Bioorganic & Medicinal Chemistry Letters 22 (2012) 6126–6135

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Ago-allosteric modulators of human glucagon-like peptide 2 receptor

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ARTICLE INFO

Article history:
Received 20 May 2012
Revised 18 July 2012
Accepted 7 August 2012
Available online 14 August 2012

Keywords:
Glucagon-like peptide 2
Agonist
Positive allosteric modulator

ABSTRACT

Glucagon-like peptide 2 (GLP-2) is an intestinotropic peptide that binds to GLP-2 receptor (GLP-2R), a class-B G protein-coupled receptor (GPCR). Few synthetic agonists have been reported so far for class-B GPCRs. Here, we report the first scaffold compounds of ago-allosteric modulators for human GLP-2R, derived from methyl 2-{[(2*Z*)-2-(2,5-dichlorothiophen-3-yl)-2-(hydroxyimino)ethyl]sulfanyl}benzoate (compound **1**).

Glucagon-like peptide 1 (GLP-1) and GLP-2 are peptides that are encoded, together with glucagon, by a proglucagon gene.[1] They are produced in the intestine by tissue-specific posttranslational regulation. GLP-1 and GLP-2 exert their effects through GLP-1 receptor (GLP-1R) and GLP-2 receptor (GLP-2R), respectively, both of which are $G\alpha_s$ G protein-coupled receptors (GPCRs). Both peptides are liberated by stimulation of nutrient intake, and secreted GLP-1 and GLP-2 are rapidly degraded by dipeptidyl peptidase IV (DPPIV) cleaving His-Pro dipeptides from the N-terminus. GLP-1 potentiates glucose-dependent insulin secretion,[2] and GLP-2 promotes intestinal mucosal growth.[3] Therefore, GLP-1 and GLP-2 are therapeutic targets for diabetes and intestinal damage, respectively. In fact, DPPIV-resistant peptide GLP-1R agonists (e.g., exenatide, liraglutide)[4] and DPPIV inhibitors (e.g., vildagliptin, sitagliptin) are employed clinically for the treatment of diabetes.[5] A DPPIV-resistant peptide GLP-2R agonist, teduglutide, is under clinical development for short bowel syndrome[6] and Crohn's disease.[7]

GLP-1R and GLP-2R have ~40% identity, and both receptors belong to the glucagon-secretin class B of the GPCRs.[8] Although several small-molecule GPCR agonists have been found in classes A and C,[9] there had been no reports regarding agonists in class B. However, in 2007, two reports on non-peptide agonists for GLP-1R were published. Chen et al. showed that a substituted cyclobutane compound, Boc5, increased intracellular cAMP levels in HEK293 cells stably expressing rat GLP-1R and cAMP response element (CRE)-driven luciferase; the response to this compound was blocked by exendin(9–39), a peptide antagonist of GLP-1R.[10] Thus, Boc5 acted on the orthosteric site of GLP-1R. In the other paper, Knudsen et al. reported that quinoxaline derivatives acted on the allosteric site of GLP-1R, because exendin(9–39) did not antagonize their effects.[11] Intriguingly, these compounds worked as both allosteric activators and agonists for GLP-1R. Therefore, the quinoxaline derivatives were categorized as ago-allosteric modulators.

Unlike the case with GLP-1R, there have been no reports of small molecules with agonistic activity for GLP-2R. However, we

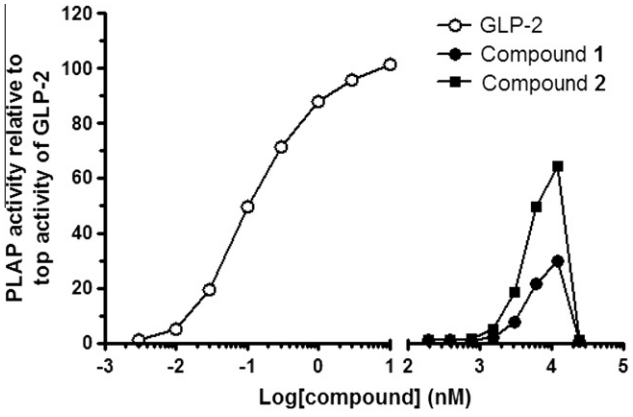

Figure 1. Placental alkaline phosphatase (PLAP) activity induced by human glucagon-like peptide 2 (GLP-2), or compound **1** or **2** in HEK293 cells stably overexpressing human GLP-2 receptor and PLAP driven by cAMP response element. Note that the concentration–activity curves are bell-shaped. Values are expressed as means. $n = 3$.

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http://dx.doi.org/10.1016/j.bmcl.2012.08.026

recently discovered compounds with interesting activity toward GLP-2R. This is the first report on structure–activity relationships (SARs) of the compounds having both agonist and allosteric positive modulator activity toward human GLP-2R.

In preliminary screening, we found that methyl 2-{[(2*Z*)-2-(2,5-dichlorothiophen-3-yl)-2-(hydroxyimino)ethyl]sulfanyl}benzoate (compound **1**) showed agonistic activity toward human GLP-2R in cell reporter assays using CRE-driven placental alkaline phosphatase (PLAP) and human GLP-2R-overexpressing cells (hGLP-2R/SE302).[12,13] Compound **2**, a de-esterified derivative of compound **1**, had stronger activity than compound **1** (Fig. 1). Their concentration–activity curves were bell-shaped owing to their cell toxicity.[14] PLAP reporter activity was not observed in CRE-driven PLAP-overexpressing cells (SE302) or CRE-driven PLAP and human GLP-1R-overexpressing cells (hGLP-1R/SE302). The same results were obtained for intracellular cAMP levels[15]: compound **2** did not elevate cAMP levels in SE302 or hGLP-1R/SE302 cells (Fig. 2A and B). However, this compound increased cAMP contents in hGLP-2R/SE302 cells (Fig. 2C). These results showed that compound **2** was a GLP-2R agonist.

