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Discovery of diacylphloroglucinols as a new class of GPR40 (FFAR1) agonists

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ABSTRACT

In this letter, we report discovery of diacylphloroglucinol compounds as a new class of GPR40 (FFAR1) agonists. Several diacylphloroglucinols with varying length of acyl functionality and substitution on aromatic hydroxyls were synthesized and evaluated for GPR40 agonism using functional calcium-flux assay. Out of 17 compounds evaluated, **14**, **17**, **19** and **25** exhibited good GPR40 agonistic activity with EC_{50} values ranging from 0.07 to 8 μM (pEC_{50} 7.12–5.09), respectively, with maximal agonistic response of 84–102%.

The G-protein coupled receptor GPR40 (also known as free fatty acid receptor 1, FFAR1) is highly expressed in human and rodent pancreatic islets and is activated by medium and long-chain free fatty acids (e.g., linoleic and palmitic acids) resulting in stimulation of downstream signaling pathways which activate protein kinase C (PKC) and elevate intracellular [Ca^{2+}].[1,2] Since both activation of PKC and increasing intracellular [Ca^{2+}] play a key role in insulin exocytosis in pancreatic β-cells,[3] GPR40 may serve as a signaling mechanism through which free fatty acids directly affect insulin secretion. Treatment of MIN6 pancreatic β-cells with small interfering RNA (siRNA) specific for GPR40 prevents fatty acid stimulation of insulin secretion. This study further supported that GPR40 may serve as an attractive target to mediate insulin secretion. Furthermore, agents that serve as GPR40 agonists may be useful for the treatment of type-2 diabetes.[4–7]

In the literature, different classes of GPR40 agonists are reported (beyond the fatty acids) as shown in Figure 1. These include aminophenyl propionic acid derivative GW9508 (**1**, pEC_{50} 7.1, GlaxoSmithcline),[8–10] 4-alkoxy phenyl propionic acid derivative **2** (EC_{50} 0.3 μM, Johnson and Johnson),[11] bicyclic compound **3** (EC_{50} 0.1–100 nM, Merck),[12] 4,5-diphenyl-pyrimidinylamino substituted carboxylic acid **4** (104% activation at 100 μM, Sanofi-Aventis),[13] phenylamino-benzoxazole substituted carboxylic acid **5** (111% activation at 100 μM, Sanofi-Aventis),[14] oxadiazolidinedione compound **6** (EC_{50} 0.26 μM, Astellas Pharma),[15] bicyclic carboxylic acid derivative **7** (EC_{50} < 0.1 μM, Amgen Inc.),[16] thiazolidinedione **8** (pEC_{50} 5.1, Heptahelix AB),[17] conformationally constrained 3-(4-hydroxy-phenyl)-substituted propanoic acid **9** (EC_{50} 0.01 μM, Amgen Inc.),[18] cyclopropane carboxylic acid compound **10** (119% activation at 100 μM, Takeda Pharmaceuticals)[19] and phenylpropanoic acid derivative **11** (EC_{50} 19 nM, Takeda Pharmaceuticals).[20] Besides these reports, Tikhonoca et al. have recently identified different new scaffolds active at FFAR1 as full agonists, partial agonists or pure antagonists by virtual screening based on two-dimensional (2D) similarity, three-dimensional (3D) pharmacophore searches and docking studies using structure of known agonists.[21,22] Most of these compounds possess terminal carboxylic acid group with a tether linking to an aromatic nucleus. This letter describes our research efforts towards identification of diacylphloroglucinols as a new series of GPR40 agonists.

