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Short communication

Discovery of gemfibrozil analogues that activate PPARα and enhance the expression of gene CPT1A involved in fatty acids catabolism

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ABSTRACT

A new series of gemfibrozil analogues conjugated with α-asarone, *trans*-stilbene, chalcone, and their bioisosteric modifications were synthesized and evaluated to develop PPARα agonists. In this attempt, we have removed the methyls on the phenyl ring of gemfibrozil and introduced the above scaffolds in para position synthesizing two series of derivatives, keeping the dimethylpentanoic skeleton of gemfibrozil unaltered or demethylated. Four compounds exhibited good activation of the PPARα receptor and were also screened for their activity on PPARα-regulated gene CPT1A.

1. Introduction

Peroxisome proliferator activated receptors (PPARs) are transcriptional factors belonging to the ligand-activated nuclear receptor superfamily; they consist of isoforms α (NR1C1), γ (NR1C3), and δ/β (NR1C2), encoded by separate genes [1]. The PPARs are essentially dietary fat sensors that maintain lipid and glucose homeostasis through control of a network of genes involved in metabolism [2]. PPARs bind as heterodimers with another nuclear receptor, the retinoid X receptor, to specific response elements termed peroxisome proliferator response elements (PPRE) and alter the transcription rate of target genes [3]. PPARα mainly regulates genes implicated in lipid oxidation, being expressed in cells with high catabolic rates of fatty acids, such as hepatocytes [4]. PPARγ, which was identified as an isoform highly expressed in adipose tissue, is related to body energy storage, and is indeed a key transcription factor involved in terminal differentiation of adipocytes [5]. PPARδ is expressed in many tissues, albeit at low levels in the liver; its primary functions are the control of fatty acid catabolism and energy homeostasis [6]. Fibrates are widely prescribed as hypolipidemic agents, that elicit biological effects by activating the PPARs, acting mainly on the PPARα isoform [7,8]. As a consequence of fibrates administration, marked changes are produced in the gene expression of key enzymes involved in the synthesis of several apolipoproteins and lipoprotein metabolism. The α-asarone (Fig. 1a) is a substance with potent hypolipidemic activity, mainly found in the medicinal plant *Guatteria gaumeri* [9]. It has been found to elevate high density lipoprotein–cholesterol (HDL–cholesterol) fraction and to reduce low density lipoprotein–cholesterol (LDL–cholesterol), by inhibiting cholesterol biosynthesis parallel to the statin mechanism, with inhibitory effect on hepatic 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) [10]. Several synthetic studies of α-asarone have been carried out, providing evidence that the hypocholesterolemic activity was enhanced when the methyl in C-2 methoxy group was changed into an acetic group, miming the pharmacophore of classical fibrates, like clofibrate, and the propenyl side chain was unconjugated, by moving the double bond from the original position to the vicinal carbon [11]. Stilbenes [12] and chalcones [13] (Fig. 1b), originally isolated from natural plant sources, are molecules with relatively simple structure widely known for antiinflammatory and antioxidant properties. Some their synthetic derivatives have also shown to lower plasma lipid levels and a significant PPARα activation was observed [14,15]. Among them, some gemfibrozil derivatives based on the resveratrol scaffold, the

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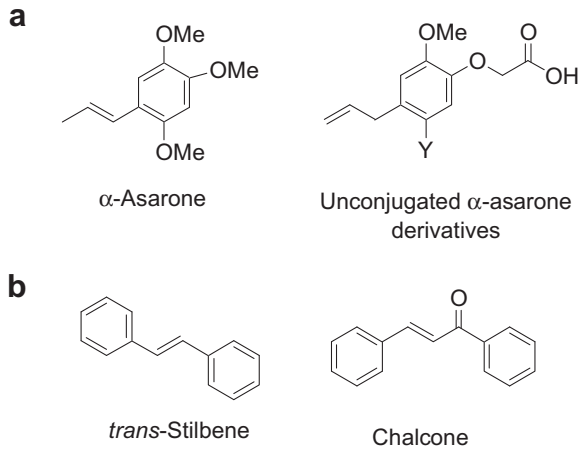


Fig. 1. (a) α-Asarone and unconjugated derivatives; (b) *trans*-stilbene and chalcone.

most studied stilbene with beneficial cardiovascular effects, appeared a good starting point for the development of novel pan-PPAR agonists for the treatment of metabolic diseases [16].

Since fibrates are weak PPARα agonists, we were interested in profiling compounds with increased potency and selectivity for PPARα as lipid-lowering drugs. We previously reported the pharmacological evaluation of a series of compounds related to clofibric acid [17], the active metabolite of clofibrate, and gemfibrozil [18], with different PPARα agonistic activity. The novel structural features of described compounds coupled with their interesting *in vitro* biological profile prompted us to embark on further investigation. In this study, we wish to report the combination of gemfibrozil with α-asarone, stilbene, chalcone, and other bioisosteric modifications, to develop new PPARα agonists and verify the hypolipidemic effect via PPARα pathway.

In this attempt, we have removed the methyls on phenyl ring and introduced the above scaffolds in *para*-position; we synthesized two series of derivatives, branched or linear, keeping the dimethylpentanoic skeleton of gemfibrozil unaltered or demethylated (Fig. 2). The *in vitro* transactivation assay was used to test the PPARα activation of new compounds, and for a preliminary evaluation of selectivity over PPARγ; compounds with best EC_{50} were also selected for the *in vitro* evaluation of CPT1A gene activation by real-time quantitative PCR analysis.