We performed chemical development based on the structure of compounds **1** and **2** to enhance GLP-2R agonistic activity. In the SAR tables described below, we summarize the maximum PLAP reporter activities and concentrations at which the maximum PLAP activities were observed, as representing the activities of the compounds, because the concentration–activity curves were bell-shaped as a result of cellular toxicity (Fig. 1).[14] The PLAP activities of the compounds were comparatively weak, particularly at the beginning of chemical development; the maximum PLAP activities were then expressed as values relative to those of 10 pM GLP-2, or of 100 pM GLP-2 when the PLAP activities became stronger.

By synthesizing derivatives of compound **1** bearing an oxime moiety, we obtained regioisomers with high or low polarity in general. Table 1 shows the SARs of the polarities for compound **1**, and phenyl (compounds **2** and **3**) and methoxyphenyl (compounds **4**–**8**) derivatives. These results showed a clear-cut correlation

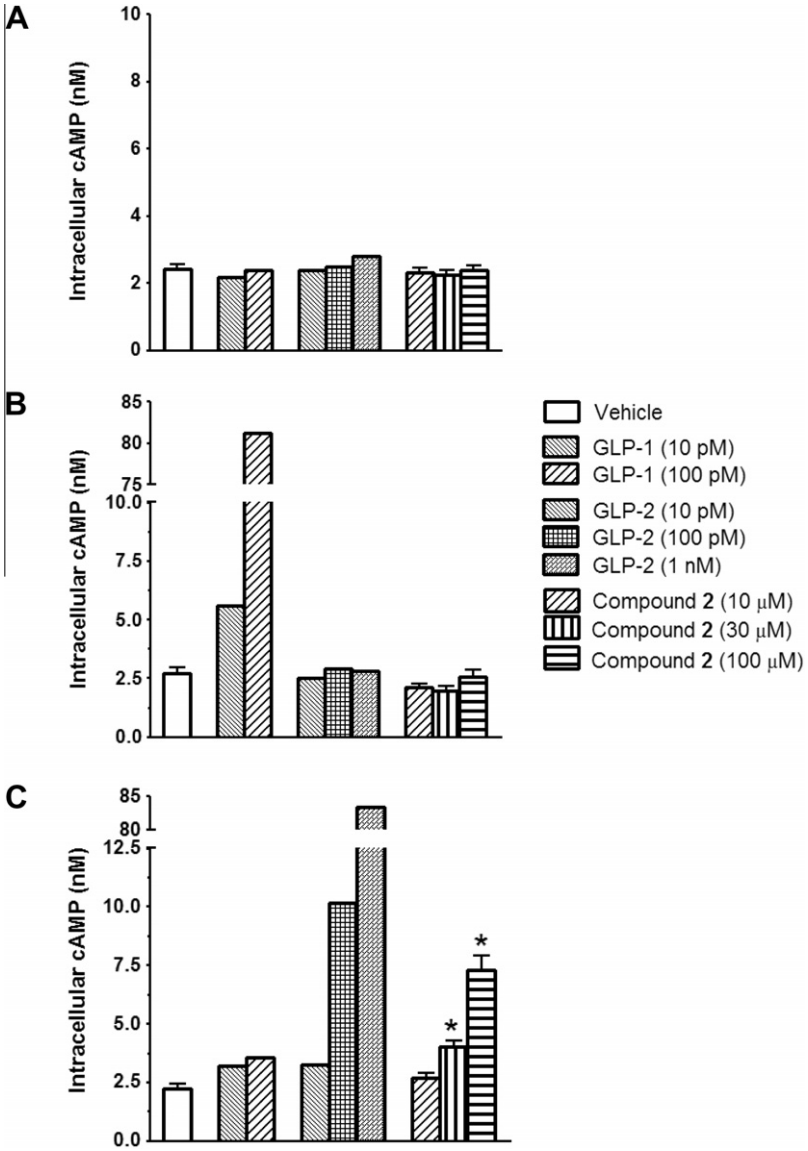
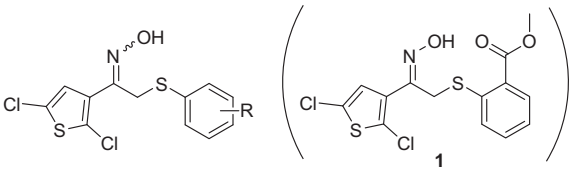


Figure 2. Changes in intracellular cAMP levels in HEK293 cells stably overexpressing placental alkaline phosphatase (PLAP) regulated by cAMP response element (CRE) (SE302 line) (A); in HEK293 cells overexpressing CRE-driven PLAP and human glucagon-like peptide 1 (GLP-1) receptor (hGLP-1R/SE302 line) (B); and in HEK293 cells overexpressing CRE-driven PLAP and human glucagon-like peptide 2 (GLP-2) receptor (hGLP-2R/SE302 line) (C) treated with human GLP-1, GLP-2, or compound **2**. Values are expressed as means for GLP-1 and GLP-2 treatment, and as means ± S.E.M. for vehicle and compound **2** treatment. n = 6, 2, 2, and 6 for vehicle, GLP-1, GLP-2, and compound **2** treatment, respectively. $^{*}p$ <0.05 compared with the vehicle-treated group.[18]

Table 1
Structure–activity relationships with low and high polarities of compound **1** and its derivatives



Compound	R	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to GLP-2 (%)	
					10 pM GLP-2	100 pM GLP-2
1	2-CO_2Me	Low	Active	25	284	39
2	/	Low	Active	25	701	55
3	/	High	Inactive			
4	2-OMe	Low	Active	50	78	—
5	2-OMe	High	Inactive			
6	3-OMe	Low	Active	25	144	—
7	3-OMe	High	Inactive			
8	4-OMe	Low	Active	50	217	—

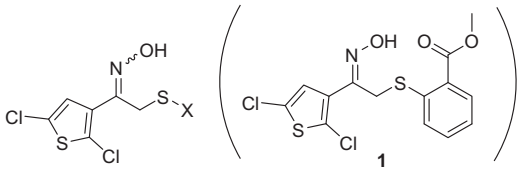
Maximum activity values are expressed as means of ⩾3 samples.
—, Not determined.
[a] Products were separated by using silica gel column chromatography.