Acyl phloroglucinol compounds widely occur in nature in Myrtaceae family and are reported to possess wide range of biological activities viz. antimicrobial, antileishmanial, antimalarial, cancer chemopreventive, antifouling, anti-HIV and plant growth regulatory activity.[23–25] Apart from these reports, this class of compounds are also reported to possess antidiabetic activities viz. macrocarpals isolated from *Eucalyptus grandis* inhibit aldose reductase which is the target enzyme for the control of diabetic complications such as cataracts, retinopathy, neuropathy and nephropathy[26]; achyrofuran has been isolated from *Achyrocline satureioides* by bioactivity-guided fractionation using the db/db mouse model for type-2 diabetes and it significantly lowered blood glucose levels in this model when administered orally at 20 mg/kg/day[27]; and

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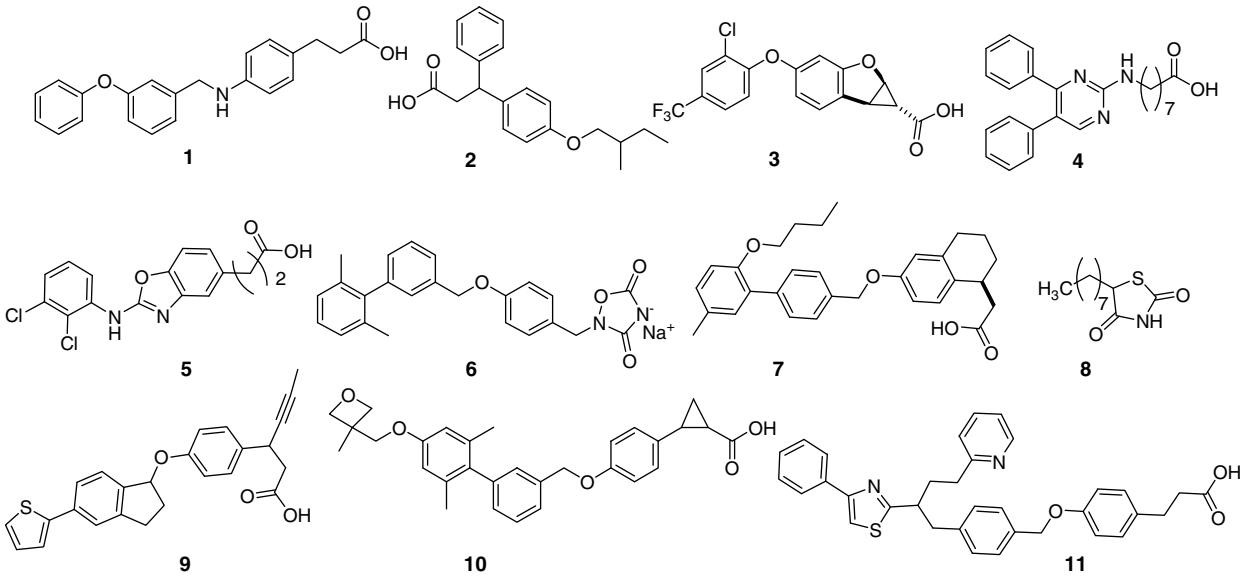


Figure 1. Structures of GPR40 agonists reported in literature.

phlorotannins, such as eckols isolated from *Eisenia bicyclis* exhibited inhibitory activity on glycation and R-amylase and thus possess the potential for the effective treatment of diabetic complications.[28] But this class of compounds have never been evaluated for their GPR40 agonistic potential.[23]

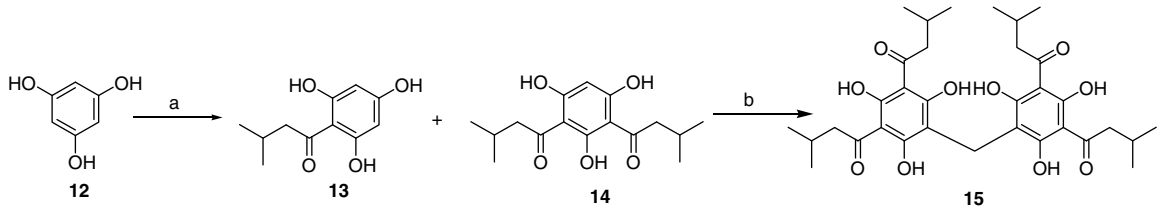
As a part of our continuing efforts towards discovery of new class of compounds against different therapeutic areas[29] and based on the literature reports, we designed a dual pharmacophore which possess a long aliphatic chain of free fatty acids and a phenyl propanoid part of known GPR40 agonists. Herein, we report synthesis of several diacylphloroglucinol compounds using simple synthetic strategy and evaluation of these compounds for GPR40 agonistic activity.