2. Results and discussion

2.1. Chemistry

The acids **17**–**24** and **32**–**38** were easily obtained in good yields by standard esterification procedures followed by hydrolysis. The hydrophobic building blocks phenols **1**–**8** were commercially

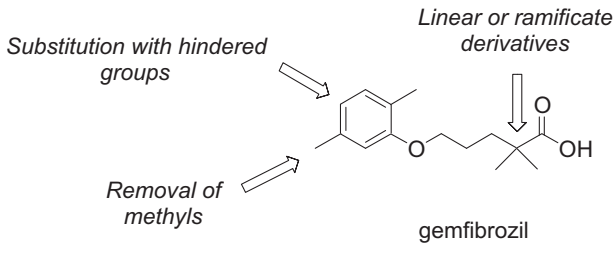


Fig. 2. Chemical modifications of gemfibrozil.

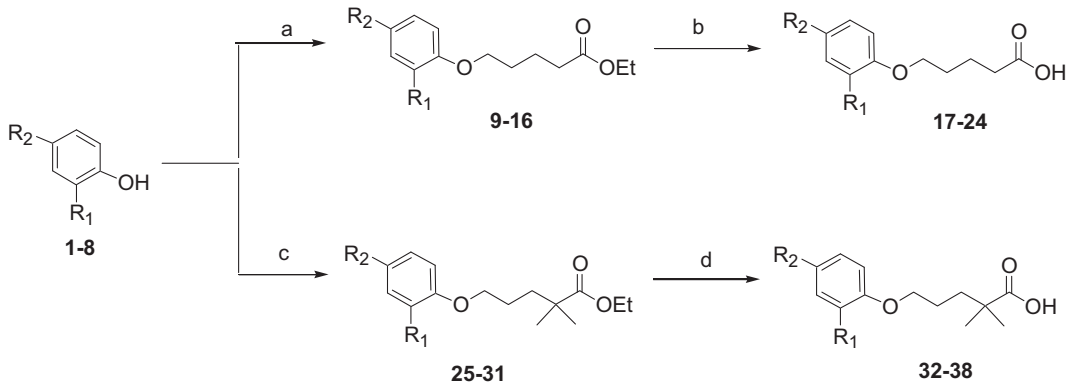
available or obtained according to literature procedure [19,20] (Scheme 1). Ethylic or isobutylic esters **9**–**14**, **16** and **25**–**31** were obtained by S_N2 reaction of nucleophiles **1**–**8** with proper 5-bromo- or 5-chloroesters, in the presence of sodium in absolute ethanol at reflux; only ester **15** was obtained under mild conditions, in the presence of sodium in absolute ethanol at room temperature. The branched ester containing the ureidic function was instable at room temperature, therefore was not obtained. The 5-bromo-2,2-dimethylpentanoic acid ethyl ester was synthesized as reported in literature [21], while the other nucleophiles are commercial reagents. The basic hydrolysis gave the desired acids **17**–**24** and **32**–**38**.

2.2. Biology

All new compounds were evaluated for *in vitro* potency and subtype selectivity in a cell-based transactivation assay in eukaryotic cells [22]; our reporter systems utilizes firefly luciferase reporter gene technology [23] to provide optimal assay sensitivity, dynamic range when quantifying nuclear receptor activity and good correlation with *in vivo* activity. We used two compounds as positive controls: gemfibrozil (150 μM) and GW7647 (1 μM), an ureido-based fibric acid with PPARα agonistic activity in the nanomolar range compared to the micromolar range for the fibrates. The results of our investigation are summarized in Table 1. The agonist responses were expressed as EC_{50}, defined as the concentration of test compound to produce 50% of maximal reporter activity. The efficacy was measured against that of reference compound gemfibrozil, normalized to 100%.

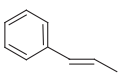
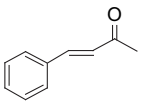
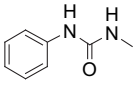
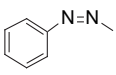
All compounds showed a significant activation of the receptor, except for **18**, **22**, **24**, and **33**, that were found inactive. Combination of gemfibrozil with chalcone scaffold exhibited detrimental effects, which are evident from the activity of both derivatives **20** and **35** (EC_{50} 109.3 and 61.2 μM, respectively). All other tested compounds exhibited better values of EC_{50} than the classical fibrate gemfibrozil. The analogues containing the dimethylpentanoic chain of gemfibrozil were found to be more potent PPARα activators than gemfibrozil, except **35**. The highest agonistic activity was seen with the *trans*-stilbene derivative **34** ($EC_{50} = 1.0$ μM), showing that the wide electron delocalization could be an important factor to determine agonistic PPARα activity; moreover, based on docking studies for similar molecules [14,16], we hypothesize that our compounds fit very well into the receptor Y-shaped pocket, establishing hydrophobic interactions in comparison with gemfibrozil. Compounds containing the unconjugated α-asarone (**32**), benzophenone (**36**), phenoxy (**37**), and the diazocompound **38**, showed EC_{50} values ranging between 6.6 and 43.3 μM. When the methyls in α to the carboxylic group of gemfibrozil were deleted, generating linear derivatives, it resulted in a weaker agonistic activity as compared to all ramificated compounds; for this reason, we envisioned that dimethylpentanoic chain of gemfibrozil plays an important role in the modulation of potency of the test compounds. Among linear derivatives, compounds **17**, **19**, **21**, and **23**, containing respectively asarone, stilbene, benzophenone, and diazophenyl *para*-substituents on the aromatic system, were found more potent of gemfibrozil. Only compound **20**, in which the aromatic ring was substituted with the chalcone skeleton, failed to retain its potency toward lead compound ($EC_{50} = 109.3$ μM).