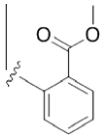
between their human GLP-2R reporter activity and polarity: low-polarity compounds had activity (compounds **1**, **2**, **4**, **6** and **8**), whereas high-polarity ones did not (compounds **3**, **5** and **7**). Our *E*/*Z* analysis clarified that compound **1** was a *Z*-stereoisomer,[16] which has low polarity.

When we replaced the 2-methoxycarbonyl-benzene in compound **1**, we observed activity in compounds having phenyl (compound **2**), 1-naphthyl (compound **14**), 3- and 4-pyridyl (compounds **16** and **17**, respectively), and 2-thienyl (compound **18**) groups (Table 2). It seemed that a planar aromatic structure was needed in this portion, because alkyl or cycloalkyl group substitution did not give activity (compounds **9**, **10** and **11**). Addition of one or three methylenes between the sulfanyl group and phenyl group resulted in loss of potency (compounds **12** and **13**), suggesting that the distance between the two groups was important for exhibition of activity. Contrary to 3- and 4-pyridyl substitution, 2-pyridyl substitution (compound **15**) did not hold the activity. 4-Pyridine gave about double the maximum activity provided by 3-pyridine.

Phenyl SARs are shown in Table 3. Single substitution was divided into two groups: 2-substitution showed higher activity than 4-substitution for a methyl (compounds **20**–**22**), chloro (compounds **29**–**31**), and trifluoromethyl (compounds **32** and **33**) moiety, and vice versa for a methoxy (compounds **4**, **6** and **8**), and

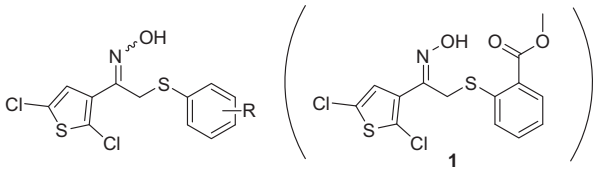
Table 2
Structure–activity relationships with substitutions of methylcarbonyl-benzene in compound **1**



Compound	X	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to GLP-2 (%)	
					10 pM GLP-2	100 pM GLP-2
1		Low	Active	25	284	39
2	Ph	Low	Active	25	701	55
9	Et	Low	Inactive			
10	*t*-Bu	Low	Inactive			
11	*c*-Hexyl	Low	Inactive			
12	Bn	Low	Inactive			
13	$(CH_2)_3$Ph	Low	Inactive			
14	1-Naphthyl	Low	Active	25	—	23
15	2-Pyridyl	Low	Inactive			
16	3-Pyridyl	Low	Active	50	—	27
17	4-Pyridyl	Low	Active	50	—	55
18	2-Thienyl	Low	Active	25	—	40

Maximum activity values are expressed as means of ⩾3 samples.
—, Not determined.
[a] Products were separated by using silica gel column chromatography.

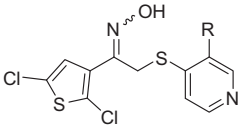
Table 3
Structure–activity relationships for the phenyl part of compound **1**



Compound	R	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to GLP-2 (%)	
					10 pM GLP-2	100 pM GLP-2
1	2-CO_2Me	Low	Active	25	284	39
2	/	Low	Active	25	701	55
4	2-OMe	Low	Active	50	78	—
6	3-OMe	Low	Active	25	144	—
8	4-OMe	Low	Active	50	217	—
19	2-CO_2H	Low	Inactive			
20	2-Me	Low	Active	25	—	90
21	3-Me	Low	Active	25	—	30
22	4-Me	Low	Active	50	65	3
23	2-Et	Low	Active	25		63
24	2-*t*-Bu	Low	Active	12.5		40
25	2-Ph	Low	Active	12.5		84
26	2-F	Low	Active	25	—	80
27	3-F	Low	Active	25	—	73
28	4-F	Low	Active	25	945	134
29	2-Cl	Low	Active	25	—	60
30	3-Cl	Low	Active	25	—	53
31	4-Cl	Low	Active	25	293	17
32	2-CF_3	Low	Active	12.5	—	58
33	4-CF_3	Low	Active	25	—	16
34	2,3-di-Me	Low	Active	25	—	54
35	2,4-di-Me	Low	Active	12.5	—	58
36	2,5-di-Me	Low	Active	12.5	—	40
37	2,6-di-Me	Low	Active	25	—	10
38	2-Me,4-F	Low	Active	25	—	124
39	2-Me,3-OMe	Low	Active	25	—	31

Maximum activity values are expressed as means of ⩾3 samples.
—, Not determined.
[a] Products were separated by using silica gel column chromatography.

