.52, 129.88, 129.09, 128.90, 126.14, 119.12, 117.96, 114.35, 107.65. Purity: 95.66%. HRMS for $C_{20}H_{10}O_8$: calcd, $[M + H]^+$ 379.0448, found 379.0448.

*6-(2-Methoxyphenyl)-2,8-dioxo-2,8-dihydropyrano[2,3-f]-chromene-3,9-dicarboxylic Acid (**13d**).* As described in method C, compound **13d** was obtained from **12a** and (2-methoxyphenyl)boronic acid with 51% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.85 (s, 1H), 8.82 (s, 1H), 8.17 (s, 1H), 7.52–7.47 (m, 1H), 7.33 (dd, J = 7.4, 1.4 Hz, 1H), 7.21 (d, J = 8.3 Hz, 1H), 7.11 (t, J = 7.4 Hz, 1H), 3.75 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.21, 163.83, 157.24, 155.85, 155.60, 155.53, 151.98, 148.77, 141.35, 136.30, 131.60, 130.86, 123.74, 123.33, 121.08, 118.74, 117.81, 114.16, 112.25, 107.25, 56.11. Purity: 96.92%. HRMS for $C_{21}H_{12}O_9$: calcd, $[M + H]^+$ 409.0554, found 409.0545.

*6-(3-Methoxyphenyl)-2,8-dioxo-2,8-dihydropyrano[2,3-f]-chromene-3,9-dicarboxylic Acid (**13e**).* As described in method C, compound **13e** was obtained from **12a** and (3-methoxyphenyl)boronic acid with 67% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.82 (s, 1H), 8.32 (s, 1H), 7.47 (t, J = 7.9 Hz, 1H), 7.21 (d, J = 7.1 Hz, 2H), 7.09–7.05 (m, 1H), 3.83 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.18, 163.86, 159.69, 155.78, 155.50, 155.16, 151.91, 148.92, 141.19, 135.76, 135.53, 130.19, 125.94, 122.09, 118.93, 117.81, 115.70, 114.29, 107.62, 55.71. Purity: 95.57%. HRMS for $C_{21}H_{12}O_9$: calcd, $[M + H]^+$ 409.0554, found 409.0548.

*6-(4-Methoxyphenyl)-2,8-dioxo-2,8-dihydropyrano[2,3-f]-chromene-3,9-dicarboxylic Acid (**13f**).* As described in method C, compound **13f** was obtained from **12a** and (4-methoxyphenyl)boronic acid with 55% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.81 (s, 1H), 8.76 (s, 1H), 8.23 (s, 1H), 7.58 (d, J = 8.7 Hz, 2H), 7.12 (d, J = 8.8 Hz, 2H), 3.84 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.74, 161.42, 159.02, 157.97, 156.08, 150.01, 132.10, 130.77, 129.15, 126.95, 114.05, 113.22, 111.37, 102.41, 55.59. Purity: 98.67%. HRMS for $C_{21}H_{12}O_9$: calcd, $[M + H]^+$ 409.0554, found 409.0548.

*6-(2-Fluorophenyl)-2,8-dioxo-2,8-dihydropyrano[2,3-f]-chromene-3,9-dicarboxylic Acid (**13g**).* As described in method C, compound **13g** was obtained from **12a** and (2-fluorophenyl)boronic acid with 42% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.93 (s, 1H), 8.89 (s, 1H), 8.36 (s, 1H), 7.64 (dtd, J = 9.2, 7.4, 1.7 Hz, 2H), 7.48 (ddd, J = 11.6, 8.5, 5.0 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.14 (s), 163.76 (s), 159.80 (d, J = 247.0 Hz), 155.69 (s), 155.34 (s), 155.20 (s), 152.46 (s), 148.62 (s), 141.16 (s), 136.30 (s), 132.48 (d, J = 2.5 Hz), 131.65 (d, J = 8.4 Hz), 125.33 (d, J = 3.3 Hz), 122.06 (d, J = 15.4 Hz), 120.46 (s), 119.07 (s), 118.08 (s), 116.35 (d, J = 21.7 Hz), 114.37 (s), 107.55 (s). Purity: 98.90%. HRMS for $C_{20}H_9FO_8$: calcd, $[M + H]^+$ 397.0354, found 397.0366.

