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Discovery of 2*H*-chromen-2-one Derivatives as G Protein-Coupled Receptor-35 Agonists

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ABSTRACT

A family of 2*H*-chromen-2-one derivatives was identified as G protein-coupled receptor-35 (GPR35) agonists using dynamic mass redistribution assays in HT-29 cells. The compounds with 1*H*-tetrazol-5-yl in 3-substituted position displayed higher potency than the corresponding carboxyl analogs, and the hydroxyl group on 7-position also played an important role in GPR35 agonistic activity. 6-Bromo-7-hydroxy-8-nitro-3-(1*H*-tetrazol-5-yl)-2*H*-chromen-2-one (**50**) was found to be the most potent GPR35 agonist with an EC_{50} of 5.8 nM. Calculating the physicochemical property of compounds with moderate to high potency suggests that compounds **30**, **50** and **51** showed good druggability. This study provides a novel series of GPR35 agonists and compound **50** may be a powerful tool to study GPR35..

INTRODUCTION

Historically, G protein-coupled receptors (GPCRs) are proven to be the largest family of druggable targets, as 30-40% of all approved small molecule drugs directly target this receptor family.[1] GPCRs still remain to be one of the most active target classes in drug discovery programs today, as these receptors play critical roles in a great number of (patho)physiological processes. Given the historical success of GPCRs as drug targets, there are strong interests in discovering lead compounds for orphan GPCRs whose natural agonists remain to be unidentified.[2] The orphan G protein-coupled receptor-35 (GPR35), first described in 1998,[3] has been implicated in a number of diseases such as coronary artery disease,[4] pain,[5, 6] cancers,[7] and hypertension.[8]

Kynurenic acid (**1**), an endogenous tryptophan metabolite, was the first GPR35 agonist described in literature.[9] However, the weak potency of this compound in the high micromolar range challenges the notion that kynurenic acid is the endogenous agonist for GPR35.[5, 10-12] Several other ligands were also postulated to be the natural agonists for GPR35, including 2-acyl lysophosphatidic acid,[13] guanosine-3′,5′-cyclic monophosphate,[14] multiple tyrosine metabolites,[13-15] and the mucosal chemokine CXCL17[16]. Although the true natural agonist for GPR35 remains to be controversial, several families of synthetic agonists have been identified including zaprinast (**2**) which has become the most widely used reference agonist for GPR35.[17, 18] Several approved drugs were also found to be GPR35 agonists; for instance, the anticoagulant dicoumarol (**6**),[3] and the antiallergic drugs cromolyn (**3**), lodoxamide (**4**), amlexanox

(**5**), and bufrolin (**7**) **(Figure 1)**.[14, 19-21] Pamoic acid (**9**), a salt component in several pharmaceutical products, was also found to be a partial GPR35 agonist.[3, 5] Recently, several new families of GPR35 agonists, such as YE210 (**8**), were identified through high throughput screening.[22] However, there are a limited number of structure activity relationship (SAR) studies reported in literature; for instance, chromen-4-one-2-carboxylic acid derivatives (**10**). [23, 24]

Here we report the synthesis and characterization of a series of coumarin (2H-chromen-2-one) derivatives as GPR35 agonists using label-free dynamic mass redistribution (DMR) assays. The detailed SARs study exhibited that 3-carboxyl or tetrazol coumarin derivatives could be novel potent GPR35 agonists.

RESULTS AND DISCUSSION

Structure Considerations

The natural products ellagic acid and dicoumarol were reported to activate the GPR35 with moderate potency.[3, 25] Several studies also suggested the importance of carboxylic acid group or a phenolic structure for the agonistic activity of a compound at the receptor.[22] Thus, we designed and synthesized a series of derivatives depending on the coumarin scaffold. By introducing an acidic group and a phenolic hydroxyl group in the molecule, we finally obtained three subclasses of 3-substituted coumarin derivatives.

Chemistry

A series of 3-substituted coumarin derivatives were synthesized. All these compounds were prepared from salicylaldehyde derivatives through Knoevenagel condensation.[26] The salicylaldehyde derivatives were reacted with bromine to obtain compounds **11a** and **11b** (1:1 ratio)**,**[27, 28] or **12a** and **12b** (1:2.5 ratio) **(Scheme 1)**.[29] The nitrosalicylaldehyde derivatives **13a** and **13b** were obtained from **12a** and **12b**, respectively, through nitration at 85^{o}C.

The 2-oxo-2*H*-chromene-3-carboxylic acid derivatives **21-30** were prepared with a good yield from 84% to 93% through the Knoevenagel condensation reaction of salicylaldehyde derivatives and Meldrum's acid **(Scheme 2)**.[26]

Scheme 3 shows the synthesis route of 3-tetrazolcoumarin compounds. First, 3-cyano-coumarins **31-40** were prepared in one-pot method as reported in literature.[30] The 3-(1H-tetrazol-5-yl)-2*H*-chromen-2-one derivatives **41, 42, 44-49** were synthesized from 3-cyano-cooumarins via the [3+2] cycloaddition of nitriles and sodium azide catalyzed by aluminium chloride at 90^{o}C with excellent yields (85~97%).[31] The compound **43** was obtained in a good yield of 94% by demethylation of **44**. To introduce nitro group into 3-tetrazolcoumarin to synthesize compounds **50** and **51**, the phenolic hydrxoy group of **35** was first reacted with choromethyl methyl ether, followed by [3+2] cycloaddition of nitriles and sodium azide to form compound **49**, which was subject to nitration to form compound **50** (1:1 with nitric acid) and **51** (1:3 with nitric acid), respectively**.** The purity of all these compounds was found to be greater than 95% by analysis of high performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (LC/ESI-MS).

Pharmacological Evaluation

DMR assays afforded by label-free resonant waveguide grating biosensors were applied to profile compound activity at the GPR35 endogenously expressed in human colorectal adenocarcinoma cell line HT-29.[32] In previous studies, zaprinast was used as a full agonist to perform DMR desensitization assay.[32] As expected, zaprinast triggered a robust DMR in HT-29 cells with an EC_{50} of 0.34±0.025 μM (n=4). Furthermore, all 3-substituted coumarin derivatives not only gave rise to a dose-dependent DMR in HT-29, but also desensitized the cells responding to 1 μM zaprinast. For all 3-substituted coumarins, the potency to trigger DMR was found to be almost equivalent that to desensitize the zaprinast response **(Table 1)**, suggesting that these compounds acted as GPR35 agonists.

DMR antagonist assay using the known GPR35 antagonist compound **11** (ML-145) **(Figure.1)** further showed that compound **11** dose-dependently and completely blocked the DMR arising from all 3-substituted coumarins, each at its respective EC_{80} to EC_{100} dose.[33] The potency of compound **11** to block the coumarin-induced DMR was mostly similar (**Table 1**), suggesting that the DMR of these cormarin compounds were specific to the activation of GPR35.

Structure-Activity Relationship analysis

Given the importance of introducing acidic group in determining the GPR35 agonistic activity,[22, 34-36] we synthesized a series of 3-substituted coumarin derivatives. These compounds can be divided into three families according to the substituent

group in R_4, namely 3-carboxylic acid-coumarins, 3-cyano-coumarins, and 3-1*H*-tetrazol-5-yl-coumarins **(Table 1)**. For the parent compounds of each family, compound **31** was inactive, while compound **41** had the highest potency (EC_{50} of 6.76 μM), and compound **21** had the relatively low potency (EC_{50} of 92.26 μM) (**Figure 2**). The tetrazole and carboxy are isosteres in drug design. Since the carboxyl group has certain stimulative activity in stomach, it is considered to be not a good druggable group. In contrast, tetrazolyl has much lower stimulative activity than carboxy acid, although it is also an acidic group because of its ionizable hydrogen ions.[37] Because of this, tetrazole has been used in antihypertensive,[38] antiallergic,[39] antiactrial[40] and antispasmodic drugs[41].