Series of diacyl phloroglucinol compounds were synthesized starting from commercially available phloroglucinol (**12**) by varying acyl functionality. Phloroglucinol (**12**) on treatment with isovaleric acid in presence of boron trifluoride etherate under reflux for 2 h resulted in formation of phloroisovalerophenone (**13**) and 2,4-diisovaleryl phloroglucinol (**14**) in 90% (30:70–mono:di) yield.[30] Compound **14** was further treated with formaldehyde solution in dichloromethane at 60 °C for 8–10 h resulting in formation of dimer **15** in 85% yield (Scheme 1).

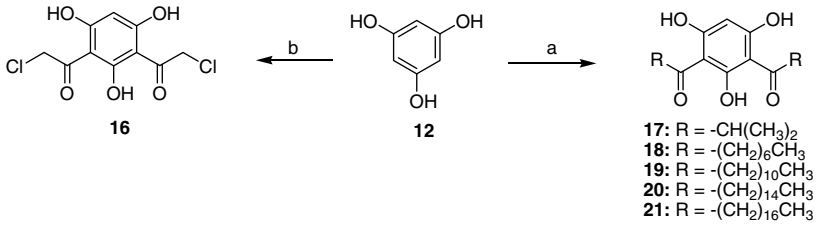
Phloroglucinol (**12**) on treatment with chloroacetyl chloride in presence of $AlCl_3$ in nitrobenzene led to formation of desired 2,4-di-(chloroacetyl) phloroglucinol (**16**) in 50% yield. Further, several diacyl phloroglucinol compounds were synthesized using different short to long-chain fatty acids. Treatment of phloroglucinol (**12**) with isobutyric acid, caprylic acid, lauric acid, stearic acid and palmitic acid in presence of boron trifluoride etherate under reflux for 2 h resulted in formation of desired diacyl phloroglucinol compounds **17–21** in 50–75% yield (Scheme 2). Compound **18** was earlier reported in literature as an antagonist of thromboxane A2 and Leukotriene D4.[31]

In order to develop structure–activity relationship, O- and C-alkylated analogues **22–25** of 2,4-diisovaleryl phloroglucinol (**14**) were synthesized (Scheme 3). Treatment of **14** with *n*-butyl bromide in presence of potassium carbonate in acetone at room temperature for 10 h resulted in formation of three products **22–24** in 50% yield (ratio of **22**:**23**:**24** = 50:30:20). Compound **25** was synthesized by refluxing **14** with *n*-butyl bromide in presence of freshly prepared sodium methoxide for 2 h.[32] Further, analogues with terminal carboxylic acid group on acyl chain (**26** and **28**) were synthesized in order to study the effect of negatively charged functionality on GPR40 agonistic activity. Compounds **26** and **28** were synthesized by treatment of **12** or **13** with glutaric anhydride in presence of BF_3 etherate at room temperature for 2 h in 40–45% yield. However, under similar reaction conditions, when phloroglucinol (**12**) was treated with succinic anhydride in presence of BF_3 etherate, desired product with terminal carboxylic acid group was not formed; instead a condensed product **27** was obtained. Further, a hydroxyethyl substituted analogue **29** was synthesized by reaction of **14** with bromoethanol in presence of potassium carbonate in acetone at room temperature for 10 h in 50% yield. Synthesis of analogues **26–29** is depicted in Scheme 4.

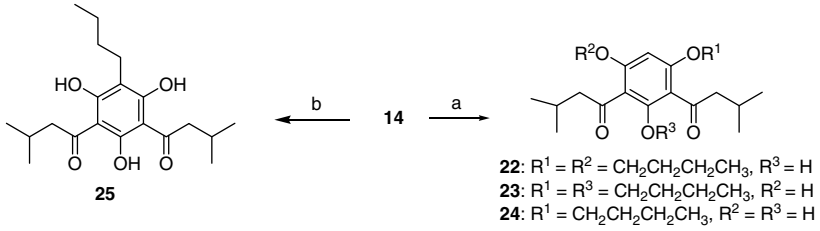
All compounds were evaluated for GPR40 agonistic activity using a functional calcium-flux assay.[1,33] Linoleic acid was used as a standard GPR40 agonist. The agonist activity was measured as the fold increase of calcium flux over control in CHO cells expressing the hGPR40 vector using the Flex Station. The activity of all compounds was compared with that of linoleic acid. Linoleic