Table 4
Structure–activity relationships of position 3 of pyridine of compound **17**



Compound	R	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to 100 pM GLP-2 (%)
17	H	Low	Active	50	55
40	Me	Low/high mixture	Active	50	44
41	CF_3	Low	Active	25	143
42	Et	Low/high mixture	Active	25	59
43	Ph	Low	Active	12.5	102

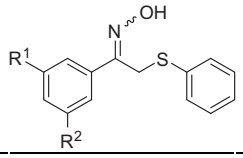
Maximum activity values are expressed as means of ⩾3 samples.
[a] Products were separated by using silica gel column chromatography.

fluoro (compounds **26**–**28**) moiety. Especially, 2-methyl (compound **20**), 2-trifluoromethyl (compound **32**), 2-phenyl (compound **25**), 2-*ter*-butyl (compound **24**), and 4-fluoro (compound **28**) groups displayed relatively high activity. On the other hand, 2-substitution with a carboxy group caused a complete loss of activity (compound **19**), suggesting that polar groups were not tolerated at this position. It appeared that no clear additive or synergic increase in the activity was generated by double substitution, comparing compounds **20**, **22** and **35**, compounds **20**, **28** and **38**, and compounds **6**, **20** and **39**.

As 4-pyridyl substitution maintained the potency, the SAR of position 3 of the 4-pyridine ring was examined (Table 4). Incorporation of a trifluoromethyl (compound **41**) or phenyl (compound **43**) group resulted in higher reporter activity than that of a methyl (compound **40**) or ethyl (compound **49**) group. This SAR seemed to be similar to that of position 2 of the benzene ring.

We found that it was possible to replace the thiophene in compound **1** with benzene (compound **44**), so we then investigated the SARs of this benzene (Table 5). Compound **44** had a very weak activity, but single substitution at position 3 increased the activity

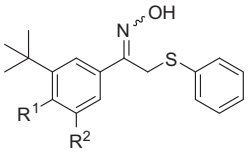
Table 5
Structure–activity relationships with substitution of benzene for thiophene in compound **2** (Part I)



Compound	R^1	R^2	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to GLP-2 (%)	
						10 pM GLP-2	100 pM GLP-2
44	H	H	Low	Active	50	4	—
45	OH	H	Low	Inactive			
46	CN	H	Low	Active	50	8	—
47	OMe	H	Low	Active	25	—	8
48	Propoxy	H	Low	Active	25	—	9
49	*i*-Propoxy	H	Low	Active	25	—	14
50	F	H	Low	Active	25	54	—
51	Cl	H	Low	Active	25	46	—
52	Br	H	Low	Active	25	42	—
53	CF_3	H	Low	Active	25	95	—
54	CF_3	CF_3	Low	Active	25	517	29
55	*t*-Bu	H	Low	Active	25	244	18
56	*t*-Bu	*t*-Bu	Low	Active	25	—	31

Maximum activity values are expressed as means of ⩾3 samples.
—, Not determined.
[a] Products were separated by using silica gel column chromatography.

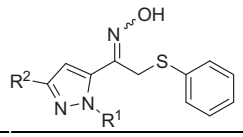
Table 6
Structure–activity relationships with substitution of benzene for thiophene in compound **2** (Part II)



Compound	R^1	R^2	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to 100 pM GLP-2 (%)
55	H	H	Low	Active	25	18
56	H	*t*-Bu	Low	Active	25	31
57	OH	*t*-Boc	Low	Active	25	4
58	OMe	CO_2H	Low	Inactive		
59	OMe	*t*-Boc	Low	Active	12.5	12
60	OMe	NMe_2	Low	Active	12.5	43
61	OMe	Br	Low	Active	12.5	43

Maximum activity values are expressed as means of ⩾3 samples.
[a] Products were separated by using silica gel column chromatography.

Table 7
Structure–activity relationships with substitution of pyrazole for thiophene in compound **2**



Compound	R^1	R^2	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to 100 pM GLP-2 (%)
62	Me	Me	Low	Inactive		
63	Me	*c*-Pr	Low	Inactive		
64	Me	*t*-Bu	Low	Inactive		
65	Me	Ph	Low	Active	25	14
66	Me	CF_3	Low	Active	25	13
67	Me	CF_3	High	Inactive		
68	*n*-Pr	CF_3	Low	Active	25	71
69	*i*-Pr	CF_3	Low	Active	25	87
70	*s*-Bu	CF_3	Low	Active	12.5	110
71	1-Et-Pr	CF_3	Low	Active	12.5	31
72	Bn	CF_3	Low	Active	12.5	59
73	*c*-Bu	CF_3	Low	Active	25	99
74	*c*-Pentyl	CF_3	Low	Active	25	127

Maximum activity values are expressed as means of ⩾3 samples.
[a] Products were separated by using silica gel column chromatography.

(compounds **46**–**53**, and **55**), except for a hydroxyl group (compound **45**). Particularly, high activity was caused by substitution with a trifluoromethyl (compound **53**) or *ter*-butyl (compound **55**) group at position 3. Double substitution at positions 3 and 5 made the reporter activity higher than with 3-substitution alone, as shown by comparing compound **53** with compound **54**, and compound **55** with compound **56**.

Additionally, we replaced positions 4 and 5 of 3-*ter*-butyl-benzene (Table 6). Comparison between compounds **57** and **59** suggested that 4-substitution of a methoxy group resulted in higher activity than that of a hydroxy group. When we fixed the methoxy group at position 4, derivatives having a dimethylamino (compound **60**), or bromo (compound **61**) group at position 5 had comparatively high potency. Incorporation of a carboxy group at position 5 (compound **58**) led to no activity, suggesting that this position did not tolerate polar groups.

Thiophene was also able to be replaced by pyrazole, and we examined its 1,3-substitution (Table 7). Fixing a methyl group at position 1, no activity was observed in compounds having a methyl (compound **62**), cyclopropyl (compound **63**), or *ter*-butyl (compound **64**) moiety at position 3. However, incorporation of a phenyl (compound **65**), or trifluoromethyl (compound **66**) group generated the PLAP activity. Then, we incorporated an alkyl, cycloalkyl, or benzyl group at position 1, with the trifluoromethyl group at position 3 fixed (compounds **68**–**74**). Among these compounds, *sec*-butyl (compound **70**) and cyclopentyl (compound **74**) substituents produced higher activity. Compound **67**, which was the high-polarity version of compound **66**, showed a loss of potency. Therefore, the correlation between polarity and activity also seemed true for pyrazole derivatives.