For 3-carboxyl-coumarin derivatives, consistent with a previous study[23] was that introducing halogen atoms (chlorine or bromine) in 6 or 6/8 positions of coumarins, as indicated by compounds **22, 23, 24, 26**, led to increased potency to activate the GPR35. The potency rank order was found as follows for 3-carboxyl coumarin derivatives: 6,8-dibromo (**26**, EC_{50} of 1.75 μM)＞6,8-dichloro (**24**, 2.02 μM)＞6-bromo (**23**, 10.76 μM)＞6-chloro (**22**, 13.52 μM)**.** This suggests that a lipophilic pocket may exist in GPR35 and can be fitted with halogen atom(s).

Furthermore, introducing a hydroxyl group in 7-position of coumarins also had a clear impact on their GPR35 agonistic activity, as indicated by the 10-fold increase in potency after introducing a hydroxyl group to the 7-position of compound **26** (**27**, EC_{50} of 0.15 μM). In contrast, replacing the hydroxyl group of compound **27** with the methoxyl group led to decreased potency (**28**, EC_{50} of 0.89 μM). Removal the

8-bromo of compound **27** also reduced its potency (**25**, EC_{50} of 4.34 μM). Furthermore, replacing 8-bromine of compound **26** with nitro group also decreased its potency (**29**, EC_{50} of 4.27 μM). Unexpectedly, introducing hydroxyl group in 7-position of compound **29** greatly increased its potency (**30**, EC_{50} of 0.051 μM), which was much higher than compound **27**.

For 3-cyano-coumarin derivatives, a different potency rank order was obtained for halogen-modified compounds: 6,8-dichloro (**33**, EC_{50} of 8.61 μM)＞6,8-dibromo (**34**, EC_{50} of 17.70 μM)＞6-chloro (**32**, EC_{50} of 21.43 μM). Furthermore, unlike 3-carboxyl-coumarins, replacing the bromo group with nitro group at 8-position of compound **34** significantly increased its potency by about 25-fold (**38**, EC_{50} of 0.70 μM). However, similar to 3-carboxyl-coumarin compounds, a potency increase was found for introducing hydroxyl group in 7-position of compound **34** (**36**, EC_{50} of 2.33 μM), while a potency decrease was found when the bromo group at 8-position of compound **36** was removed to yield compound **35**, or the hydroxyl group was replaced with methoxy group at 7-position to yield compound **37**.

For 3-tetrazyl coumarin derivatives, the potency rank order among compounds with varying substituents in the 6, 7, 8-postisions was: 6-dromo-8-nitro-7-hydroxy (**50**, EC_{50} of 0.0058 μM)＞6,8-dinitro-7-hydroxy (**51**, 0.014 μM)＞6,8-dibromo-7-hydroxy (**43**, 0.068 μM)＞6-dibromo-7-hydroxy (**49**, 0.71 μM) ≈ 6,8-dibromo (**42**, 0.93 μM) ≈ 6,8-dibromo-7-methoxy (**44**, 1.41 μM)＞6-dromo-8-nitro-7-methoxy (**46**, 2.66 μM)＞6-dibromo-7-methoxy (**47**, 14.76 μM). The trend in potency for 6, 7, 8 position substitutions was almost identical to those found for 3-cyano-coumarin derivatives.

β-Arrestin Translocation Induced by Selected Compounds

Next, Tango GPR35 β-arrestin assay was used to examine the agonistic activity of selected compounds (**27**, **30**, **42**, **43**, **44**, **50** and **51**) to cause *β*-arrestin translocation via GPR35. This assay uses Tango GPR35-*bla* U2OS cells to detect GPR35 agonist-induced recruitment of TEV protease tagged *β*-arrestin molecules to the GPR35-Gal4-VP16 transcription factor fusion protein linked by a TEV protease cleavage site. Results showed that all these selected compounds resulted in a dose-dependent response with a right-shifted potency, compared to DMR assay results (**Figure 4, Table 2**). Compounds **27, 30, 43, 50** and **51** all showed higher potency than zaprinast in this translocation assay. In addition, the potency rank order (in EC_{50}) was **50** (0.20μM) ＞ **51** (0.29μM) ＞ **30** (0.48μM) > **43** (1.54 μM) > **27** (3.63 μM) > **42** (7.05 μM) > **44** (14.06 μM), which is to large degree the same as the potency rank order obtained using DMR assays. These results suggested that these compounds were agonists in the *β*-arrestin translocation assay.

Receptor Selecivity

To assess the selectivity of these new GPR35 agonists versus other GPCRs, we tested the representative compound **50** using DMR assays on other cell lines. The results showed that **50** did not display activity on histamine H_1 receptor, GPR109a, β_2-adrenergic receptor, angiotensin AT_1 receptor and bradykinin B_2 receptor (Figure S1 and Figure S2). Therefore, **50** had selectivity agonistic activity on GPR35.

Physicochemical Properties of Selected Compounds

Physicochemical parameters have been proposed to predict drug-like properties including the partition coefficient (clogP), the ligand efficiency (LE) and the ligand-lipophilicity efficiency (LLE).[42-44] We calculated these parameters for compounds with relatively high potency, including compounds **26, 27, 28, 30, 36, 38, 42, 43, 44, 45, 50** and **51 (Table 3)**. Lipinski's rule of five considered that the clogP value should be lower than 5 if a compound is druggable.[45, 46] The clogP values of all these selected compounds were within this range. For perorally administered drugs, the clogP value is often between 2 and 3. Most of these selected compounds exhibited a clogP value between 2 and 3 or closed to this range.

The LE is calculated as follows: LE = pEC_{50}/N (N= non-hydrogen atoms). This parameter represents the binding force between per atom and receptors. For all of these selected compounds except compound **44**, a LE value is lower than 0.3. Another useful parameter LLE which combines the potency and lipophilicity is also calculated (LLE = pEC_{50}-clogP). Among these compounds, **30, 50** and **51** showed a good ligand lipophilicity efficiency with a LLE value of 5.009, 6.495 and 6.935, respectively. For the suitable drug candidates, LE＞0.3 and LLE＞5 could be optimal. Combining all of these parameters, the highly potent compounds **30, 50** and **51** showed very good clogP value, and suitable LE and LLE values.

Conclusion

In this article, a series of coumarin derivatives were synthesized based on the

structure characteristics of GPR35 agonists reported previously. SAR analysis showed that 6-bromo substituent, 8-nitro substituent, 7-hydroxyl substituent and 3-tetrazyl substituent were found to significantly increase the potency of coumarin compounds to activate the GPR35. Combining these substitutions resulted in compound **50** with the highest potency found within this class of compounds (EC_{50}, 5.8nM). Compound **50** also exhibited good physicochemical properties. Together, this study provides a probe ligand to elucidate the biology and functions of GPR35.

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 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 spectra (HRMS) 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 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 10 min and maintained 5/95 for another 10min. The flow rate was 200μL/min. Peaks were detected at 290nm or 254nm.

Synthesis of compounds 11a,11b, 12a, 12b, 13a and 13b

Compounds **11a, 11b, 12a** and **12b** were synthesized as described in literature.[47] The filtrate was purified with column chromatography (8:2 DCM/PE).

5-Bromo-2-hydroxy-4-hydroxy-3-nitrobenzaldehyde (13a) and 5-bromo-2-hydroxy-4-methoxy-3-nitrobenzaldehyde (13b). To synthesize **13a** and **13b**, nitric acid (65%) (1.0 equiv) was added dropwise to a solution of 5-bromo-2,4-dihydroxybenzaldehyde or 5-bromo-2-hydroxy-4-methoxybenzaldehyde (1.0 equiv), respectively, in acetic acid at 85 °C for 3h. Catalytic amount of sulfuric was added. The resulting mixture was poured into ice water. The precipitated product was filtered, washed with water, and dried under vacuum. The crude solids were recrystallized from methyl alcohol. The products were filtered off, and dried under vacuum at 50 °C. The NMR data of all these benzaldehyde derivatives was shown in support information.

General procedures of method A for the synthesis of compounds 21-30

The appropriate salicylaldehyde derivatives (1.0 equiv) and Meldrum's acid (1.5 equiv) were mixed in 20-40 mL of water at room temperature. Catalytic amount of ammonium acetate were added, and the reaction mixture was stirred under the room temperature for 2-3h. Hydrochloric acid solution (2M) was added to adjust pH to 4-5. The resulted solid was filtered, washed three times with 5 ml of methanol, and dried under vacuum at 50 ^{o}C.