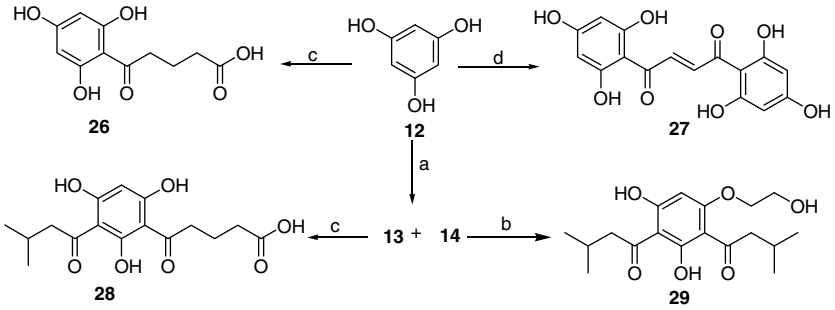
Scheme 1. Reagents and conditions: (a) $(CH_3)_2CHCH_2COOH$, BF_3–Et_2O, 100 °C, 2 h, 90% (mono:di = 30:70); (b) HCHO, CH_2Cl_2, 60 °C, 8–10 h, 85%.



Scheme 2. Reagents and conditions: (a) RCOOH, BF_3–Et_2O, 100 °C, 2 h, 50–75%; (b) $ClCOCH_2Cl$, $AlCl_3$, $PhNO_2$, 60 °C, 2 h, 50%.



Scheme 3. Reagents and conditions: (a) K_2CO_3, acetone, $CH_3CH_2CH_2CH_2Br$ (4 equiv), rt, 10 h, 50%; (b) Na/MeOH, $CH_3CH_2CH_2CH_2Br$, 90 °C, 2 h, 60%.



Scheme 4. Reagents and conditions: (a) $(CH_3)_2CHCH_2COOH$, BF_3–Et_2O, 100 °C, 2 h, 90% (mono:di = 30:70); (b) K_2CO_3/acetone, $BrCH_2CH_2OH$, rt, 10 h, 50%; (c) glutaric anhydride, BF_3–Et_2O, rt, 2 h, 40–45%; (d) succinic anhydride, BF_3–Et_2O, rt, 2 h, 60%.

acid exhibited GPR40 agonism with an EC_{50} of 0.54 μM. Previously, Itoh et al. have reported comparable calcium flux response in the FLIPR for linoleic acid (EC_{50} = 1.8 μM) with similarly hGPR40 transfected CHO cells.[2] Out of the 17 compounds tested, compounds **14**, **17**, **19** and **25** showed good agonistic activity at 10 μM (Table 1).

Table 1
GPR40 agonistic activity of diacylphloroglucinols.

Entry	GPR40 agonist activity (at 10 μM)		
	Fold increase	Calcium flux	Comments
13	1.116	Not observed	Not active
14	1.893	Observed	Active
15	1.115	Not observed	Not active
16	1.211	Not observed	Not active
17	2.313	Observed	Active
18	1.211	Not observed	Not active
19	2.326	Observed	Active
20	1.024	Not observed	Not active
21	1.185	Not observed	Not active
22	1.347	Not observed	Not active
23	1.459	Not observed	Not active
24	1.024	Not observed	Not active
25	2.121	Observed	Active
26	1.000	Not observed	Not active
27	1.006	Not observed	Not active
28	1.003	Not observed	Not active
29	1.018	Not observed	Not active
Linoleic acid	2.547	Observed	Active

Specificity of compounds for GPR40 was confirmed by running parallel Ca^{2+} flux assay in CHO cells with the vector lacking the hGPR40 transfection. From results (Table 1), it was noticed that substitution of phenolic hydroxyls with alkyls (**22–24**) resulted in loss of activity while alkylation on aromatic ring (**25**) did not alter GPR40 agonistic potential. Dimerization (compound **15**) of active compound **14** resulted in loss of activity. Analogues with terminal carboxylic acid group on acyl chain (**26** and **28**) did not showed GPR40 agonistic activity. As well as hydroxyethyl substituted analogue **29** was inactive.