*6-(3-Fluorophenyl)-2,8-dioxo-2,8-dihydropyrano[2,3-f]-chromene-3,9-dicarboxylic Acid (**13h**).* As described in method C, compound **13h** was obtained from **12a** and (3-fluorophenyl)boronic acid with 58% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.83 (s, 1H), 8.81 (s, 1H), 8.34 (s, 1H), 7.61 (td, J = 8.0, 6.3 Hz, 1H), 7.55–7.52 (m, 1H), 7.51–7.48 (m, 1H), 7.37–7.32 (m, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.10 (s), 163.79 (s), 162.48 (d, J = 243.5 Hz), 155.66 (s), 155.36 (s), 155.08 (s), 152.12 (s), 148.75 (s), 141.07 (s), 136.70 (d, J = 8.4 Hz), 135.62 (s), 131.14 (d, J = 8.5 Hz), 126.04 (d, J = 2.5 Hz), 124.67 (s), 119.04 (s), 117.91 (s), 116.85 (s), 115.79 (d, J = 20.9 Hz), 114.29 (s), 107.65 (s). Purity: 96.12%. HRMS for $C_{20}H_9FO_8$: calcd, $[M + H]^+$ 397.0354, found 397.0358.

*6-(4-Fluorophenyl)-2,8-dioxo-2,8-dihydropyrano[2,3-f]-chromene-3,9-dicarboxylic Acid (**13i**).* As described in method C, compound **13i** was obtained from **12a** and (4-fluorophenyl)boronic acid with 55% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.82 (s, 1H), 8.30 (s, 1H), 7.70 (dd, J = 8.0, 5.8 Hz, 2H), 7.42 (t, J = 8.8 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.16 (s), 163.83 (s), 162.67 (d, J = 246.3 Hz), 155.75 (s), 155.44 (s), 155.13 (s), 151.91 (s), 148.82 (s), 141.13 (s), 135.47 (s), 132.02 (d, J = 8.4 Hz), 130.86 (d, J = 3.4 Hz), 125.08 (s), 119.00 (s), 117.90 (s), 116.08 (d, J = 21.5 Hz), 114.30 (s), 107.64 (s). Purity: 95.81%. HRMS for $C_{20}H_9FO_8$: calcd, $[M + H]^+$ 397.0354, found 397.0367.

*6-(4-Isopropylphenyl)-2,8-dioxo-2,8-dihydropyrano[2,3-f]-chromene-3,9-dicarboxylic Acid (**13j**).* As described in method C, compound **13j** was obtained from **12a** and (4-isopropylphenyl)boronic acid with 63% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.82 (s, 1H), 8.28 (s, 1H), 7.57 (d, J = 8.2 Hz, 2H), 7.43 (d, J = 8.2 Hz, 2H), 3.02–2.96 (m, 1H), 1.28 (d, J = 6.9 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.12, 162.78, 154.75, 154.50, 154.06, 150.65, 148.17, 147.76, 143.25, 140.08, 134.25, 130.90, 128.75, 125.98, 125.07, 116.81, 113.27, 106.56, 32.68, 23.23. Purity: 97.91%. HRMS for $C_{23}H_{16}O_8$: calcd, $[M + H]^+$ 421.0918, found 421.0930.

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*2,8-Dioxo-6-(thiophen-2-yl)-2,8-dihydropyrano[2,3-f]chromene-3,9-dicarboxylic Acid (**13k**).* As described in method C, compound **13k** was obtained from **12a** and naphthalen-2-ylboronic acid with 48% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.89 (s, 1H), 8.81 (s, 1H), 8.61 (s, 1H), 7.78 (dd, J = 5.1, 1.1 Hz, 1H), 7.74 (dd, J = 3.7, 1.1 Hz, 1H), 7.28 (dd, J = 5.1, 3.7 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.03, 162.66, 154.58, 154.09, 152.76, 150.41, 147.68, 140.19, 133.82, 132.23, 127.56, 127.20, 127.10, 118.09, 117.80, 116.99, 113.44, 106.75. Purity: 97.83%. HRMS for $C_{18}H_8O_8S$: calcd, $[M + H]^+$ 385.0013, found 385.0004.