With the aim to achieve preliminary information on selectivity PPARα/γ, we performed the transactivation assay on both the isoforms, testing selected compounds at concentration of PPARα EC_{50}. In these conditions, gemfibrozil showed a moderate selectivity for PPARγ isoform (0.7), whereas the other tested compounds were found to be essentially equipotent dual activators, with selectivity around 1. An increase of selectivity was seen only with GW7647 (2.9) and chalcone derivatives **20** and **35** (3.0 and 2.1, respectively). This



Scheme 1. a) $Br(CH_2)_4COOEt$, Na, EtOH, reflux, 10–15 h; only for ester **15**, $Br(CH_2)_4COOEt$, Na_2CO_3, acetone, rt, 10–15 h; b) KOH, EtOH, reflux, 6–10 h; only for acid **23**, 10% KOH, EtOH, rt, 6 h; c) $Cl(CH_2)_3C(CH_3)_2COOEt$ or $Br(CH_2)_3C(CH_3)_2COOiBu$, Na, EtOH, reflux, 10–15 h; d) 10% KOH or 2 N NaOH, EtOH, reflux, 6–10 h.

Table 1
In vitro PPAR transactivation of test and reference compounds.

Compound	R_1	R_2	Linear (L) or branched (B)	EC_{50}[a]	Efficacy (%)[b]	Selectivity PPARα/γ
17	OCH_3		L	11.3 ± 1.1	94 ± 11	0.8
32			B	6.6 ± 0.6	117 ± 4	1.0
18	H		L	ia[c]	ia	–[d]
33			B	ia	ia	–
19	H		L	18.8 ± 1.2	206 ± 12	1.1
34	H		B	1.0 ± 0.02	223 ± 18	1.4
20	H		L	109.3 ± 3.0	235 ± 10	3.0
35	H		B	61.5 ± 2.2	165 ± 14	2.1
21	H		L	27.1 ± 0.3	123 ± 11	1.0
36			B	9.3 ± 0.9	82 ± 10	0.9
22	H		L	ia	ia	–
37			B	9.1 ± 0.9	118 ± 12	1.4
23	H		L	9.3 ± 0.9	106 ± 4	1.3
24	H		L	ia	ia	–
38			B	43.7 ± 0.4	76 ± 3	0.8
Gemfibrozil				59.0 ± 3.9	100 ± 3	0.7
GW7647				0.2 ± 0.02	164 ± 13	2.9

[a] Compounds were tested in at least three separate experiments at five concentrations ranging from 1 to 150 μM. The results are expressed ± SEM.
[b] Compounds were tested at least three separate experiments at 150 μM. Only 1 was tested at 1 μM. Efficacy values were calculated as percentage of the maximum obtained fold induction with the reference compound gemfibrozil.
[c] ia: inactive at tested concentration.
[d] Not determined.

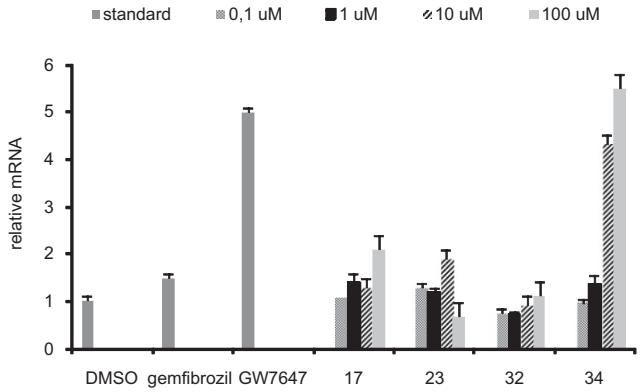


Fig. 3. CPT1A expression in HepG2 following treatment. RTqPCR was performed to measure CPT1A mRNA levels. Values shown represent the mean ± SEM of four independent determinations performed in duplicate. Cyclophilin was used as reference gene, and values were normalized to data obtained from vehicle treated cells.

evaluation was only a qualitative overview, but furnished a selectivity profile in order to explore a possible activation of PPARγ.

In the liver, PPARα has been shown to affect fatty acid import into hepatocyte mitochondria by up-regulating expression of the liver-predominant mitochondrial carnitine palmitoyl acyl-CoA transferase 1 (CPT1A) [24]; the expression pattern of this gene is a well established *in vitro* model to study PPARα activation [25]. Human hepatocellular carcinoma cell line HepG2 was used to analyze the impact of selected compounds **17**, **23**, **32**, and **34** (with best EC_{50} values) on PPARα expression; measurement of mRNA concentration was done as a quantitative analysis by RTqPCR. HepG2 cells were stimulated with increasing amounts of selected compounds (from 1 to 100 μM) and compared with gemfibrozil and GW7647 at EC_{50} concentration; control cells were treated with DMSO alone (Fig. 3).

Stimulation of HepG2 cells with gemfibrozil showed a slight increase of mRNA level, while compounds **17**, **23**, and **32** did not influence the CPT1A expression. Incubation of cells with increasing amounts of **34** led to a significant and concentration-dependent increase of mRNA levels; indeed, at intermediate concentration of 10 μM the mRNA induction was doubled with respect to gemfibrozil and at 100 μM the relative mRNA was about 6.0. This value of mRNA level is a little higher than the potent PPAR activator GW7647 (5.0). The up-regulation of CPT1A gene by **34** well also corresponds with the best EC_{50} value compared to other tested compounds, showing that the combination of stilbene scaffold and gemfibrozil enhances the PPARα agonistic activity.