Table 2
EC_{50} and pEC_{50} values of active compounds.*

Entry	EC_{50}[a] (pEC_{50}[b]) (μM)	% Maximal response[c]
14	6.0 ± 0.1 (5.22 ± 0.01)	102.2 ± 5.3%
17	8.0 ± 0.2 (5.09 ± 0.01)	88.7 ± 6.2%
19	0.07 ± 0.004 (7.12 ± 0.03)	100.2 ± 1.9%
25	0.97 ± 0.06 (6.01 ± 0.03)	84.5 ± 2.3%
Linoleic acid	0.54 ± 0.02 (6.26 ± 0.02)	—

[a] Concentration of the samples that produce 50% of the maximal response, and was calculated from dose–response curves.
[b] Negative log of the molar concentration required to elicit a half-maximal response.
[c] The maximal agonistic response elicited by the compound as a percentage of the maximal response evoked by Linoleic acid at 10 μM.
* Data represents mean value ± SEM.

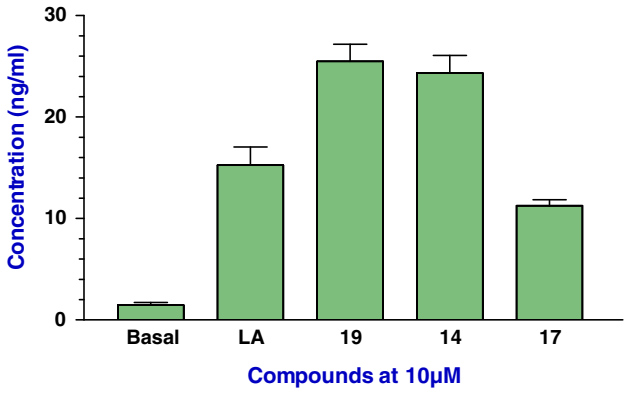


Figure 2. Effect of compounds **14**, **17**, **19** and linoleic acid on insulin secretion from the hamster pancreatic insulinoma cell line (HIT-T15).

Active compounds **14**, **17**, **19** and **25** were further tested and EC_{50} and pEC_{50} values were determined as shown in Table 2. Compound **25** showed EC_{50} of 0.97 μM (pEC_{50} 6.01) with 84.5% maximal response, which suggests that introduction of alkyl chain on aromatic nucleus of **14**, resulted in improved GPR40 agonistic activity than that of **14** (EC_{50} 6 μM, pEC_{50} 5.22, 102.2% maximal response). Compound **19** showed potent activity with EC_{50} value of 0.07 ± 0.004 μM (pEC_{50} 7.12, 100.2% maximal response). The efficacy of the compound is shown as a percentage of the maximal agonistic response elicited by the test compound with respect to the maximal response evoked by the internal standard (linoleic acid) at a dose of 10 μM. In order to determine the selectivity, compound **19** was also tested for hPPAR-γ agonistic activity and it showed very weak agonistic activity for hPPAR-γ. DMPK study on one of representative of the scaffold (compound **14**) was performed and pharmacokinetic parameters (T_{max} 0.25 h, C_{max} 0.88 μg/mL, AUC_{last} 3–13 h μg/mL, $AUC_{0\text{-infinity}}$ 3.41 h μg/mL and half-life 6.59 h) showed that this scaffold has good PK properties.