*6-(2-Fluoro-4-methoxyphenyl)-2,8-dioxo-2,8-dihydropyrano-[2,3-f]chromene-3,9-dicarboxylic Acid (**13l**).* As described in method C, compound **13l** was obtained from **12a** and (2-fluoro-4-methoxyphenyl)boronic acid with 38% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.84 (s, 1H), 8.82 (s, 1H), 8.17 (s, 1H), 7.37 (t, J = 7.6 Hz, 1H), 7.14 (d, J = 11.5 Hz, 1H), 6.96 (t, J = 8.1 Hz, 1H), 3.76 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.21 (s), 163.96 (d, J = 246.13 Hz), 163.83 (s), 158.69 (d, J = 10.6 Hz), 155.84 (s), 155.57 (s), 155.52 (s), 152.01 (s), 148.63 (s), 141.12 (s), 136.35 (s), 132.75 (d, J = 10.2 Hz), 122.74 (s), 119.57 (d, J = 3.1 Hz), 117.94 (s), 114.16 (s), 107.57 (s), 107.43 (s), 107.31 (s), 100.67 (d, J = 26.1 Hz), 56.65 (s). Purity: 95.24%. HRMS for $C_{21}H_{11}FO_9$: calcd, $[M + H]^+$ 427.0460, $[M + H_2O + H]^+$ 445.0566, found 427.0467, 445.0569.

*Preparation of 6-Bromo-2,8-dioxo-3-(1H-tetrazol-5-yl)-2, 8-dihydropyrano[2,3-f]chromene-9-carboxylic Acid (**13m**).* A mixture of **19b** (1.0 equiv) and Meldrum's acid (1.5 equiv) were mixed in 10 mL of ethanol at room temperature. A catalytic amount of ammonium acetate was added, and the reaction mixture was stirred at the room temperature for 2 h. Hydrochloric acid solution (2 M) was added to adjust pH to 4−5. Ethanol was evaporated under reduced pressure. The resulting solid was filtered, washed three times with 2 mL of methanol, and dried under vacuum at 50 °C. The target product was obtained with 83% yield as a gray solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.08 (s, 1H), 8.79 (s, 1H), 8.69 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.60, 157.26, 155.02, 154.09, 150.68, 143.28, 140.53, 136.89, 119.90, 116.03, 112.97, 108.89, 105.32. Purity: 95.31%. HRMS for $C_{14}H_5BrN_4O_6$: calcd, $[M + H]^+$ 404.9465, found $[M + H]^+$ 404.9471, $[M + H + 2]^+$ 406.9463.

*Preparation of 2,8-Dioxo-3-(1H-tetrazol-5-yl)-2,8-dihydropyrano[2,3-f]chromene-9-carbonitrile (**13n**).* A mixture of **19a** (1.0 equiv) and malononitrile (1.5 equiv) were mixed in 20 mL of water at room temperature. A catalytic amount of ammonium acetate was added, and the reaction mixture was stirred at room temperature for 4 h. Hydrochloric acid solution (2 M) was added to adjust pH to 4−5. The resulting solid was filtered, washed three times with 2 mL of methanol, and dried under vacuum at 50 °C. The target product was obtained with 78% yield as a gray solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.28 (s, 1H), 9.14 (s, 1H), 8.37 (d, J = 8.8 Hz, 1H), 7.60 (d, J = 8.8 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 156.31, 156.08, 155.52, 150.03, 145.33, 143.03, 135.14, 114.41, 113.47, 113.40, 111.48, 106.42, 102.69. Purity: 97.4%. HRMS for $C_{14}H_5N_5O_4$: calcd, $[M - H]^-$ 306.0269, found 306.0283.

*7-Hydroxy-2-oxo-6-phenyl-2H-chromene-3-carboxylic Acid (**15b**).* As described in method A, compound **15b** was obtained from **14** and phenylboronic acid with 80% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.85 (s, 1H), 7.57−7.53 (m, 2H), 7.44 (t, J = 7.7 Hz, 2H), 7.36 (t, J = 7.4 Hz, 1H), 6.90 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.73, 161.47, 157.91, 156.38, 149.92, 137.02, 132.57, 129.65, 128.61, 127.73, 127.26, 113.36, 111.38, 102.50. Purity: 96.76%. HRMS for $C_{16}H_{10}O_5$: calcd, $[M + H]^+$ 283.0601, found 283.0600.