3. Conclusions

In the course of our study with gemfibrozil derivatives, we constructed our structure–activity relationships with data derived from cell-based functional assay. This investigation led to the identification of several active compounds; among them, the stilbene derivative **34** was found more effective than gemfibrozil in ability to activate PPARα. Noticeably, its profile of transactivation, selectivity and CPT1A gene induction are similar to full PPARα agonist GW7647, providing a good starting point for development of new lead compounds in the treatment of lipid disorders.

4. Experimentals

4.1. Chemistry

4.1.1. Materials and methods

Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Infrared spectra were recorded on an FT-IR 1600 Perkin–Elmer spectrometer. NMR spectra were run at 300 MHz on a Varian instrument; chemical shifts (δ) are reported in ppm. Microanalyses were carried out with a Eurovector Euro EA 3000 model analyzer. GC analyses were run on an autosystem GC Perkin Elmer apparatus using a fused silica capillary column (30 m, 0.53 mm ID), SPB-5 Supelco. Commercial reagents were used as received from Aldrich or Fluka. We report only chemical data for PPARα activators more potent than the gemfibrozil ($EC_{50} < 59$ μM) and their previous esters.

4.1.2. General procedure for the preparation of esters ***9*–*16*** and ***25*–*31***

A solution of phenol (5 mmol) was added to a solution of sodium (114 mg, 5 mmol) in absolute EtOH (5 mL) at room temperature. After stirring for 30 min, a solution of suitable ethyl or isobutyl ester (5-bromovalerate, 5-bromo-2,2-dimethylpentanoic acid ethyl ester or 5-chloro-2,2-dimethylpentanoic acid isobutyl ester) (5 mmol) in absolute EtOH (5 mL) was added and the solution was stirred at reflux for 10–15 h. The solvent was removed under reduced pressure and the residue was dissolved into CH_2Cl_2 or ethyl acetate (25 mL) and washed with NaOH 2 N (3 × 25 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to yield the crude product that was purified by column chromatography to give the ester **9**–**14**, **16** or **25**–**31**. Ester **15** was obtained with the same procedure, but under milder conditions: K_2CO_3 (1.1 g, 8.38 mmol) was added to a solution of *N*-(4-hydroxyphenyl)-*N*′-phenylurea (531 mg, 2.33 mmol) in acetone (10 mL) at room temperature. After stirring for 30 min, a solution of ethyl 5-bromovalerate (488 mg, 2.33 mmol, 0.37 mL) in acetone (10 mL) was added. After 30 h the solvent was removed under reduced pressure and the residue was treated as above to give the compound **15**.

*4.1.2.1. Ethyl 5-(4-allyl-2-methoxyphenoxy)pentanoate (**9**).* Yellow oil, 90 mg, 3.2 mmol, 65% yield. IR (neat) 3076, 2937, 2872, 1732, 1589, 1513, 1260, 1036 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.24 (t, $J = 7.4$ Hz, 3H, CH_3CH_2), 1.61–1.90 (m, 4H, CH_2CH_2), 2.37 (t, $J = 7.4$ Hz, 2H, CH_2CO), 3.32 (d, $J = 6.6$, 2H, CH_2C_{Ar}), 3.83 (s, 3H, CH_3O), 3.99 (t, $J = 6.0$ Hz, 2H, CH_2CH_3), 4.11 (q, $J = 7.2$ Hz, 2H, CH_2O), 5.06 (t, $J = 6.9$ Hz, 2H, $H_2C{=}CH$), 5.90–5.96 (m, 1H, $H_2C{=}CH$), 6.70 (s, 2H, $CH_{Ar}CH_2$), 6.79 (d, $J = 8.7$ Hz, 1H, $CH_{Ar}CO$); ^{13}C NMR ($CDCl_3$) δ 14.47 (CH_3CH_2), 21.79 (CH_2CH_2CO), 28.90 (CH_2CH_2O), 34.22 (CH_2CO), 40.04 ($CH_2C{=}C$), 56.14 (CH_3O), 60.52 (CH_2CH_3), 68.82 (CH_2O), 112.54 ($C_{Ar}HCOCH_3$), 113.45 ($C_{Ar}HC$), 115.83 ($CH_2{=}C$), 120.65 ($C_{Ar}HCCH_2$), 133.10 ($C_{Ar}CH_2$), 137.92 ($CH{=}CH_2$), 147.11 ($C_{Ar}O$), 149.58 ($C_{Ar}OMe$), 173.74 (C=O). Anal. Calcd for $C_{17}H_{24}O_4$: C, 69.84; H, 8.27. Found: C, 69.82; H, 8.30.