On the basis of the above-mentioned substitution SARs of two rings in compound **1**, we prepared compounds combined with rings showing higher potency (Table 8), namely, 3-*ter*-butyl-4-methoxy-5-bromo-phenyl (compound **75**) or 1-cyclopentyl-3-trifluoromethyl-pyrazol-5-yl (compound **76**) in place of the 2,5-dichloro-thiophen-3-yl group of compound **1**; both of these compounds had 2-trifluoromethyl-phenyl in place of the 2-methoxycarbonyl-phenyl group. Comparison with compound **1** revealed that the concentrations at maximum activity were decreased by about fourfold. Thus, we were able to potentiate GLP-2R agonistic activity by suitable combination of the two rings. Figure 3 shows concentration–activity curves of compounds **1**, **75** and **76**. Outline of the synthesis of these potent compounds is indicated in Scheme 1.

To investigate the mode of action of our GLP-2R agonists, we examined whether GLP-2(11–33) antagonized the PLAP reporter activity of compound **1**. GLP-2(11–33) was a novel partial agonist for human GLP-2R, having 88% binding activity at 1 μM and 100% at 10 μM,[17] and 11% of intrinsic PLAP reporter activity (EC_{50}, 1.3 μM) compared with GLP-2. GLP-2(11–33) was able to antago-

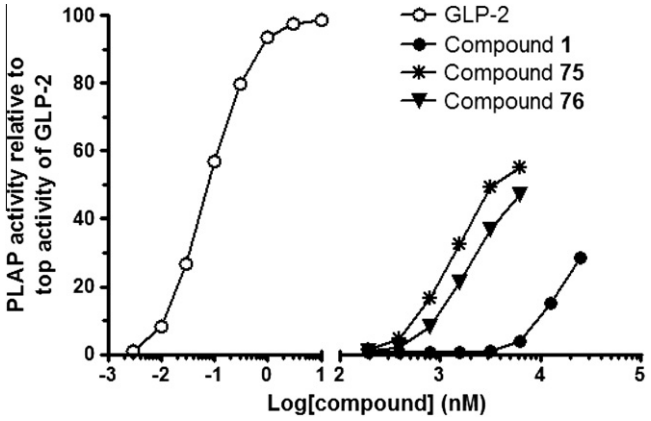
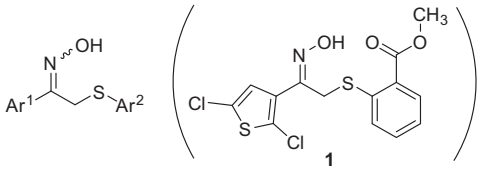
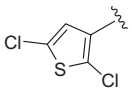
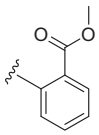
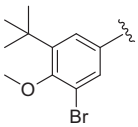
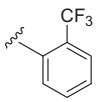
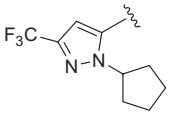
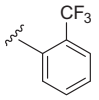


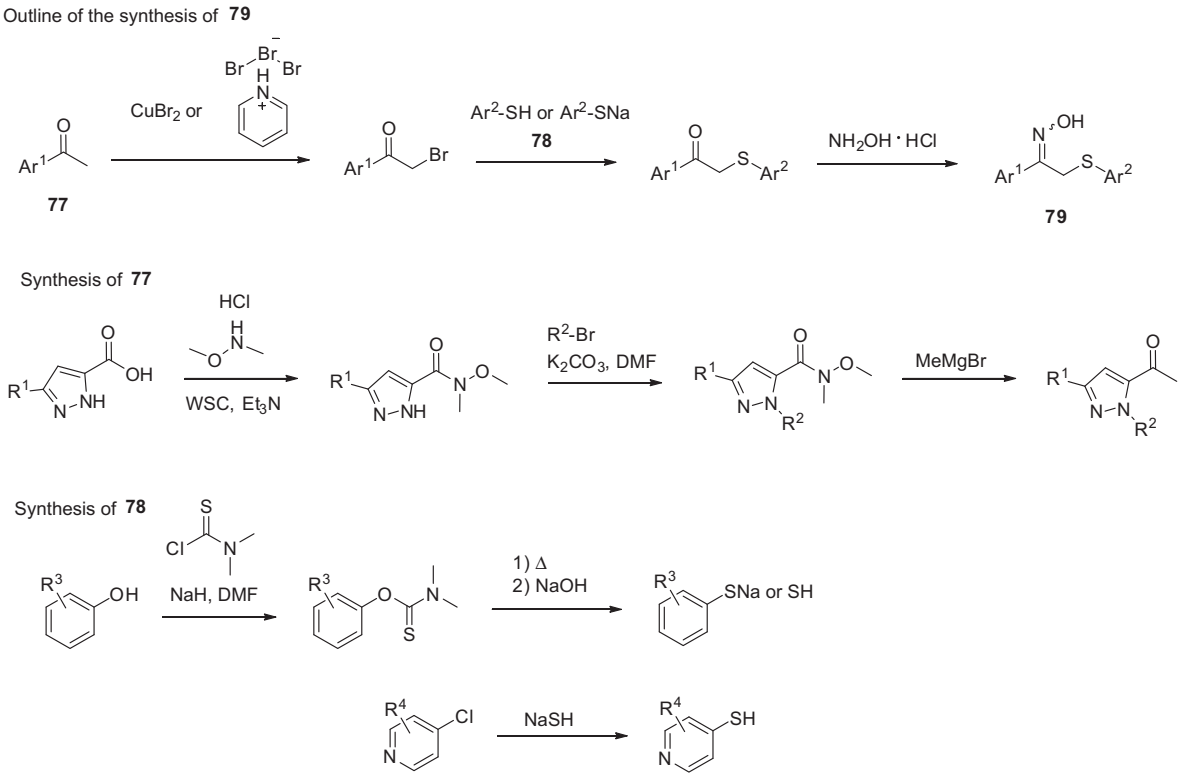
Figure 3. Placental alkaline phosphatase (PLAP) activity induced by human glucagon-like peptide 2 (GLP-2), and by compounds **1**, **75** and **76** in HEK293 cells stably overexpressing human GLP-2 receptor and PLAP driven by cAMP response element. Concentration–activity curves are drawn up until the maximum activity of the compounds. Values are expressed as means. $n = 3$.