General procedure of method B for the synthesis of compounds 31-40

The appropriate salicylaldehyde derivatives (1.0 equiv) and malononitrile (1.5 equiv) were mixed in 20-40 mL of water at room temperature. Catalytic amount of ammonium acetate were added, and the reaction mixture was stirred under the room temperature for 2-3h. The resulted solid was filtered and washed three times with 10 mL of water. Next, the products were added in 20 mL 3M hydrochloric acid solution, and the reaction mixture was stirred at 75 ^{o}C for 30 min. After reaction, the material was cooled to room temperature. The resulted solid was filtered, and washed three times with 5 mL of methanol. If necessary, the filtrate was separated by column chromatography (40:1 DCM/MeOH) to yield the pure compound and dried under vacuum at 50 ^{o}C.

General procedure of method C for the synthesis of compounds 41-47

Aluminum chloride (3.0 equiv) was added to 20 mL of anhydrous tetrahydrofuran. Subsequently, sodium azide (6.0 equiv) and 3-cyano coumarin derivatives (1.0 equiv) were added in order. The mixure was refluxed for 5 hours with agitation under an argon atmosphere. After the completion of the reaction, the reaction mixture was extracted three times with excessive diluted hydrochloric acid to remove Al^{3+}. The organic phase was dried with anhydrous Na_2SO_4 and was concentrated under reduced pressure until the product started to crystallize and cooled in ice-bath to complete the crystallization. The product was filtered off, washed with modicum methyl alcohol (MeOH), and dried under vacuum at 50 ^{o}C.

2-oxo-2*H*-chromene-3-carboxylic acid (21). **21** was obtained from 2-hydroxybenzaldehyde and Meldrum's acid as described for method A. 86% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.27 (s, 1H), 8.76 (s, 1H), 7.92 (d, J = 7.5 Hz, 1H), 7.74 (t, J = 7.5 Hz, 1H), 7.56 – 7.32 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.43, 157.16, 154.92, 148.81, 134.72, 130.64, 125.26, 118.80, 118.44, 116.57. Purity: 99.30%. High resolution MS (HRMS) for $C_{10}H_6O_4$: calcd, 190.0281; found, 189.0208 $[M-H]^-$, 145.0317 $[M-COOH]^-$.

6-chloro-2-oxo-2*H*-chromene-3-carboxylic acid (22). **22** was obtained from 6-chloro-2-hydroxybenzaldehyde and Meldrum's acid as described for method A. 91% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) ^{1}H NMR (600 MHz, DMSO) δ 13.39 (s, 1H), 8.70 (s, 1H), 8.04 (s, 1H), 7.76 (d, J = 8.6 Hz, 1H), 7.48 (d, J = 8.6 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.22, 156.58, 153.59, 147.47, 134.09,

129.46, 128.89, 120.06, 119.84, 118.65. Purity: 98.64%. HRMS for $C_{10}H_5ClO_4$: calcd, 223.9882; found, 222.9809 $[M-H]^-$, 224.9775 $[M-H+2]^-$, 178.9915 $[M-COOH]^-$, 180.9876 $[M-COOH+2]^-$.

6-bromo-2-oxo-2*H*-chromene-3-carboxylic acid (23). 23 was obtained from 6-bromo-2-hydroxybenzaldehyde and Meldrum's acid as described for method A. 84% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.66 (s, 1H), 8.16 (d, J = 2.4 Hz, 1H), 7.86 (dd, J = 8.8, 2.4 Hz, 1H), 7.41 (d, J = 8.8 Hz, 1H).^{13}C NMR (151 MHz, DMSO-d_6) δ 164.29, 156.61, 153.93, 146.93, 136.70, 132.38, 120.41, 118.87, 116.66. Purity: 99.01%. HRMS for $C_{10}H_5BrO_4$: calcd, 267.9379; found, 266.9306 $[M-H]^-$, 268.9286 $[M-H+2]^-$, 222.9408 $[M-COOH]^-$, 224.9388 $[M-COOH+2]^-$.

6,8-dichloro-2-oxo-2*H*-chromene-3-carboxylic acid (24). 24 was obtained from 6,8-dichloro-2-hydroxybenzaldehyde and Meldrum's acid as described for method A. 86% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.71 (s, 1H), 8.07 – 8.02 (m, 2H). ^{13}C NMR (151MHz, DMSO-d_6): 163.91, 155.56, 149.43, 147.29, 133.36, 128.73, 128.58, 121.20, 120.92, 120.69. Purity: 100%. HRMS for $C_{10}H_4Cl_2O_4$: calcd, 257.9497; found, 256.9425 $[M-H]^-$, 258.9392 $[M-H+2]^-$, 212.9542 $[M-COOH]^-$, 214.9502 $[M-COOH+2]^-$.

6-bromo-7-hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid (25). 25 was obtained from **11a** and Meldrum's acid as described for method A. 84% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.02 (s, 1H), 11.92 (s, 1H), 8.65 (d, J = 3.6 Hz, 1H), 8.14 (d, J = 8.2 Hz, 1H), 6.88 (d, J = 3.6 Hz, 1H).^{13}C NMR (151 MHz, DMSO-d_6) δ 164.48, 160.04, 157.28, 156.16, 148.71, 134.37, 114.36, 112.34, 107.11,

102.94. Purity: 99.46%. HRMS for $C_{10}H_5BrO_5$: calcd, 283.9320; found, 282.9347 $[M-H]^-$, 284.9327 $[M-H+2]^-$.

6,8-dibromo-2-oxo-2*H*-chromene-3-carboxylic acid (26). 26 was obtained from 6,8-dibromo-2-hydroxybenzaldehyde and Meldrum's acid as described for method A. 89% yield as a white solid; ^{1}H NMR(600 MHz, DMSO-d_6) δ 13.52(s, 1H), 8.65(s, 1H), 8.36 – 8.08(m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.93, 155.79, 150.87, 147.01, 138.59, 132.08, 121.40, 120.90, 116.67, 110.50. Purity: 99.17%. HRMS for $C_{10}H_4Br_2O_4$: calcd, 345.8501; found, 344.8419 $[M-H]^-$, 346.8411 $[M-H+2]^-$, 348.8380 $[M-H+4]^-$.

6,8-dibromo-7-hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid (27). 27 was obtained from **12a** and Meldrum's acid as described for method A. 93% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.05 (s, 1H), 8.66 (s, 1H), 8.19 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.23, 157.00, 156.64, 153.09, 148.52, 132.89, 114.91, 113.18, 108.19, 99.13. Purity: 96.25%. HRMS for $C_{10}H_4Br_2O_5$: calcd, 361.8460; found, 360.8374 $[M-H]^-$, 362.8373 $[M-H+2]^-$, 364.8335 $[M-H+4]^-$.

6,8-dibromo-7-methoxy-2-oxo-2*H*-chromene-3-carboxylic acid (28). 28 was obtained from **12b** and Meldrum's acid as described for method A: 87% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.69 (s, 1H), 8.30 (s, 1H), 3.91 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.93, 158.12, 155.84, 152.56, 147.56, 133.15, 118.57, 117.51, 112.93, 106.25, 61.51. Purity: 99.20%. HRMS for $C_{11}H_6Br_2O_5$: calcd, 375.8582; found, 374.8516 $[M-H]^-$, 376.8502 $[M-H+2]^-$, 378.8477 $[M-H+4]^-$, 330.8630$[M-COOH]^-$, 332.8629 $[M-COOH+2]^-$, 334.8590 $[M-COOH+4]^-$.

6-bromo-8-nitro-2-oxo-2*H*-chromene-3-carboxylic acid (29). 29 was obtained from 5-bromo-2-hydroxy-3-nitrobenzaldehyde and Meldrum's acid as described for method A. 89% yield as a wihite solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.65 (s, 1H), 8.75 (s, 1H), 8.56 (d, J = 2.3 Hz, 1H), 8.51 (d, J = 2.3 Hz, 1H). ^{13}C NMR (151MHz, DMSO-d_6): 163.63, 154.50, 146.81, 146.46, 137.64, 137.45, 131.36, 122.32, 120.91, 115.28. Purity: 100%. HRMS for $C_{10}H_4BrNO_6$: calcd, 312.9234; found, 311.9161 $[M-H]^-$, 313.9142 $[M-H+2]^-$, 267.9342$[M-COOH]^-$, 269.9321 $[M-COOH+2]^-$.