*7-Hydroxy-6-(2-methoxyphenyl)-2-oxo-2H-chromene-3-carboxylic Acid (**15c**).* As described in method A, compound **15c** was obtained from **14** and (2-methoxyphenyl)boronic acid with 82% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.70 (s, 1H), 7.66 (s, 1H), 7.39−7.34 (m, 1H), 7.17 (dd, J = 7.4, 1.7 Hz, 1H), 7.08 (d, J = 8.1 Hz, 1H), 7.00 (td, J = 7.4, 0.8 Hz, 1H), 6.84 (s, 1H), 3.71 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.69, 161.09, 156.99, 156.21, 155.40, 148.89, 132.13, 130.48, 128.54, 125.01, 124.02, 119.51, 111.88, 110.63, 109.73, 100.87, 54.75. Purity: 97.44%. HRMS for $C_{17}H_{12}O_6$: calcd, $[M + H]^+$ 313.0707, found 313.0710.

*7-Hydroxy-6-(3-methoxyphenyl)-2-oxo-2H-chromene-3-carboxylic Acid (**15d**).* As described in method A, compound **15d** was obtained from **14** and (3-methoxyphenyl)boronic acid with 78% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.87 (d, J = 4.8 Hz, 1H), 7.36 (dd, J = 15.0, 7.1 Hz, 1H), 7.12 (d, J = 7.8 Hz, 1H), 7.10−7.08 (m, 1H), 6.95−6.92 (m, 1H), 6.89 (s, 1H), 3.79 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.63, 160.33, 158.35, 156.76, 155.29, 148.89, 137.21, 131.55, 128.55, 125.93, 120.92, 114.32, 112.31, 112.05, 110.26, 101.42, 54.46. Purity: 95.38%. HRMS for $C_{17}H_{12}O_6$: calcd, $[M + H]^+$ 313.0707, found 313.0706.

*7-Hydroxy-6-(4-methoxyphenyl)-2-oxo-2H-chromene-3-carboxylic Acid (**15e**).* As described in method A, compound **15e** was obtained from **14** and (4-methoxyphenyl)boronic acid with 82% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.81 (s, 1H), 7.51−7.45 (m, 2H), 7.01 (t, J = 10.8 Hz, 2H), 6.88 (s, 1H), 3.80 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.73, 161.43, 159.02, 157.99, 156.07, 149.99, 132.10, 130.76, 129.16, 126.96, 114.05, 113.23, 111.37, 102.42, 55.59. Purity: 96.79%. HRMS for $C_{17}H_{12}O_6$: calcd, $[M + H]^+$ 313.0707, found 313.0708.

*7-Hydroxy-6-(2-fluorophenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxylic Acid (**15f**).* As described in method A, compound **15f** was obtained from **14** and (2-fluorophenyl)boronic acid with 64% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.73 (d, J = 8.3 Hz, 1H), 7.80 (s, 1H), 7.47−7.43 (m, 1H), 7.40 (td, J = 7.5, 1.5 Hz, 1H), 7.28 (t, J = 8.1 Hz, 2H), 6.90 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.68 (s), 161.71 (s), 159.96 (d, J = 245.9 Hz), 157.77 (s), 156.91 (s), 149.75 (s), 133.30 (s), 132.40 (d, J = 3.3 Hz), 130.33 (d, J = 8.1 Hz), 124.82 (d, J = 7.8 Hz), 124.75 (d, J = 4.8 Hz), 121.96 (s), 115.92 (d, J = 22.0 Hz), 113.56 (s), 111.09 (s), 102.19 (s). Purity: 98.76%. HRMS for $C_{16}H_9FO_5$: calcd, $[M + H]^+$ 301.0507, found 301.0512.