Table 8
Combination of Ar^1 and Ar^2 rings



Compound	Ar^1	Ar^2	Low/high polarity[a]	Active/inactive	Concentration at maximum activity (μM)	Maximum activity relative to GLP-2 (%)	
						10 pM GLP-2	100 pM GLP-2
1			Low	Active	25	284	39
75			Low	Active	6.3	714	97
76			Low	Active	6.3	610	83

Maximum activity values are expressed as means of ⩾3 samples.
[a] Products were separated by using silica gel column chromatography.



Scheme 1. General synthesis of human GLP-2 receptor ago-allosteric modulators.

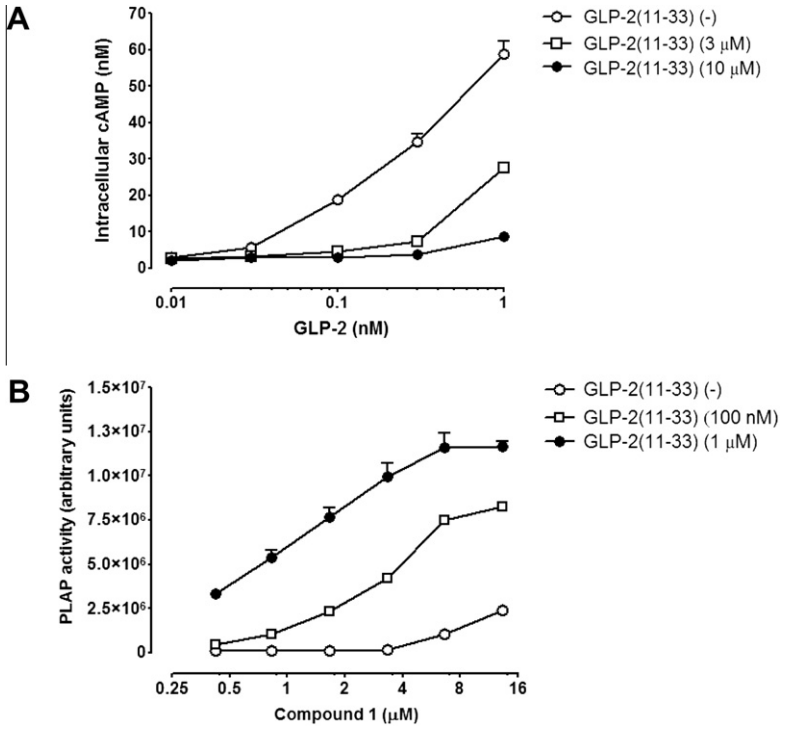


Figure 4. Effects of a truncated human glucagon-like peptide 2 (GLP-2), GLP-2(11-33), on intracellular cAMP produced by GLP-2 (A), and on placental alkaline phosphatase (PLAP) activity induced by compound **1** (B) in HEK293 cells stably overexpressing human GLP-2 receptor and PLAP driven by cAMP response element. Values are expressed as means ± S.E.M. $n = 3$.

nize GLP-2 activity, resulting in a concentration-dependent decrease in the intracellular cAMP concentration generated by GLP-2 (Fig. 4A). This examination gave the unexpected result that the PLAP activity of compound **1** was dramatically increased by the addition of GLP-2(11–33), in a concentration-dependent manner (Fig. 4B).

Next, we investigated whether our compounds enhanced the reporter activity of GLP-2, GLP-2(11–33), and GLP-2(3–33), a trun-

cated GLP-2 peptide produced by DPPIV in vivo (its intrinsic PLAP reporter activity was 58% of that of GLP-2 and its EC_{50} was 31 nM). Addition of compound **2** shifted the concentration–reporter response curves for GLP-2 and its truncated peptides to the left or upward, or both, in a concentration-dependent fashion: this addition increased the PLAP activity of GLP-2 additively (Fig. 5A). EC_{50} values were not affected by compound **2**, but maximum PLAP activity was increased by ~10% with the addition of compound **2** at 10 μM. For GLP-2(3–33), compound **2** at 1 and 10 μM decreased EC_{50} values by ~1.2-fold and ~3.3-fold, and increased maximum activity by ~110% and ~150%, respectively (Fig. 5B). More notable was the effect of compound **2** on the PLAP activity of GLP-2(11–33)