6-bromo-7-hydroxy-8-nitro-2-oxo-2*H*-chromene-3-carboxylic acid (30). 30 was obtained from **13a** and Meldrum's acid as described for method A. 84% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.59 (s, 1H), 8.15 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.85, 164.55, 158.06, 156.91, 149.01, 148.94, 134.24, 129.89, 113.01, 108.78, 107.37. Purity: 99.51%. HRMS for $C_{10}H_4BrNO_7$: calcd, 328.9171; found, 327.9241 $[M-H]^-$, 329.9222 $[M-H+2]^-$.

2-oxo-2*H*-chromene-3-carbonitrile (31). 31 was obtained from 2-hydroxybenzaldehyde and malononitrile as described for method B. 58% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.95 (s, 1H), 7.84 – 7.78 (m, 2H), 7.53 – 7.45 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 157.35, 154.53, 153.93, 135.92, 130.45, 125.95, 117.98, 117.27, 115.07, 102.63. Purity: 98.69%. HRMS for $C_{10}H_5NO_2$: calcd, 171.0320; found, 172.0393 $[M+H]^+$.

6-chloro-2-oxo-2*H*-chromene-3-carbonitrile (32). 32 was obtained from 6-chloro2-hydroxybenzaldehyde and malononitrile as described for method B. 64% yield as a light yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 1H), 7.92 (d, J

= 2.6 Hz, 1H), 7.84 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.57 (d, *J* = 8.9 Hz, 1H). ^{13}C NMR (151MHz, DMSO-d_6): 156.92, 153.20, 152.56, 135.20, 129.56, 129.17, 119.33, 119.27, 114.80, 104.03. Purity: 98.99%. HRMS for $C_{10}H_4ClNO_2$: calcd, 204.9931; found, 238.0272 $[M+CH_3OH+H]^+$, 240.0239 $[M+CH_3OH+H+2]^+$.

6,8-dichloro-2-oxo-2*H*-chromene-3-carbonitrile (33). 33 was obtained from 6,8-dichloro-2-hydroxybenzaldehyde and malononitrile as described for method B. 71% yield as a light yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.87 (s, 1H), 8.17 (d, *J* = 2.4 Hz, 1H), 7.90 (d, *J* = 2.4 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 156.13, 152.20, 149.02, 134.52, 129.48, 128.30, 121.89, 120.34, 114.47, 104.94. Purity: 96.28%. HRMS for $C_{10}H_3Cl_2NO_2$: calcd, 238.9541; found, 271.9897 $[M+CH_3OH+H]^+$, 273.9858 $[M+CH_3OH+H+2]^+$.

6,8-dibromo-2-oxo-2*H*-chromene-3-carbonitrile (34). 34 was obtained from 6,8-dibromo-2-hydroxybenzaldehyde and malononitrile as described for method B. 65% yield as a light yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 1H), 7.92 (d, *J* = 2.6 Hz, 1H), 7.84 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.57 (d, *J* = 8.9 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 156.32, 152.17, 150.51, 139.84, 131.80, 120.77, 117.40, 114.48, 111.22, 104.79. Purity: 96.42%. HRMS for $C_{10}H_3Br_2NO_2$: calcd, 326.8531; found, 359.8871 $[M+CH_3OH+H]^+$, 361.8861 $[M+CH_3OH+H+2]^+$, 363.8831$[M+CH_3OH+H+4]^+$.

6-bromo-7-hydroxy-2-oxo-2*H*-chromene-3-carbonitrile (35). 35 was obtained from 6-bromo-2-hydroxybenzaldehyde and malononitrile as described for method B. 58% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.67 (s, 1H), 7.97 (s, 1H),

6.87 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.34, 157.60, 155.83, 152.58, 133.98, 115.43, 111.69, 108.11, 103.50, 97.56. Purity: 98.64%. HRMS for $C_{10}H_4BrNO_3$: calcd, 264.9393; found, 263.9320 $[M-H]^-$, 265.9300 $[M-H+2]^-$.

6,8-dibromo-7-hydroxy-2-oxo-2*H*-chromene-3-carbonitrile (36). 36 was obtained from **12a** and malononitrile as described for method B. 52% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.68 (s, 1H), 8.03 (s, 1H). (151 MHz, DMSO-d_6) δ 158.81, 157.37, 152.96, 152.21, 132.45, 115.36, 112.26, 109.35, 99.82, 97.53. Purity: 95.19%. HRMS for $C_{10}H_3Br_2NO_3$: calcd, 342.8511; found, 341.8433 $[M-H]^-$, 343.8420 $[M-H+2]^-$, 345.8393 $[M-H+4]^-$.

6,8-dibromo-7-methoxy-2-oxo-2*H*-chromene-3-carbonitrile (37). 37 was obtained from **12b** and malononitrile as described for method B: 69% yield as a light yellow solid; ^{1}H NMR (600MHz, DMSO-d_6): δ 8.83(s, 1H), 8.19(d, 1H), 3.93(s, 3H). ^{13}C NMR (151MHz, DMSO-d_6): 159.02, 156.30, 152.19, 152.11, 132.88, 117.08, 114.61, 113.67, 107.05, 102.83, 61.70. Purity: 98.81%. HRMS for $C_{11}H_5Br_2NO_2$: calcd, 356.8636; found, 389.8973 $[M+CH_3OH+H]^+$, 391.8958 $[M+CH_3OH+H+2]^+$, 393.8935 $[M+CH_3OH+H+4]^+$.

6-bromo-8-nitro-2-oxo-2*H*-chromene-3-carbonitrile (38). 38 was obtained from 5-bromo-2-hydroxy-3-nitrobenzaldehyde and malononitrile as described for method B. 54% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.04 (s, 1H), 8.84 (d, J = 2.5 Hz, 1H), 8.77 (d, J = 2.3 Hz, 1H).^{13}C NMR (151 MHz, DMSO-d_6) δ 155.93, 154.93, 152.30, 144.41, 132.30, 125.16, 119.23, 114.27, 110.86, 105.64. Purity: 97.94%. HRMS for $C_{10}H_3BrN_2O_4$: calcd, 293.9276; found, 326.9613

$[M+CH_3OH+H]^+$, 328.9593 $[M+CH_3OH+H+2]^+$.

6-bromo-7-methoxy-8-nitro-2-oxo-2*H*-chromene-3-carbonitrile (39). 39 was obtained from **13b** and malononitrile as described for method B. 58% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.40 (s, 1H), 4.04 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 155.14, 152.86, 151.49, 145.82, 135.94, 134.02, 116.62, 114.36, 112.75, 103.73, 63.97. Purity: 95.76%. HRMS for $C_{11}H_5BrN_2O_5$: calcd, 323.9382; found, 356.9723 $[M+CH_3OH+H]^+$, 358.9704 $[M+CH_3OH+H+2]^+$.

3-(1H-tetrazol-5-yl)-2*H*-chromen-2-one (41). 41 was obtained from **31** as described for method C. 87% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.09 (s, 1H), 8.06 (dd, J = 7.7, 1.0 Hz, 1H), 7.83 – 7.78 (m, 1H), 7.58 (d, J = 8.3 Hz, 1H), 7.54 – 7.48 (m, 1H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 158.56, 154.11, 150.10, 144.82, 134.41, 130.47, 125.70, 118.90, 116.85, 112.85. Purity: 100%. HRMS for $C_{10}H_6N_4O_2$: calcd, 214.0497; found, 213.0424 $[M-H]^-$.