*7-Hydroxy-6-(3-fluorophenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxylic Acid (**15g**).* As described in method A, compound **15g** was obtained from **14** and (3-fluorophenyl)boronic acid with 75% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.70 (s, 1H), 8.26 (s, 1H), 7.26 (s, 1H), 5.46 (s, 2H), 4.29 (q, J = 7.1 Hz, 2H), 3.44 (s, 3H), 1.30 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.66 (s), 162.36 (d, J = 242.8 Hz), 161.30 (s), 157.71 (s), 156.60 (s), 149.81 (s), 139.29 (d, J = 8.3 Hz), 132.75 (s), 130.56 (d, J = 8.6 Hz), 125.75 (d, J = 2.2 Hz), 125.70 (s), 116.35 (d, J = 22.0 Hz), 114.53 (d, J = 20.6 Hz), 113.63 (s), 111.40 (s), 102.61 (s). Purity: 97.20%. HRMS for $C_{16}H_9FO_5$: calcd, $[M + H]^+$ 301.0507, found 301.0510.

*7-Hydroxy-6-(4-fluorophenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxylic Acid (**15h**).* As described in method A, compound **15h** was obtained from **14** and (4-fluorophenyl)boronic acid with 79% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.72 (s, 1H), 7.85 (s, 1H), 7.61−7.55 (m, 2H), 7.28 (dd, J = 12.4, 5.4 Hz, 2H), 6.89 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.70 (s), 161.96 (d, J = 244.62 Hz), 161.42 (s), 157.87 (s), 156.42 (s), 149.83 (s), 133.32 (d, J = 3.1 Hz), 132.51 (s), 131.62 (d, J = 8.2 Hz), 126.17 (s), 115.46 (d, J = 21.3 Hz), 113.39 (s), 111.35 (s), 102.52 (s). Purity: 98.67%. HRMS for $C_{16}H_9FO_5$: calcd, $[M + H]^+$ 301.0507, found 301.0538.

*7-Hydroxy-6-(4-isopropylphenyl)-2-oxo-2H-chromene-3-carboxylic Acid (**15i**).* As described in method A, compound **15i** was obtained from **14** and (4-isopropylphenyl)boronic acid with 83% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.72 (s, 1H), 7.83 (s, 1H), 7.46 (d, J = 8.1 Hz, 2H), 7.31 (d, J = 8.2 Hz, 2H), 6.89 (s, 1H), 2.93 (dp, J = 13.7, 6.8 Hz, 2H), 1.24 (d, J = 6.9 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.74, 161.51, 157.92, 156.26, 149.97, 147.92, 134.48, 132.39, 129.59, 127.26, 126.54, 113.27, 111.37, 102.44, 33.68, 24.37. Purity: 96.37%. HRMS for $C_{19}H_{16}O_5$: calcd, $[M + H]^+$ 325.1071, found 325.1132.

*7-Hydroxy-2-oxo-6-(thiophen-2-yl)-2H-chromene-3-carboxylic Acid (**15j**).* As described in method A, compound **15j** was obtained from **14** and thiophen-2-ylboronic acid with 68% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.80 (s, 1H), 8.29 (s, 1H), 7.67 (d, J = 3.6 Hz, 1H), 7.64 (d, J = 5.1 Hz, 1H), 7.21 (dd, J = 6.3, 2.3 Hz, 1H), 6.95 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.64, 160.43, 156.84, 155.24, 148.83, 136.53, 135.89, 131.46, 129.14, 127.40, 127.27,

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126.33, 125.73, 112.22, 110.26, 101.38, 20.50. Purity: 95.93%. HRMS for $C_{14}H_8O_5S$: calcd, $[M + H]^+$ 289.0165, found 289.0153.

*Preparation of 2,11-Dioxo-2,11-dihydrobenzo[f]pyrano[3,2-h]-chromene-3,10-dicarboxylic Acid (**20**).* A mixture of **13** (1.0 equiv) and Meldrum's acid (3.0 equiv) were mixed in 10 mL of water at room temperature. A catalytic amount of ammonium acetate was added, and the reaction mixture was stirred at the room temperature for 6 h. Hydrochloric acid solution (2 M) was added to adjust pH to 4−5. The resulting solid was filtered, washed three times with 2 mL of methanol, and dried under vacuum at 50 °C. The target product was obtained with 91% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.35 (s, 2H), 8.65 (dd, J = 6.0, 3.1 Hz, 2H), 7.79 (dd, J = 6.2, 3.0 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.39, 155.59, 143.15, 142.93, 128.79, 125.74, 123.73, 121.13, 116.99. Purity: 95.22%. HRMS for $C_{18}H_8O_8$: calcd, $[M + Na]^+$ 375.0111, found 375.0087.