*4.1.2.2. Ethyl 5-{4-[(E)-2-phenylvinyl]phenoxy}pentanoate (**11**).* White solid, 97 mg, 2.9 mmol, 60% yield, mp 107–109 °C. IR (KBr) 3076, 2937, 1732, 1513, 1260, 1036 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.26 (t, $J = 7.2$ Hz, 3H, CH_3CH_2), 1.68–1.80 (m, 4H, CH_2CH_2), 2.39 (m, 2H, CH_2CO), 3.99 (t, $J = 4.5$ Hz, 2H, CH_2O), 4.13 (q, $J = 7.2$ Hz, 2H, CH_2CH_3), 6.8 (d, $J = 8.7$ Hz, 2H, CH_{Ar}), 7.0 (q, $J = 14.1$ Hz, 2H, $HC{=}CH$), 7.17 (m, 1H, CH_{Ar}), 7.34 (t, $J = 7.2$ Hz, 2H, CH_{Ar}), 7.44 (d, $J = 9.0$ Hz, 2H, CH_{Ar}), 7.49 (d, $J = 7.8$ Hz, 2H, CH_{Ar}); ^{13}C NMR (CD_3OD) δ 14.49 (CH_3), 21.88 (CH_2CH_2CO), 28.90 (CH_2CH_2O), 34.19 (CH_2CO), 60.57 (CH_2CH_3), 67.65 (CH_2O), 114.89 ($C_{Ar}H$), 126.46 ($CH{=}CH$), 126.76 ($C_{Ar}H$), 127.42 ($C_{Ar}H$), 127.93 ($C_{Ar}H$), 128.45 ($CH{=}CH$), 128.87 (C_{Ar}), 130.29 (C_{Ar}), 137.88 (C_{Ar}), 158.91 ($C_{Ar}O$), 173.70 ($C{=}O$). Anal. Calcd for $C_{21}H_{24}O_3$: C, 77.75; H 7.46. Found: C, 77.80; H 7.41.

*4.1.2.3. Ethyl 5-(4-benzoylphenoxy)pentanoate (**13**).* Uncolored oil, 1.19 g, 3.4 mmol, 70% yield. IR (neat) 3061, 2941, 1732, 1653, 1601, 1255 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.25 (t, $J = 7.2$ Hz, 3H, CH_3CH_2),

1.84–1.82 (m, 4H, CH_2CH_2), 2.36–2.40 (m, 2H, CH_2COO), 4.02–4.10 (m, 2H, CH_2O), 4.12 (q, J = 7.2 Hz, 2H, CH_2CH$_3$), 6.90 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.47 (d, J = 7.2 Hz, 2H, CH_{Ar}), 7.51–7.54 (m, 1H, CH_{Ar}), 7.74 (d, J = 6.6 Hz, 2H, CH_{Ar}), 7.8 (d, J = 9 Hz, 2H, CH_{Ar}); ^{13}C NMR ($CDCl_3$) δ 14.48 (*C*H$_3$CH$_2$), 21.78 (*C*H$_2$CH$_2$CO), 28.72 (*C*H$_2$CH$_2$O), 34.09 (*C*H$_2$CO), 60.61 (*C*H$_2$CH$_3$), 67.85 (*C*H$_2$O), 114.92 (*C*$_{Ar}$), 128.16 (*C*$_{Ar}$), 129.8 (*C*$_{Ar}$), 132.31 (*C*$_{Ar}$), 138.22 (*C*$_{Ar}$CO), 162.17 (*C*$_{Ar}$O), 173.71 (*C*OO), 195.98 (*C*O). Anal. Calcd for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79. Found: 73.58; H, 6.80.

*4.1.2.4. Ethyl 5-{4-[(anilinocarbonyl)amino]phenoxy}pentanoate (**15**).* White solid, 500 mg, 1.40 mmol, 60% yield, mp 152–153 °C. IR (KBr) 1729 cm^{-1}; ^{1}H NMR (Ac_D) δ 1.20 (t, J = 7.2 Hz, 3H, CH_3CH$_2$), 1.76–1.78 (m, 4H, CH_2CH_2), 2.36–2.40 (m, 2H, CH_2COO), 3.95–3.98 (m, 2H, CH_2O), 4.08 (q, J = 7.2 Hz, 2H, CH_2CH$_3$), 6.85 (d, J = 9 Hz, 2H, CH_{Ar}), 6.94–6.98 (m, 1H, C$_{Ar}$), 7.25 (t, J = 7.8 Hz, 2H, C$_{Ar}$), 7.41 (d, J = 9 Hz, 2H, C$_{Ar}$), 7.51 (d, J = 8.1 Hz, 2H, C$_{Ar}$), 7.90 (s, 1H, N*H*CONH), 8.01 (s, 1H, NHCON*H*); ^{13}C NMR (Ac_D) δ 14.48 (*C*H$_3$CH$_2$), 21.78 (*C*H$_2$CH$_2$CO), 28.72 (*C*H$_2$CH$_2$O), 34.09 (*C*H$_2$CO), 60.61 (*C*H$_2$CH$_3$), 67.85 (*C*H$_2$O), 116.60 (*C*$_{Ar}$), 120.43 (*C*$_{Ar}$), 118.6 (*C*$_{Ar}$), 122.65 (*C*$_{Ar}$), 129.26 (*C*$_{Ar}$), 134.13 (*C*$_{Ar}$NH), 139.42 (*C*$_{Ar}$NH), 152.14 (*C*$_{Ar}$O), 115.57 (*C*ONH), 173.71 (*C*OO). Anal. Calcd for $C_{20}H_{24}N_2O_4$: C, 67.40; H, 6.79; N, 7.86. Found: C, 67.38; H, 6.77; N, 7.89.