6,8-dibromo-3-(1H-tetrazol-5-yl)-2*H*-chromen-2-one (42). **42** was obtained from **34** as described for method C. 89% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.86 (s, 1H), 7.92 (d, J = 2.6 Hz, 1H), 7.84 (dd, J = 8.9, 2.6 Hz, 1H), 7.57 (d, J = 8.9 Hz, 1H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 157.49, 150.89, 149.90, 142.20, 138.00, 131.72, 121.94, 117.15, 116.03, 110.68. Purity: 99.44%. HRMS for $C_{10}H_4Br_2N_4O_2$: calcd, 369.8726; found, 368.8651 $[M-H]^-$, 370.8634 $[M-H+2]^-$, 372.8612 $[M-H+4]^-$.

Preparation of 6,8-dibromo-7-hydroxy-3-(1H-tetrazol-5-yl)-2H-chromen-2-one

(43).The compound **44 (1.0 equiv)** was dissolved in 10mL DCM, cooled to -78 ^{o}C; BBr_3(3.0 equiv) was added dropwise. The reaction mixture was stirred in room temperature overnight. After the reaction, 25mL of water was added, and the layers were separated. And then extracted with 2 × 20 mL DCM. The combined organic layers were dried over Na_2SO_4 and was concentrated under reduced pressure. The product was washed with modicum MeOH, and dried in vacuum at 50 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.75 (s, 1H), 8.10 (s, 1H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.60, 158.80, 153.34, 150.52, 143.84, 131.99, 111.71, 110.12, 103.43, 99.43. Purity: 100%. HRMS for $C_{10}H_4Br_2N_4O_3$: calcd, 385.8679; found, 384.8603 $[M-H]^-$, 386.8587 $[M-H+2]^-$, 388.8564 $[M-H+4]^-$.

6,8-dibromo-7-methoxy-3-(1H-tetrazol-5-yl)-2*H*-chromen-2-one (44). **44** was obtained from **37** as described for method C. 96% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.92 (s, 1H), 8.37 (s, 1H), 3.92 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 157.64, 157.62, 151.49, 150.45, 142.58, 132.76, 118.05, 113.74, 113.40, 106.52, 61.56. Purity: 98.94%. HRMS for $C_{11}H_6Br_2N_4O_3$: calcd, 399.8834; found, 398.8760 $[M-H]^-$, 400.8741 $[M-H+2]^-$, 402.8720 $[M-H+4]^-$.

6-bromo-8-nitro-3-(1H-tetrazol-5-yl)-2*H*-chromen-2-one (45). 45 was obtained from **38** as described for method C. 91% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.06 (s, 1H), 8.65 – 8.59 (m, 2H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 156.42, 145.43, 142.38, 137.73, 137.35, 131.01, 122.68, 115.92, 115.53. Purity: 96.18%. HRMS for $C_{10}H_4BrN_5O_4$: calcd, 336.9467; found, 335.9393 $[M-H]^-$, 337.9375 $[M-H+2]^-$.

6-bromo-7-methoxy-8-nitro-3-(1H-tetrazol-5-yl)-2H-chromen-2-one (46). **46** was obtained from **39** as described for method C. 89% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.02 (s, 1H), 8.63 (s, 1H), 4.04 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 156.32, 151.74, 149.89, 145.22, 142.27, 135.95, 134.04, 117.77, 114.06, 112.58, 63.93. Purity: 97.62%. HRMS for $C_{11}H_6BrN_5O_5$: calcd, 366.9585; found, 365.9513 $[M-H]^-$, 367.9493 $[M-H+2]^-$.

6-bromo-7-methoxy-3-(1H-tetrazol-5-yl)-2*H*-chromen-2-one (47). 47 was obtained from **40** as described for method C. 87% yield as a white solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.31 (s, 1H), 7.36 (s, 1H), 4.01 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.11, 158.51, 155.53, 150.05, 143.87, 133.51, 113.52, 109.95, 107.97, 101.22, 57.94. Purity: 97.97%. HRMS for $C_{11}H_7BrN_4O_3$: calcd, 321.9723; found, 320.9650 $[M-H]^-$, 322.9630 $[M-H+2]^-$.

6-bromo-7-hydroxy-3-(1H-tetrazol-5-yl)-2H-chromen-2-one (49). 49 was obtained from **48** as described for method C. 93% yield as a yellow solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.87 (s, 1H), 8.19 (s, 1H), 7.00 (s, 1H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 159.69, 158.57, 155.12, 150.15, 144.14, 133.99, 112.69, 108.78, 107.56, 103.25. Purity: 100%. HRMS for $C_{10}H_5BrN_4O_3$: calcd, 307.9576; found, 306.9503 $[M-H]^-$, 308.9484 $[M-H+2]^-$.

6-bromo-7-hydroxy-8-nitro-3-(1H-tetrazol-5-yl)-2H-chromen-2- one (50). Nitric acid (65%) (1.0 equiv) was added dropwise to a solution of **49** (1.0 equiv) in acetic acid at 85 ^{o}C for 1h. Catalytic amount of sulfuric was added. The resulting mixture was concentrated under reduced pressure to give yellow solid. Then, the solid was

collected by filtration and purified by recrystallization from petroleum ether/ethyl acetate (1:2) to give title compounds as yellow solid and dried in vacuum at 50 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.85 (s, 1H), 8.49 (s, 1H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.20, 157.62, 150.38, 149.95, 146.00, 139.60, 133.58, 129.61, 102.38, 100.73. Purity: 97.99%. HRMS for $C_{10}H_4BrN_5O_5$: calcd, 352.9422; found, 351.9349 $[M-H]^-$, 353.9330 $[M-H+2]^-$.

7-hydroxy-6,8-dinitro-3-(1H-tetrazol-5-yl)-2H-chromen-2-one (51). Nitric acid (65%) (1.0 equiv) was added dropwise to a solution of **50** (1.0 equiv) in acetic acid at 85 °C for 1h. Catalytic amount of sulfuric was added. The resulting mixture was concentrated under reduced pressure to give yellow solid. Then, the solid was washed with modicum petroleum ether to give the compound as yellow solid and dried in vacuum at 50 °C. ^{1}H NMR (600 MHz, DMSO) δ 9.02 (s, 1H), 8.75 (s, 1H); ^{13}C NMR (151 MHz, DMSO) δ 157.79, 155.49, 155.36, 149.95, 144.16, 135.73, 127.04, 110.91, 110.06, 101.82. Purity: 98.99%. HRMS for $C_{10}H_4N_6O_7$: calcd, 320.0141; found, 319.0107 $[M-H]^-$.

Materials and Cell Culture

Zaprinast was obtained from Sigma-aldrich. ML-145 was obtained from Tocris. Epic® 384-well biosensor microplates were obtained from Corning Incorporated (Corning, NY). HT-29 cells were cultured at 37 °C, 5% CO_2 in McCoy's 5A Medium modified with 10% FBS, 50 μg/mL penicillin and 100 μg/mL streptomycin.

DMR Assays Using Epic BT System

All DMR assays were performed using Epic BT system (Corning Incorporated). Epic

is a swept wavelength interrogation reader system tailored for resonant waveguide grating biosensors in microtiter plates[48]. 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 1μM. 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).

Tango *β*-Arrestin Translocation Gene Reporter Assays

Tango GPR35-bla U2OS cells was used. This cell line stably expresses two fusion proteins: human GPR35 linked to a TEV protease site and a Gal4-VP16 transcription factor and *β*-arrestin/TEV protease fusion protein. The cell line also stably expresses the *β*-lactamase reporter gene under the control of a UAS response element. The activation of GPR35 by agonists leads to the recruitment of *β*-arrestin/TEV protease fusion proteins to the activated GPR35. As a result, the protease cleaves the Gal4-VP16 transcription factor from the receptor, which then translocates to the nucleus and activates the expression of *β*-lactamase. Briefly, 10000 cells per well

were seeded in 384-well, black-wall, clear bottom assay plates with low fluorescence background (Corning) and cultured in Dulbecco's Modified Eagle Medium (Invitrogen, 10569_010) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM nonessential amino acids, 25 μM Hepes (pH 7.3), 100 μg/mL penicillin, and 100 μg/mL streptomycin. After overnight culture, the cells were stimulated with ligands for 5 h in a humidified 37 ^{o}C/5% CO_2 and then loaded with the cell permeable LiveBLAzer FRET B/G substrate. After the 2 h incubation, the coumarin:fluorescein ratio was measured using Tecan Safire II microplate reader (Männedorf, Switzerland). In the absence of *β*-lactamase expression (i.e., no GPR35 activation), cells generated green fluorescence. In the presence of *β*-lactamase expression upon receptor activation, the substrate was cleaved and the cells generated blue fluorescence. The coumarin: fluorescein ratio was used as a normalized reporter response.