*10-Bromo-2,8-dioxo-2,8-dihydropyrano[3,2-g]chromene-3,7-dicarboxylic Acid (**23a**).* As described in method B, compound **23a** was obtained from **22a** with 82% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.49 (s, 2H), 8.77 (s, 2H), 8.43 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.90, 155.52, 155.15, 148.14, 131.65, 118.64, 116.44, 97.25. Purity: 97.14%. HRMS for $C_{14}O_8H_5Br$: calcd, $[M - H]^-$ 334.9197 and 336.9176, found 334.9112 and 336.9102.

*10-Methyl-2,8-dioxo-2,8-dihydropyrano[3,2-g]chromene-3,7-dicarboxylic Acid (**23b**).* As described in method B, compound **23b** was obtained from **22b** with 91% yield as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.72 (s, 2H), 8.28 (s, 1H), 2.36 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.06, 155.05, 155.00, 147.39, 129.16, 116.93, 114.39, 111.66, 7.12. HRMS for $C_{15}H_8O_8$: calcd, $[M + H]^+$ 317.0292, found 317.0305.

*10-Hydroxy-2,8-dioxo-2,8-dihydropyrano[3,2-g]chromene-3,7-dicarboxylic Acid (**23c**).* As described in method B, compound **23c** was obtained from **22c** with 82% yield as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.65 (s, 2H), 7.87 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.26, 156.17, 148.46, 146.80, 132.04, 121.75, 118.47, 115.95. Purity: 96.69%. HRMS for $C_{14}H_6O_9$: calcd, $[M + H]^+$ 319.0085, found 319.0094.

*10-(2-Methoxyphenyl)-2,8-dioxo-2,8-dihydropyrano[3,2-g]-chromene-3,7-dicarboxylic Acid (**23d**).* As described in method C, compound **23d** was obtained from **22a** and (2-methoxyphenyl)boronic acid with 55% yield as a grey solid. Purity: 95.42%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.87 (s, 2H), 8.53 (s, 1H), 7.61−7.57 (m, 1H), 7.42 (dd, J = 7.5, 1.7 Hz, 1H), 7.31 (d, J = 8.2 Hz, 1H), 7.19 (t, J = 7.4 Hz, 1H), 3.78 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.02, 156.45, 154.97, 154.40, 147.33, 131.17, 130.98, 130.02, 119.85, 117.13, 116.95, 114.66, 113.28, 111.24, 55.05. Purity: 96.63%. HRMS for $C_{21}H_{12}O_9$: calcd, $[M + H]^+$ 409.0554, found 409.0544.

*10-(3-Methoxyphenyl)-2,8-dioxo-2,8-dihydropyrano[3,2-g]-chromene-3,7-dicarboxylic Acid (**23e**).* As described in method C, compound **23e** was obtained from **22a** and (3-methoxyphenyl)boronic acid with 59% yield as a white solid. Purity: 96.19%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 2H), 8.51 (s, 1H), 7.54 (t, J = 7.9 Hz, 1H), 7.19−7.17 (m, 1H), 7.15 (ddd, J = 5.7, 4.6, 2.5 Hz, 2H), 3.87 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.05, 158.43, 154.96, 154.08, 147.26, 130.87, 129.50, 128.76, 122.27, 117.20, 115.99, 115.84, 114.82, 113.33, 54.60. Purity: 95.26%. HRMS for $C_{21}H_{12}O_9$: calcd, $[M + H]^+$ 409.0554, found 409.0565.

*10-(4-Methoxyphenyl)-2,8-dioxo-2,8-dihydropyrano[3,2-g]-chromene-3,7-dicarboxylic Acid (**23f**).* As described in method C, compound **23f** was obtained from **22a** and (4-methoxyphenyl)boronic acid with 67% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.79 (s, 2H), 8.42 (s, 1H), 7.51−7.45 (m, 2H), 7.15−7.11 (m, 2H), 3.86 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.17, 159.92, 156.13, 155.20, 148.33, 132.47, 131.49, 121.12, 118.32, 116.89, 115.94, 114.28, 55.68. Purity: 95.83%. HRMS for $C_{21}H_{12}O_9$: calcd, $[M + H]^+$ 409.0554, found 409.0541.