*4.1.2.5. Ethyl 5-(2-methoxy-4-allyl-phenoxy)-2,2-dimethyl pentanoate (**25**).* Yellow oil, 1.39 g, 4.3 mmol, 87% yield. IR (nujol) 3076, 2937, 1722, 1513, 1260, 1036 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.18 (s, 6H, CH_3), 1.24 (t, J = 7.2 Hz, 3H, CH_3CH$_2$), 1.61–1.90 (m, 4H, CH_2CH_2), 3.32 (d, J = 6.6 Hz, 2H, CH_2C$_{Ar}$), 3.83 (s, 3H, CH_3O), 3.99 (t, J = 6.0 Hz, 2H, CH_2CH$_3$), 4.11 (q, J = 7.2 Hz, 2H, CH_2O), 5.06 (t, J = 6.9 Hz, 2H, H_2C=CH), 5.90–5.96 (m, 1H, H$_2$C=C*H*), 6.70 (s, 2H, CH_{Ar}CH$_2$), 6.69 (d, J = 8.7 Hz, 1H, CH_{Ar}CO); ^{13}C NMR ($CDCl_3$) δ 14.47 (*C*H$_3$CH$_2$), 25.18 (CH$_3$), 25.34 (CH$_3$), 28.90 (*C*H$_2$CH$_2$O), 36.08 (CH$_2$*C*H$_2$CO), 40.04 (*C*CO), 42.19 (*C*H$_2$C=C), 56.01 (*C*H$_3$O), 60.52 (*C*H$_2$CH$_3$), 69.54 (*C*H$_2$O), 112.54 (*C*$_{Ar}$HCOCH$_3$), 113.45 (*C*$_{Ar}$HCO), 115.83 (*C*H$_2$=C), 120.65 (*C*$_{Ar}$HCCH$_2$), 133.10 (*C*$_{Ar}$CH$_2$), 137.92 (*C*H=CH$_2$), 147.11 (*C*$_{Ar}$O), 149.58 (*C*$_{Ar}$OMe), 173.74 (C=O). Anal. Calcd for $C_{19}H_{28}O_4$: C, 71.22; H, 8.81. Found: C, 71.19; H, 8.80.

*4.1.2.6. Ethyl 2,2-dimethyl-5-{4-[(E)-2-phenylvinyl]phenoxy}pentanoate (**27**).* White solid, 1.09 g, 3.1 mmol, 62% yield. IR (neat) 3022, 2974, 1720, 1604, 1513, 1248, 1048 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.21 (s, 6H, CH_3), 1.25 (t, J = 6.9 Hz, 3H, CH_3CH$_2$), 1.68–1.80 (m, 4H, CH_2CH_2), 3.95 (t, J = 5.7 Hz, 2H, CH_2O), 4.12 (q, J = 7.2 Hz, 2H, CH_2CH$_3$), 6.8 (d, J = 8.4 Hz, 2H, CH_{Ar}), 7.0 (q, J = 14.1 Hz, 2H, *H*C=C*H*), 7.24 (d, J = 8.1 Hz, 1H, CH_{Ar}), 7.34 (t, J = 7.2 Hz, 2H, CH_{Ar}), 7.38 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.48 (d, J = 7.2 Hz, 2H, CH_{Ar}); ^{13}C NMR (CD_3OD) δ 14.49 (*C*H$_3$), 25.17 (CH$_3$), 21.88 (*C*H$_2$CCO), 28.90 (*C*H$_2$CH$_2$O), 36.97 (*C*CO), 60.57 (*C*H$_2$CH$_3$), 67.65 (*C*H$_2$O), 114.89 (*C*$_{Ar}$H), 126.46 (*C*H=CH), 126.76 (*C*$_{Ar}$H), 127.42 (*C*$_{Ar}$H), 127.93 (*C*$_{Ar}$H), 128.45 (CH=*C*H), 128.87 (*C*$_{Ar}$), 130.29 (*C*$_{Ar}$), 137.88 (*C*$_{Ar}$H), 158.91 (*C*$_{Ar}$O), 173.70 (*C*=O). Anal. Calcd for $C_{23}H_{28}O_3$: C, 78.38; H, 8.01. Found: C, 78.36; H, 8.00.

*4.1.2.7. Isobutyl 5-(4-benzoylphenoxy)-2,2-dimethylpentanoate (**29**).* Uncolored oil, 1.2 g, 3.2 mmol, 65% yield. IR (neat) 3058, 2950, 1702, 1698, 1570 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.94 (d, J = 6.6 Hz, 6H, *C*H$_3$CH*C*H$_3$), 1.22 (s, 6H, CH_3), 1.70–1.76 (m, 4H, CH_2CH_2), 1.80–1.98 (m, 1H, C*H*CH$_2$), 3.84 (d, J = 6.3 Hz, 2H, CH_2CH), 4.01 (t, J = 4.5 Hz, 2H, CH$_2$CH_2O), 6.92 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.43–7.49 (m, 2H, CH_{Ar}), 7.53–7.59 (m, 1H, CH_{Ar}), 7.76 (d, J = 8.4 Hz, 2H, CH_{Ar}), 7.81 (d, J = 9.0 Hz, 2H, CH_{Ar}); ^{13}C NMR ($CDCl_3$) δ 19.35 (*C*H$_3$CH*C*H$_3$), 25.15 (CH$_2$), 25.45 (*C*H$_3$C*C*H$_3$), 28.02 (*C*H), 37.14 (CH$_2$*C*), 42.39 (*C*CH$_3$), 68.56 (CH$_2$*C*H$_2$O), 70.80 (OCH$_2$*C*H), 114.18 (*C*$_{Ar}$), 128.40 (*C*$_{Ar}$), 129.95 (*C*$_{Ar}$), 130.20 (*C*$_{Ar}$CO), 132.09 (*C*$_{Ar}$), 132.80 (*C*$_{Ar}$), 138.53 (*C*$_{Ar}$CO), 162.91 (*C*$_{Ar}$O), 177.90 (*C*OO*i*Bu), 196.0 (*C*=O). Anal. Calcd for $C_{24}H_{30}O_4$: C, 75.36; H, 9.91. Found: C, 75.39; H, 9.89.