ASSOCIATED COTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthetic procedures and ^{1}H and ^{13}C NMR spectral data for compounds **11a**, **11b**, **12a**, **12b**, **13a** and **13b** were shown in supporting information.

Molecular formula strings

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ABBREVIATIONS USED

GPR35, G protein-coupled receptor 35; GPCR, G protein-coupled receptor; SARs, structure activity relationships; DMR, dynamic mass redistribution; HPLC, high performance liquid chromatography; THF, tetrahydrofuran; LE, ligand efficiency; LLE, ligand-lipophilicity efficiency; HRMS, high-resolution mass spectra.

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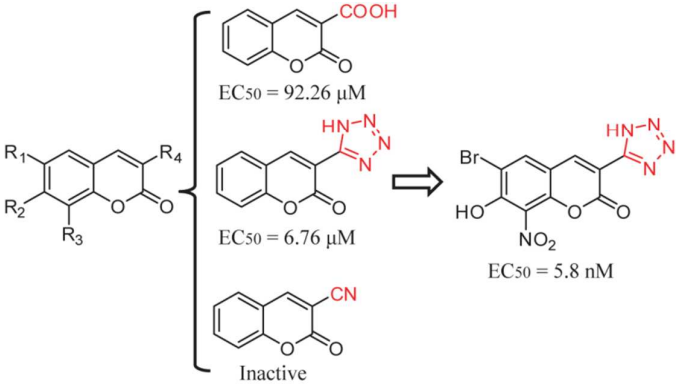


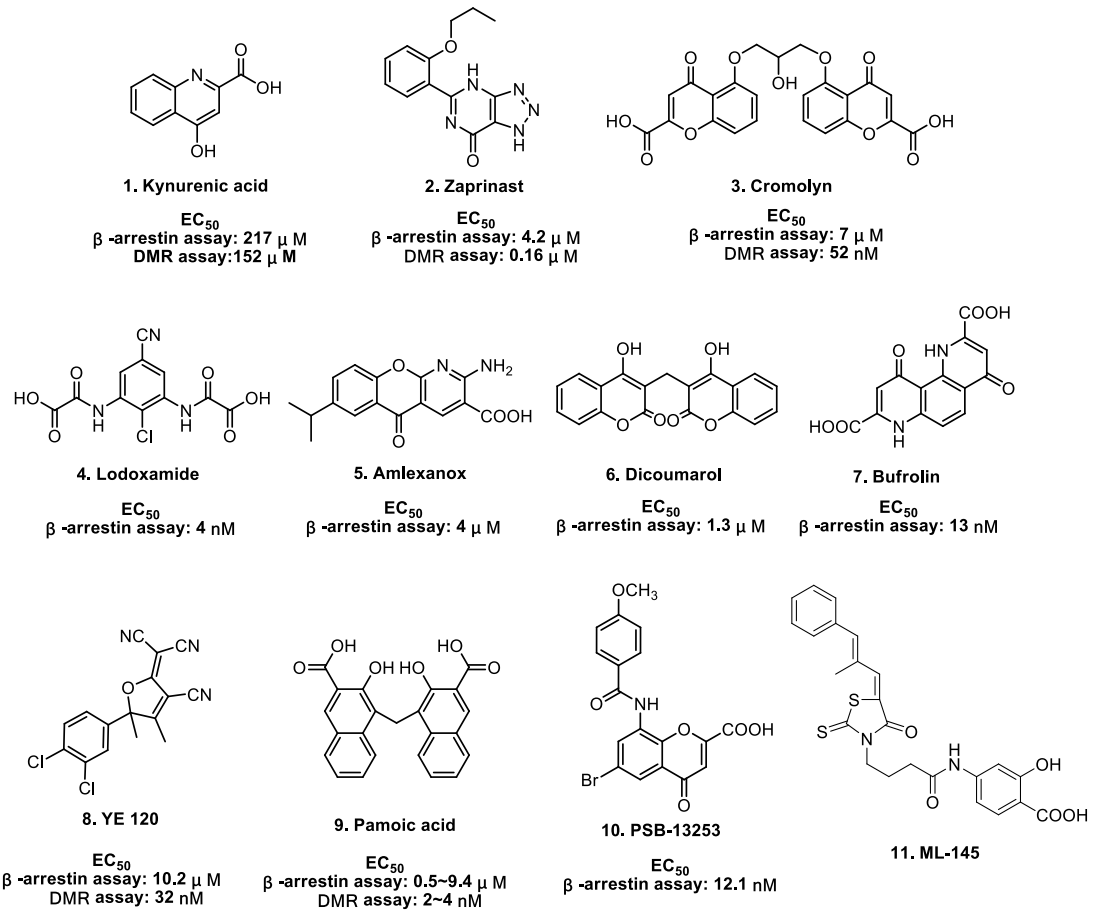
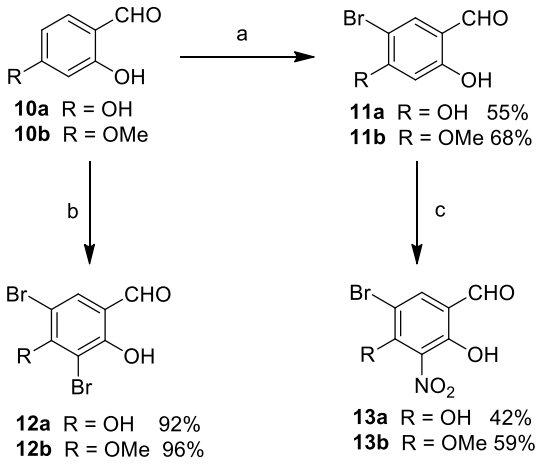


Figure.1 Selected GPR35 agonists with potencies at GPR35 (**1**~**10**). Selected GPR35 antagonist (**11**).

Scheme 1. Synthesis of Salicylaldehyde Dervaives[a]



10a R = OH
10b R = OMe

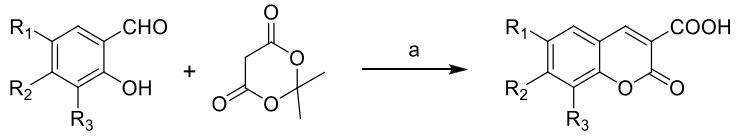
11a R = OH 55%
11b R = OMe 68%

12a R = OH 92%
12b R = OMe 96%

13a R = OH 42%
13b R = OMe 59%

[a]Reagents and conditions: (a) Br_2, AcOH, 0℃, 3h; (b) Br_2, AcOH ,rt , 3h; (c) HNO_3 (65%), H_2SO_4, AcOH, 3h, 85℃.

Scheme 2. Synthesis of 2-oxo-2*H*-chromene-3-carboxylic acid Derivatives[a]



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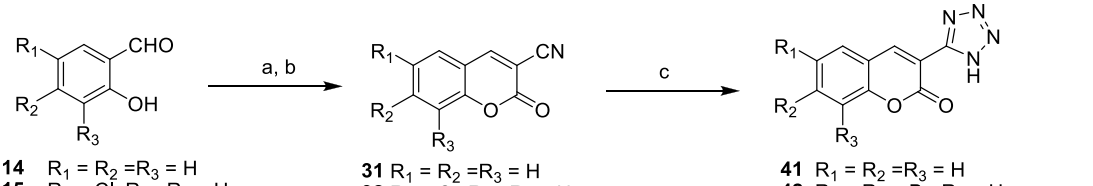
15 R_1 = Cl, $R_2 = R_3 = H$
16 R_1 = Br, $R_2 = R_3 = H$
17 $R_1 = R_3$ = Cl, $R_2 = H$
18 $R_1 = R_3$ = Br, $R_2 = H$
11a R_1 = Br, R_2 = OH, $R_3 = H$
12a $R_1 = R_3$ = Br, R_2 = OH
12b $R_1 = R_3$ = Br, R_2 = OMe
19 R_1 = Br, $R_2 = H$, $R_3 = NO_2$
13a R_1 = Br, R_2 = OH, $R_3 = NO_2$

21 $R_1 = R_2 = R_3 = H$
22 R_1 = Cl, $R_2 = R_3 = H$
23 R_1 = Br, $R_2 = R_3 = H$
24 $R_1 = R_3$ = Cl, $R_2 = H$
25 $R_1 = R_3$ = Br, $R_2 = H$
26 R_1 = Br, R_2 = OH, $R_3 = H$
27 $R_1 = R_3$ = Br, R_2 = OH
28 $R_1 = R_3$ = Br, R_2 = OMe
29 R_1 = Br, $R_2 = H$, $R_3 = NO_2$
30 R_1 = Br, R_2 = OH, $R_3 = NO_2$

[a]Reagents and conditions: (a) CH_3COONH_4, H_2O, rt, 2h, yield 84~93%.