*4,6-Dichloro-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylic Acid (**26**).* The compound **33** (1.0 equiv) were added into a solution of 2 M NaOH (2.5 equiv) in 10 mL water/MeOH (1:1). The reaction mixture was stirred at rt until a clear solution was obtained (1 h). The mixture was acidified to pH 4−5 with hydrochloric acid solution (2 M). The obtained precipitate was filtered off, washed with a modicum of MeOH (3 × 2 mL), and dried under vacuum at 50 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.75 (s, 1H), 8.21 (s, 2H), 3.29 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 165.77, 150.86, 144.74, 143.52, 141.59, 125.74, 121.58, 117.29, 13.68. Purity: 98.12%. HRMS for $C_{15}H_8Cl_2N_2O_4$: calcd, $[M + H]^+$ 350.9934 and 352.9904, found 350.9919 and 352.9892.

Materials and Cell Culture. Zaprinast, PQQ and compound **27** were obtained from Sigma-Aldrich. ML-145 was obtained from Tocris. Epic 384-well biosensor microplates were obtained from Corning Incorporated (Corning, NY). HT-29 and CHO-K1 cells were cultured using McCoy's 5A and Ham's F12K. The medium was supplemented with 10% FBS and 1% penicillin/streptomycin, and all cells were maintained in a 5% CO_2 incubator at 37 °C.

Transfection of hGPR35 Cell Line. CHO-K1 cells were transfected with 8 μg of pcDNA3.1-hGPR35 plasmid mixed with 24 μL of lipofectamine 2000 reagent (Invitrogen). After 24 h post-transfection, clones were selected using a complete medium containing 400 μg/mL zeocin (TransGen Biotech Co., Ltd., Beijing, China). Stable clones were selected with zeocin treatment for 3−4 weeks to obtain successfully transfected cell line CHO-K1-hGPR35. After cultured for 3−4 months, the stably transfected cell line CHO-K1-hGPR35 was obtained. The function expression of hGPR35 was detected every 2 weeks using zaprinast as the probe in the DMR assay.

DMR Assays Using an Epic BT System. All DMR assays were performed using an Epic BT system (Corning Incorporated). Epic is a swept wavelength interrogation reader system tailored for resonant waveguide grating biosensors in microtiter plates. Cells were directly seeded in Epic plates and cultured overnight to form a confluent monolayer in the cell culture medium. After being washed, the cells were maintained with Hank's balanced salt solution and further incubated inside the system for 1 h. For agonist profiling, a 2 min baseline was then established. After the compound addition, the cellular responses were recorded immediately. For desensitization assays, cells were initially treated with compounds for 1 h, followed by stimulation with zaprinast at 1 μM for HT29 or 400 nM for CHO-K1-hGPR35. The cellular responses were recorded throughout the assays. All EC_{50} or IC_{50} described in the main text were calculated based on the amplitudes of DMR signals at 8 min post-stimulation. All GPR35 agonists led to a sustained positive-DMR signal. The data represents mean ± sd from two independent measurement, each with four replicates (n = 8).

Modeling of GPR35 and Docking. Homology modeling of hGPR35 was performed using a MODELER protocol in Discovery Studio 2019 based on the X-ray structure of envelope protein US28. The PDB IDs of the template used for hGPR35 were 5WB1, 5WB2, and 4XT1. Homology sequence searching and alignment were conducted using sequence analysis and multiple sequence alignment modules, respectively. Energy minimization optimization was performed for the selected agonist molecules, which were treated as dianions here since they would be ionized at physiological pH. The optimized agonists were docked into the active site of the hGPR35 model using a CDOCKER protocol in Discovery Studio with default parameters. Ten binding modes were suggested for each agonist; then the highest scoring ones were selected for further analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01624.

Molecular formula strings (CSV)

hGPR35 (PDB)

Synthetic procedures and ^{1}H and ^{13}C NMR spectral data for compounds **9b**, **10a**, **10b**, **11a**, **11b**, **12a**, **12b**, **14**, **16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b**, **21a**, **21b**, **22a**, **22b**, **22c**, and **33** were shown in supporting information (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

hGPR35, human G protein-coupled receptor 35; GPCRG, protein-coupled receptor; SARs, structure activity relationships; DMR, dynamic mass redistribution; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; HRMS, high-resolution mass spectra

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