*4.1.2.8. Isobutyl 2,2-dimethyl-5-(4-phenoxyphenoxy)pentanoate (**30**).* Uncolored oil, 1.2 g, 3.5 mmol, 70% yield. IR (neat) 2963, 2930,2638, 1723, 1676, 1505, 1386, 1202, 1138 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.6 Hz, 6H, CH_3), 1.20 (s, 6H, CH_3), 1.68 (m, 4H, CH_2CH_2), 1.92 (m, 1H, C*H*), 3.84 (m, 2H, CH_2O), 6.86 (d, J = 9.0 Hz, 2H, CH_{Ar}CO), 6.99 (m, 4H, CH_{Ar}CO), 7.07 (m, 1H, CH_{Ar}), 7.2 (t, J = 6.9 Hz, 2H, CH_{Ar}C); ^{13}C NMR (CD_3OD) δ 19.35 (*C*H$_3$), 25.41 (*C*H$_3$), 28.00 (*C*H$_2$), 37.23 (*C*H$_2$C), 42.41 (*C*CO), 68.99 (*C*H), 70.72 (*C*H$_2$O), 116.20 (*C*$_{Ar}$CO$_{Al}$), 118.9 (*C*$_{Ar}$CO), 120.3 (*C*$_{Ar}$CO), 123.7 (*C*$_{Ar}$), 150.6 (*C*$_{Ar}$O), 151.10 (*C*$_{Ar}$O$_{Al}$), 157.3 (*C*$_{Ar}$O), 178.04 (*C*=O). Anal. Calcd for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16. Found: C, 74.54; H, 8.18.

*4.1.2.9. Isobutyl 2,2-dimetyl-5-{4-[(E)-phenyldiazenyl]phenoxy}pentanoate (**31**).* Orange solid, 1.14 g, 3 mmol, 60% yield, mp 46–47 °C. IR (KBr) 2964, 2873, 1719, 1602, 1501, 1469, 1255, 1192, 1142 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.94 (d, J = 6.6 Hz, 6H, CH_3CHCH_3), 1.57 (s, 6H, CH_3CCH_3), 1.74–1.76 (m, 4H, CH_2CH_2), 1.89–1.98 (m, 1H, C*H*CH$_3$), 3.85 (t, J = 6.6 Hz, 2H, CH_2O), 4.02 (t, J = 5.7 Hz, 2H, CH_2CH), 6.97 (d, J = 9.0 Hz, 2H, CH_{Ar}CO), 7.25–7.52 (m, 3H, CH_{Ar}), 7.85–7.92 (m, 4H, CH_{Ar}N=NCH_{Ar}); ^{13}C NMR ($CDCl_3$) δ 19.35 (2*C*H$_3$), 25.20 (*C*H$_3$), 25.45 (CH$_3$), 28.02 (*C*H), 31.19 (*C*H$_2$), 37.16 (CH$_2$), 42.41 (*C*CH$_3$), 68.62 (CH$_2$O), 70.79 (*C*H$_2$OCO), 114.87 (*C*$_{Ar}$CO), 122.75 (*C*$_{Ar}$CN), 124.96 (*C*$_{Ar}$CN), 129.25 (*C*$_{Ar}$C), 130.55 (*C*$_{Ar}$), 147.11 (*C*$_{Ar}$N), 161.74 (*C*$_{Ar}$O), 177.94 (*C*=O). Anal. Calcd for $C_{23}H_{30}N_2O_3$: C, 72.22; H, 7.91; N, 7.32. Found: C, 72.20; H, 7.94; N, 7.31.

*4.1.3. General procedure for the preparation of acids **17**–**24** and **32**–**38***

A solution of KOH 1% in absolute EtOH (420 mg, 7.5 mmol) was added to ester **9**–**14**, **16** or **25**–**31** (5 mmol) in absolute EtOH (5 mL), and the mixture was stirred at reflux for 6–10 h. Only ester **15** reacted at rt. The solvent was removed under reduced pressure and the residue was poured into water (10 mL) and acidified with 2 N HCl. The aqueous layer was extracted with dichloromethane (3 × 100 mL) and then the organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by crystallization in cyclohexane or by flash chromatography to give the acid **17**–**24** or **33**–**39**.

*4.1.3.1. 5-(4-Allyl-2-methoxyphenoxy)pentanoic acid (**17**).* White solid, 0.77 g, 3.07 mmol, 86% yield, mp 62 °C. IR (neat) 2957, 1713, 1514, 1259, 1135, 1016, 906 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.61–1.90 (m, 4H, CH_2CH_2), 2.37 (t, J = 7.3 Hz, 2H, CH_2CO), 3.30 (t, J = 1.8 Hz, 2H, CH_2C$_{Ar}$), 3.83 (s, 3H, CH_3O), 3.96 (q, J = 7.2 Hz, 2H, CH_2O), 4.88 (t, J = 5.7 Hz, 2H, H_2C = C), 5.90–5.96 (m, 1H, C*H*=CH$_2$), 6.70 (s, 2H, CH_{Ar}CH$_2$), 6.69 (d, J = 7.5 Hz, 1H, CH_{Ar}CO); ^{13}C NMR (CD_3OD) δ 21.79 (CH$_2$CH$_2$CO), 28.90 (*C*H$_2$CH$_2$O), 34.22 (*C*H$_2$CO), 40.04 (CH$_2$*C*=C), 56.14 (*C*H$_3$O), 68.82 (*C*H$_2$O), 112.54 (*C*$_{Ar}$HCOMe), 113.45 (*C*$_{Ar}$HCCH$_2$), 115.83 (*C*H$_2$=C), 120.65 (*C*$_{Ar}$HC), 133.10 (*C*$_{Ar}$CH$_2$), 137.92 (*C*H=CH$_2$), 147.11 (*C*$_{Ar}$O), 149.58 (*C*$_{Ar}$OMe), 173.74 (*C*=O). Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.14; H, 7.66.