Scheme 3. Synthesis of 2-oxo-2*H*-chromene-3-carbonitrile and 3-(1H-tetrazol-5-yl)-2*H*-chromen-2-one Derivatives[a]



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18 $R_1 = R_3$ = Br, $R_2 = H$
11a R_1 = Br, R_2 = OH, $R_3 = H$
12a $R_1 = R_3$ = Br, R_2 = OH
12b $R_1 = R_3$ = Br, R_2 = OMe
19 R_1 = Br, $R_2 = H$, $R_3 = NO_2$
13b R_1 = Br, R_2 = OMe, $R_3 = NO_2$
11b R_1 = Br, R_2 = OMe, $R_3 = H$

31 $R_1 = R_2 = R_3 = H$
32 R_1 = Cl, $R_2 = R_3 = H$
33 $R_1 = R_3$ = Cl, $R_2 = H$
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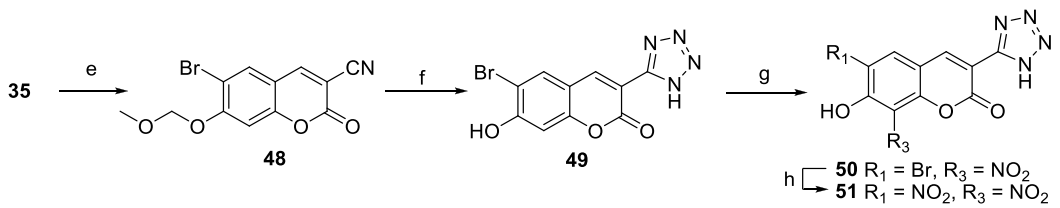
36 $R_1 = R_3$ = Br, R_2 = OH
37 $R_1 = R_3$ = Br, R_2 = OMe
38 R_1 = Br, $R_2 = H$, $R_3 = NO_2$
39 R_1 = Br, R_2 = OMe, $R_3 = NO_2$
40 R_1 = Br, R_2 = OMe, $R_3 = H$

41 $R_1 = R_2 = R_3 = H$
42 $R_1 = R_3$ = Br, $R_2 = H$
43 $R_1 = R_3$ = Br, R_2 = OH
d → **44** $R_1 = R_3$ = Br, R_2 = OMe
45 R_1 = Br, $R_2 = H$, $R_3 = NO_2$
46 R_1 = Br, R_2 = OMe, $R_3 = NO_2$
47 R_1 = Br, R_2 = OMe, $R_3 = H$

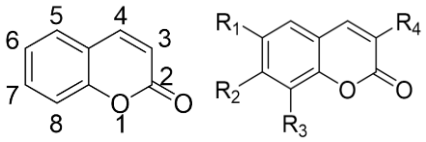


48 **49**

h → **50** R_1 = Br, $R_3 = NO_2$
51 $R_1 = NO_2$, $R_3 = NO_2$

[a]Reagents and conditions: (a,b) CH_3COONH_4, H_2O, rt, 2h, yield 66~81%; HCl(2N), H_2O, 75℃, 30min, yield 86~94%; (c) NaN_3, $AlCl_3$, THF, 90℃, 5h, yield 85~97%.; (d) BBr_3, CH_2Cl_2, -78℃ ~rt, overnight, yield 94%;(e) MOMCl, Et_3N, CH_2Cl_2, rt, 2h; (f) NaN_3, $AlCl_3$, THF, 90℃, 5h, yield 93%; (g) HNO_3 (65%), H_2SO_4, AcOH, 1h, 85℃, yield 76%; (h) HNO_3 (65%), H_2SO_4, AcOH, 1h, 85℃, yield 92%

Table 1. The Potency of Compounds in DMR Assays.(EC_{50} to Trigger DMR, IC_{50} to Desensitize Cells upon Cells Repeated Stimulation 1μM Zaprinast, IC_{50} of Known GPR35 Antagonist **11** to Block the Agonist-induced DMR)



compd	R_1	R_2	R_3	R_4	EC_{50}(μM)	Desensitization IC_{50}(μM)	Antagonist IC_{50}(μM)
21	H	H	H	COOH	92.26±6.22	100.0±18.6	0.040±0.047
22	Cl	H	H	COOH	13.52±1.09	13.24±0.72	0.13±0.10
23	Br	H	H	COOH	10.76±0.23	11.30±0.65	0.26±0.09
24	Cl	H	Cl	COOH	2.02±0.07	2.30±0.18	0.39±0.20
25	Br	OH	H	COOH	4.34±0.36	3.76±0.41	0.13±0.04
26	Br	H	Br	COOH	1.75±0.15	1.08±0.07	0.51±0.06
27	Br	OH	Br	COOH	0.15±0.02	0.056±0.002	0.79±0.08
28	Br	OMe	Br	COOH	0.89±0.04	0.75±0.04	0.61±0.26
29	Br	H	NO_2	COOH	4.27±0.16	3.75±0.16	0.49±0.09
30	Br	OH	NO_2	COOH	0.051±0.006	0.031±0.002	0.45±0.07
31	H	H	H	CN	Inactive	NA	NA
32	Cl	H	H	CN	21.43±1.39	21.43±1.39	0.34±0.18
33	Cl	H	Cl	CN	8.61±0.40	8.61±0.40	0.41±0.35
34	Br	H	Br	CN	17.70±0.96	14.86±0.89	~1.37
35	Br	OH	H	CN	9.27±0.97	9.33±0.68	~0.467
36	Br	OH	Br	CN	2.33±0.45	2.89±0.23	0.44±0.11
37	Br	OMe	Br	CN	8.22±1.21	0.59±0.06	0.75±0.14
38	Br	H	NO2	CN	0.70±0.09	0.65±0.02	2.64±0.52
39	Br	OMe	NO2	CN	2.91±0.49	1.69±0.25	0.25±0.07
41	H	H	H	1H-tetrazol-5-yl	6.76±0.42	5.08±0.29	0.13±0.08
42	Br	H	Br	1H-tetrazol-5-yl	0.93±0.08	0.38±0.02	1.77±0.28
43	Br	OH	Br	1H-tetrazol-5-yl	0.068±0.010	0.023±0.002	0.68±0.10
44	Br	OMe	Br	1H-tetrazol-5-yl	1.41±0.14	0.46±0.06	2.25±0.43
45	Br	H	NO_2	1H-tetrazol-5-yl	0.33±0.04	0.33±0.04	0.28±0.15
46	Br	OMe	NO_2	1H-tetrazol-5-yl	2.66±0.25	2.82±0.40	0.96±0.13
47	Br	OMe	H	1H-tetrazol-5-yl	14.76±1.21	14.13±0.80	0.17±0.09
49	Br	OH	H	1H-tetrazol-5-yl	0.71±0.06	0.483±0.0	0.22±0.07
50	Br	OH	NO_2	1H-tetrazol-5-yl	0.0058±0.0011	0.0086±0.0003	0.44±0.15
51	NO_2	OH	NO_2	1H-tetrazol-5-yl	0.014±0.001	0.013±0.001	1.28±0.46

Table 2. The Potency and Efficacy of Compounds in Tango Assays Relative to Zaprinast. (EC_{50} to Trigger β-Arrestin Translocation in Tango Assays)

Compd	EC_{50}(μ M)	% zaprinast
Zaprinast	3.52 ±0.41	100 ±1
27	3.63 ±0.95	168 ±3
30	0.479 ±0.073	110.8 ±0.8
42	7.05 ±1.55	81.3 ±2.0
43	1.54 ±0.14	125.7 ±7.9
44	14.06 ±1.67	86.4 ±4.0
50	0.197 ±0.038	103.7 ±2.6
51	0.288 ±0.085	107.2 ±1.6

Table.3 Physicochemical Properties of Selected Compounds.