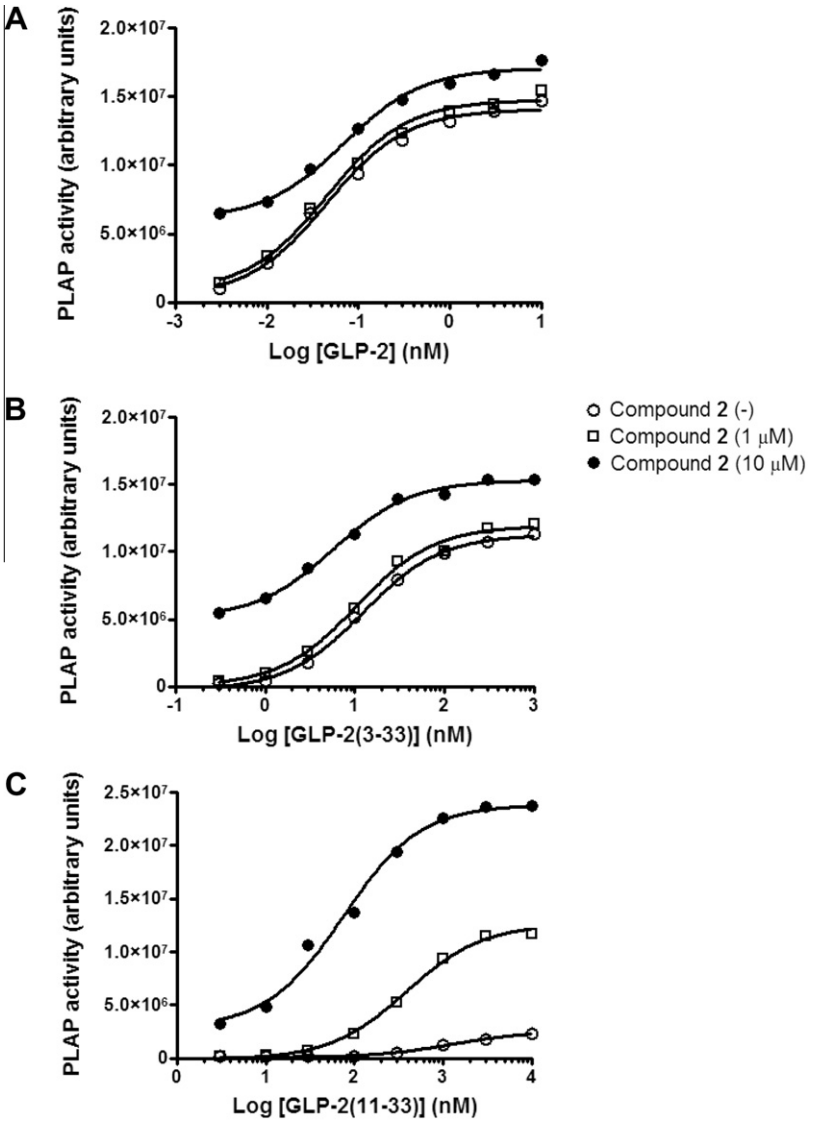


Figure 5. Effects of compound **2** on placental alkaline phosphatase (PLAP) activity induced by human glucagon-like peptide 2 (GLP-2) (A), GLP-2(3-33) (B), and GLP-2(11-33) (C) in HEK293 cells stably overexpressing human GLP-2 receptor and PLAP driven by cAMP response element.[19] Each value is the mean of three samples.

Table 9
Effects of compound **2** on placental alkaline phosphatase (PLAP) activity of GLP-2 and its truncated peptides in HEK293 cells overexpressing human GLP-2 receptor and PLAP driven by the cAMP response element

GLP-2 or truncated GLP-2 peptide	Compound **2**	EC_{50} (nM)	Maximum PLAP activity (%)	
			Relative to each control	Relative to GLP-2
GLP-2	–	0.097	100	100
	1 μM	0.084	103	103
	10 μM	0.087	114	114
GLP-2(3–33)	–	31	100	59
	1 μM	27	114	67
	10 μM	9.4	145	97
GLP-2(11–33)	–	1300	100	11
	1 μM	380	494	56
	10 μM	75	932	107