Activation of the GRP40 receptor is known to play a role in pancreatic and neurological function and the receptor is specifically localized in the brain and pancreas.[1] In the pancreas the receptor expression is restricted to insulin producing β-cells. Since the key objective of GPR40 agonists is to prime islet β-cells to respond to glucose by inducing insulin secretion, we further evaluated these active compounds for their ability to induce insulin secretion in pancreatic islet cells. Further, the GPR40 mRNA is known to be expressed significantly in most pancreatic β-cell lines with highest expression levels in MIN6, followed by β-TC and HIT-T15.[2] We therefore selected the HIT-T15 cell line for insulin secretion studies. HIT-T15 from hamsters were treated with 10 μM of compound **14**, **17** and **19**[34] Each of these induced insulin secretion comparable to the effect of linoleic acid (Fig. 2). Further, the inactive compounds did not show any antagonist effect for linoleic acid induced calcium flux (data not included).[9]

In conclusion, compounds that activate GPR40 at low submicromolar concentrations have been identified, the majority of which behave as full agonists as compared to the endogenous long-chain fatty acid ligands. This series of compounds have a potential to emerge as a lead candidate with potent GPR40 agonistic activity for the treatment of type-2 diabetes.

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30. *A typical procedure for synthesis of diacylphloroglucinols:* A mixture of phloroglucinol (**12**, 1 mmol), respective carboxylic acid (2.5 mmol) and boron trifluoride-diethyl ether (10 mL) was refluxed at 100 °C for 2 h. Reaction mixture was poured in crushed ice and stirred for 5 min and then extracted with ethyl acetate. Ethyl acetate layer was washed with brine solution and finally dried over anhydrous sodium sulphate. Solvent was removed under reduced pressure and crude product was purified by silica gel column chromatography (#100–200) using hexane/EtOAc (3:1) as eluent. 1,3-Di-(3-methyl-butanoyl)-2,4,6-trihydroxy benzene (**14**): cream colored solid; yield: 67%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.84 (s, 1H), 2.98 (d, J = 6.4 Hz, 4H), 2.25 (m, 2H), 0.99 (d, J = 6.6 Hz, 12H); CIMS: m/z 295 $[M+1]^+$. 1,3-Diisobutyryl-2,4,6-trihydroxy benzene (**17**): cream colored sticky solid; yield: 55%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.13 (s, 1H), 3.93 (m, 2H), 1.17 (d, J = 6.7 Hz, 12H); CIMS: m/z 267 $[M+1]^+$. 1,3-Didodecanoyl-2,4,6-trihydroxybenzene (**19**): cream colored solid; yield: 60%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.27 (s, 1H, OH), 5.79 (s, 1H), 3.10 (t, J = 7.5 Hz, 4H), 1.67 (m, 4H), 1.32 (m, 32H), 0.98 (t, J = 7.5 Hz, 6H); MS (ESI): m/z 489.4 $[M-1]^+$.
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32. *Procedure for synthesis of 1,3-diisopentanoyl-5-butyl-2,4,6-trihydroxybenzene (**25**):* To a solution of **14** (1 mmol) in methanol, a freshly prepared solution of sodium methoxide was added followed by addition of *n*-butyl bromide (1.2 mmol). Reaction mixture was then refluxed for 2 h. Solvent was evaporated on vacuo rotavapor and crude product was purified by silica gel column chromatography (#100–200) using hexane/EtOAc (4:1) as eluent. Yield: 60%; yellow oil; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.18 (s, 1H), 14.96 (s, 1H),

2.99 (d, J = 6.9 Hz, 4H), 2.57 (m, 2H), 2.28 (m, 2H), 1.52–1.46 (m, 4H), 0.99 (m, 15H); MS (APCI): m/z 351.1 $[M+1]^+$.

33. GPR40 assay[1]: CHO K1 cells stably transfected with hGPR40 in the vector pCDN were seeded in the density of 10,000 cells/well into poly D-lysine coated 96-well microplate, black/clr bottom. After 48 h, the cells were loaded for 1 h with 2.5 μM Fluo-4AM fluorescent indicator dye (Molecular probes, Inc.,) in assay buffer (10 mM Hepes, 200 μM Ca^{2+} and 2.5 mM probenecid in 1× HBSS, pH 7.5). Cells were washed three times with assay buffer and incubated at 37 °C for 10 min before reading in the FlexStation II. The change in fluorescence over baseline was used to determine the agonistic response. The agonistic responses for the screened actives were checked with the mock control (CHO K1 cells transfected without hGPR40 in the vector pCDN) for its specificity (data not shown). Statistical analysis was performed with Graphpad prism software, version 3.03.
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