Compd	pEC_{50}	clogP[a]	LE	LLE
26	5.757	3.280	0.36	2.477
27	6.824	2.624	0.40	4.200
28	6.052	2.818	0.34	3.234
30	7.292	1.828	0.38	5.009
36	5.632	2.303	0.35	3.329
38	6.239	1.451	0.37	4.788
42	6.031	2.841	0.34	3.190
43	7.176	2.542	0.38	4.634
44	5.851	2.738	0.29	3.113
45	6.477	1.721	0.32	4.756
50	8.238	1.743	0.39	6.495
51	7.853	0.918	0.34	6.935

[a]Calculated by the Chembiodraw Ultra 11.0.

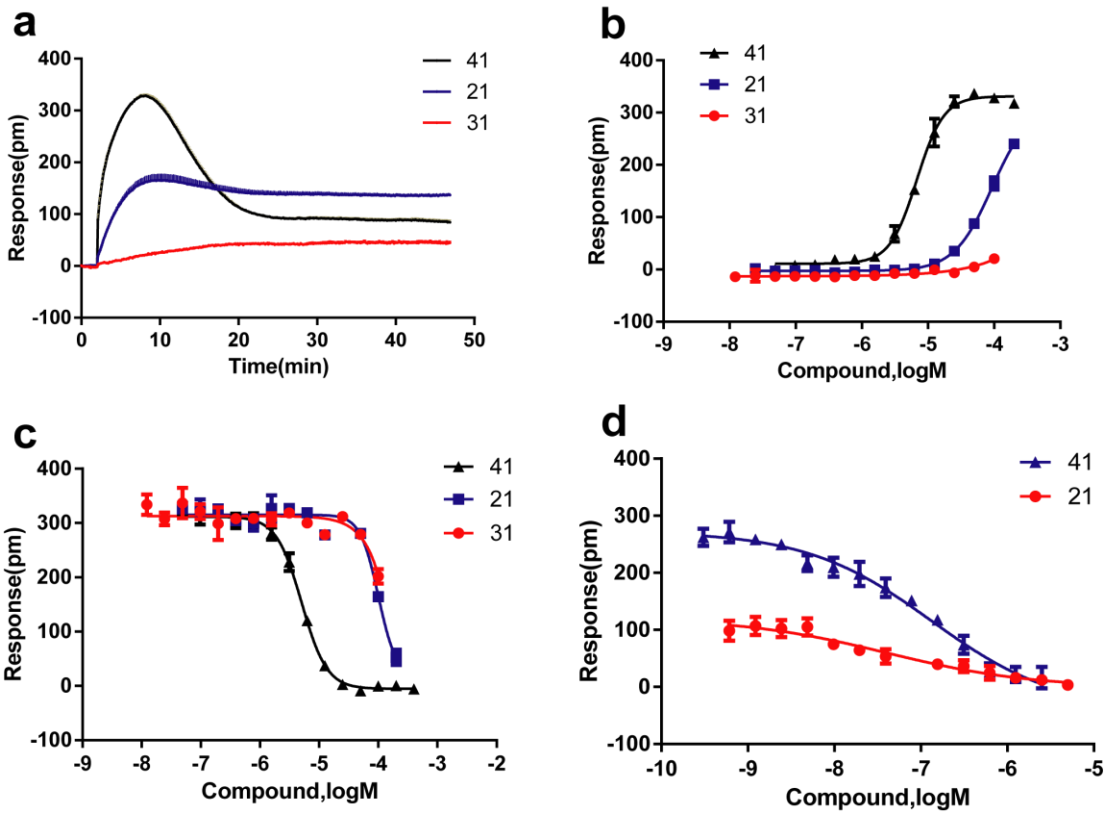


Figure 2. (a) The DMR characteristics of compound **21** (100μM), **31** (100μM), and **41**(100 μM). (b) The amplitudes of the DMR induced by compound **21**, **31** and **41**. (c) The dose-dependent desensitization by compound **21**, **31** and **41** identified to the repeated stimulation with 1μM zaprinast. (d) The DMR amplitudes of compound **21** and **41** as a function of compound **11**. The data respresents mean ± sd from two independent measurement, each with four replicates (n=8).

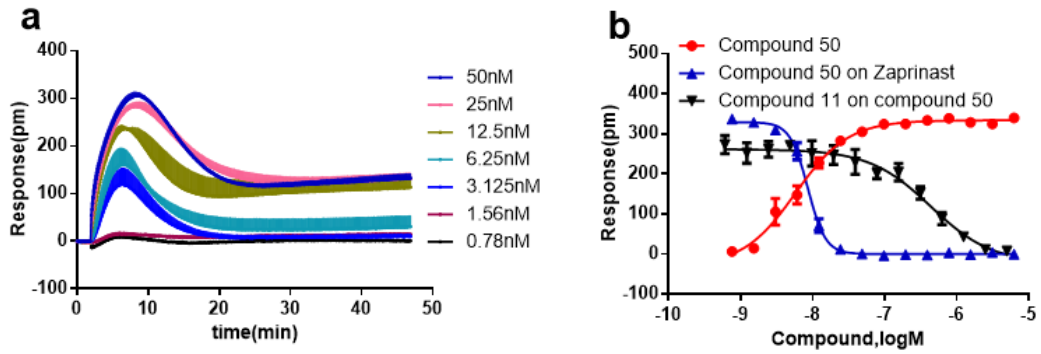


Figure 3. (a) Real time kinetic responses of **50** at different doses in HT-29 cells. (b) DMR amplitudes of compound **50** as a function of doses, in compared with the dose-dependently desensitization of the zaprinast DMR by **50**, and the dose-dependent inhibition of the DMR of 40nM compound **50** by compound **11**. The data respresents mean ± sd from two independent measurement, each with four replicates (n=8).

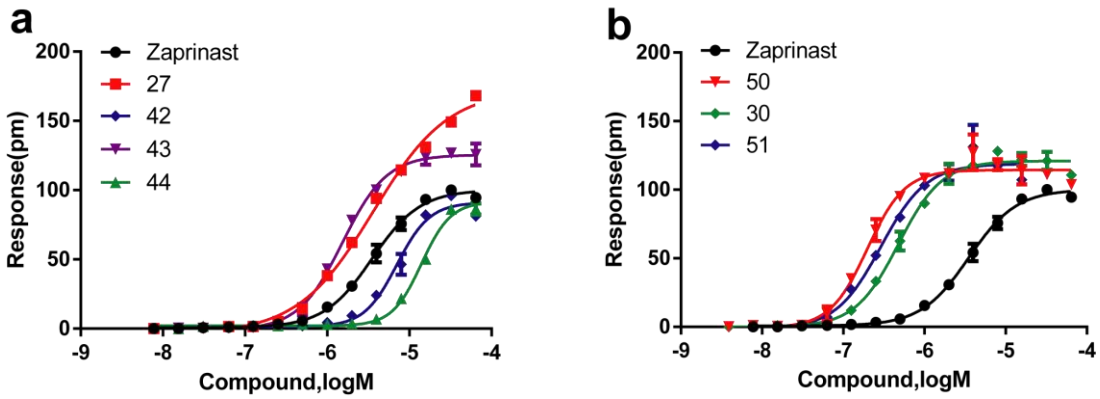


Figure 4. Dose-dependent responses of GPR35 ligands as measured using Tango β-arrestin translocation gene reporter assays. The coumarin to fluorescein ratio was plotted as a function of ligand doses. Zaprinast was included as a positive control. The data represents mean±sd from two independent measurement, each in duplicate (n=4).