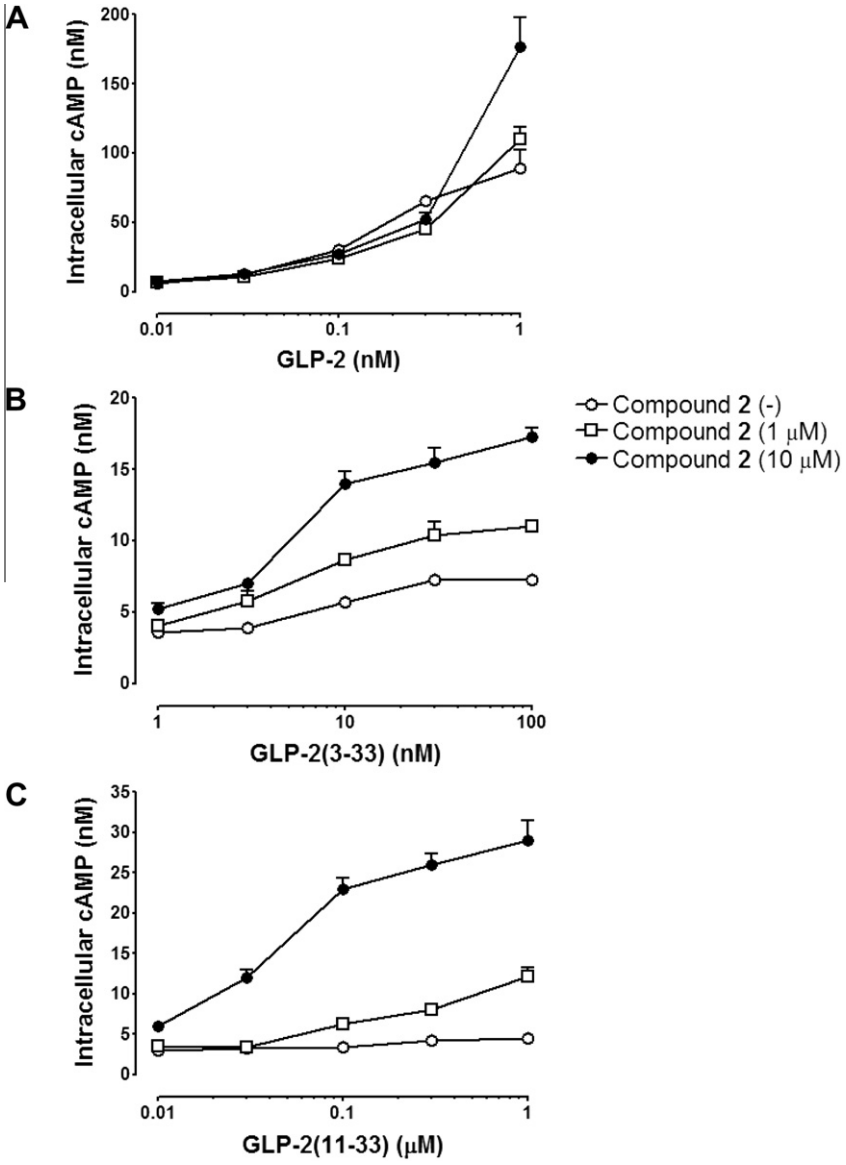


Figure 6. Changes in intracellular cAMP levels induced by human glucagon-like peptide 2 (GLP-2) (A), GLP-2(3–33) (B), and GLP-2(11–33) (C) in HEK293 cells stably overexpressing human GLP-2 receptor and placental alkaline phosphatase regulated by cAMP response element, in the present or absence of compound **2**. Values are expressed as means ± S.E.M. $n = 3$.

(Fig. 5C). Compound **2** treatment at 1 and 10 μM decreased EC_{50} values by ~3.4-fold and ~17-fold, and increased maximum activity by ~490% and ~930%, respectively. The PLAP activity of GLP-2(3–33) and GLP-2(11–33) was raised to the maximum intrinsic activity of GLP-2 by the addition of compound **2** at 10 μM. These effects are summarized in Table 9. Similar effects were also observed for compound **56** (data not shown). We also tested the effects of compound **2** on changes in intracellular cAMP levels in the presence of GLP-2, GLP-2(3–33), or GLP-2(11–33). Compound **2** at 10 μM increased intracellular cAMP levels in the presence of 1 nM GLP-2 (Fig. 6A). In the cases of GLP-2(3–33) and GLP-2(11–33), compound **2** elevated intracellular cAMP levels (Fig. 6B and C, respectively); this effect was particularly prominent with GLP-2(11–33).

We examined the binding activity of compound **17** to human GLP-2R,[17] because among the active compounds examined this compound had relatively good solubility. There was no binding activity until 100 μM. We therefore speculated that the compounds did not interact with the GLP-2-binding site of GLP-2R (i.e. the orthostatic site), and that these compounds were positive allosteric modulators of GLP-2R. These findings, together with the agonistic actions of the compounds alone, indicated that our compounds functioned as both agonists and positive allosteric modulators—that is, positive ago-allosteric modulators—of human GLP-2R. Our observations would be useful for developing small-molecule agonists and/or ago-allosteric modulators of human GLP-2R.

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12. By transduction using murine leukemia-based retrovirus into the HEK293 cell line, we generated a reporter cell line stably expressing cAMP response element-driven human placental alkaline phosphatase (PLAP). The cell line was named SE302. By the same method, human GLP-1 receptor (GLP-1R) or GLP-2 receptor (GLP-2R) was transduced into SE302 cells to produce cell lines stably expressing human GLP-1R and GLP-2R. These lines were named hGLP-1R/SE302 and hGLP-2R/SE302, respectively.
13. SE302, hGLP-1R/SE302, and hGLP-2R/SE302 cells were seeded at 2×10^4/well on to gelatin-coated 96-well plates and incubated at 37 °C overnight. Next day (day 2), the compound or peptide, or both, was added to the culture, which was then incubated at 37 °C overnight. The supernatants were collected and mixed with substrate/enhancer solution (Applied Biosystems) in the wells of 96-well plates; the plates were incubated for 1 h at room temperature on day 3, and PLAP activity was determined. In screening, both hGLP-2R/SE302 and SE302 cells were utilized. Evaluation using hGLP-1R/SE302 cells was added if needed. Cell viability was always checked using Invitrogen's alamarBlue®.
14. Decreases in the PLAP reporter activity of a compound were accompanied with decreases in fluorescence intensity in the alamarBlue assay.
15. Cells were treated with 2 mM of 3-isobutyl-1-methylxanthine for 30 min at room temperature. The cells (5×10^4) were then treated with the compound or peptide, or both, on 96-well half-area plates at 37 °C for 30 min. Intracellular cAMP was then quantified by using Cisbio's cAMP measurement kit.
16. The *E*/*Z*-stereochemistry of compound **1** was confirmed from its NOESY spectrum by using Avance 600 MHz NMR (Bruker BioSpin AG).
17. Membranes for binding experiments were prepared from confluent hGLP-2R/SE302 cells. Receptor binding studies were conducted using 100 pM [^{125}I]-Bolton Hunter labeled GLP-2 as a ligand. On the day of the experiments, the membranes were reconstituted at 100 μg protein in 0.5 mL of binding buffer. The binding was equilibrated by incubation at room temperature for 2 h. The ligand–receptor complexes were separated on a glass filter by rapid filtration at 4 °C. Nonspecific binding was assessed in the presence of 100 nM GLP-2. Each filter was collected, and the radioactivity remaining was quantified with a gamma counter.
18. In the comparison between vehicle and treated groups, Bartlett's test for equal variance was conducted, followed by one-way analysis of variance (when $p \geqslant 0.05$ in Bartlett's test) or the Kruskal-Wallis test (when $p < 0.05$ in Bartlett's test) using GraphPad Prism®. Dunnett's multiple comparison test was performed as a post-hoc test.
19. Regression curves were generated by using GraphPad Prism, and peak and EC_{50} values were calculated.