*4.1.3.2. 5-{4-[(E)-2-phenylvinyl]phenoxy}pentanoic acid (**19**).* White solid, 0.58 g, 1.9 mmol, 60% yield, mp 176–177 °C. IR (KBr) 3022, 2948, 1695, 1507, 1244, 1170, 1029 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.68–1.80 (m, 4H, CH_2CH_2), 2.39 (m, 2H, CH_2CO), 3.95 (t, J = 4.5 Hz, 2H, CH_2O), 6.8 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.0 (q, J = 13.5 Hz, 2H, *H*C=C*H*), 7.17 (m, 1H, CH_{Ar}), 7.33 (t, J = 7.2 Hz, 2H, CH_{Ar}), 7.43 (d, J = 8.4 Hz, 2H, CH_{Ar}), 7.48 (d, J = 7.5 Hz, 2H, CH_{Ar}); ^{13}C NMR (CD_3OD) δ 21.88 (*C*H$_2$CH$_2$CO), 28.90 (*C*H$_2$CH$_2$O), 34.19 (*C*H$_2$CO), 67.65 (*C*H$_2$O), 114.89 (*C*$_{Ar}$H), 126.46 (*C*H=CH), 126.76 (*C*$_{Ar}$H), 127.42 (*C*$_{Ar}$H), 127.93 (*C*$_{Ar}$H), 128.45 (CH=*C*H), 128.87 (*C*$_{Ar}$), 130.29 (*C*$_{Ar}$), 137.88 (*C*$_{Ar}$),

158.91 ($C_{Ar}O$), 173.70 (*C*=O). Anal. Calcd for $C_{19}H_{20}O_3$: C, 77.00; H, 6.80. Found: C, 77.09; H, 6.83.

*4.1.3.3. 5-(4-Benzoylphenoxy)pentanoic acid (**21**).* White solid, 0.98 g, 3.29 mmol, 87% yield, mp 103 °C. IR (KBr) 3050 (broad), 2954, 2913, 2873, 1698, 1639, 1604 cm^{-1}; ^1H NMR (CD_3OD) δ 1.84–1.82 (m, 4H, CH_2CH_2), 2.36–2.40 (m, 2H, CH_2COO), 4.02–4.10 (m, 2H, CH_2O), 7.02 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.51 (d, J = 7.8 Hz, 2H, CH_{Ar}), 7.57–7.59 (m, 1H, CH_{Ar}), 7.70 (d, J = 7.2 Hz, 2H, CH_{Ar}), 7.77 (d, J = 8.7 Hz, 2H, CH_{Ar}); ^{13}C NMR (CD_3OD) δ 21.78 (CH_2CH_2CO), 28.72 (CH_2CH_2O), 34.09 (CH_2CO), 67.85 (CH_2O), 114.92 (C_{Ar}), 128.16 (C_{Ar}), 129.8 (C_{Ar}), 132.31 (C_{Ar}), 138.22 ($C_{Ar}CO$), 162.17 ($C_{Ar}O$), 173.71 (COOH), 195.98 (CO). Anal. Calcd for $C_{18}H_{18}O_8$: C, 72.47; H, 6.08. Found: C, 72.45; H, 6.09.

*4.1.3.4. 5-{4-[(Anilinocarbonyl)amino]phenoxy}pentanoic acid (**23**).* White solid, 300 mg, 60% yield, mp 229 °C (dec). IR (KBr) 3313, 2936, 1639, 1595, 1566, 1515, 1241 cm^{-1}; ^1H NMR (Ac_D) δ 1.76–1.78 (m, 4H, CH_2CH_2), 2.36–2.40 (m, 2H, CH_2COO), 3.95–3.98 (m, 2H, CH_2O), 6.86 (d, J = 9 Hz, 2H, CH_{Ar}), 6.94–6.98 (m, 1H, C_{Ar}), 7.25 (t, J = 7.8 Hz, 2H, C_{Ar}), 7.42 (d, J = 9 Hz, 2H, C_{Ar}), 7.52 (d, J = 8.1 Hz, 2H, C_{Ar}), 7.94 (s, 1H, NHCONH), 8.05 (s, 1H, NHCONH); ^{13}C NMR (Ac_D) δ 21.78 (CH_2CH_2CO), 28.72 (CH_2CH_2O), 34.09 (CH_2CO) 67.85 (CH_2O), 116.60 (C_{Ar}), 120.43 (C_{Ar}), 118.6 (C_{Ar}), 122.65 (C_{Ar}), 129.26 (C_{Ar}), 134.13 ($C_{Ar}NH$), 139.42 ($C_{Ar}NH$), 152.14 ($C_{Ar}O$), 115.57 (CONH), 173.71 (COOH). Anal. Calcd for $C_{18}H_{20}N_2O_4$: C, 65.84; H, 6.14; N, 8.53. Found: C, 65.86; H, 6.16; N, 8.50.

*4.1.3.5. 5-(2-Methoxy-4-allylphenoxy)-2,2-dimethylpentanoic acid (**32**).* White solid, 1.02 g, 2.6 mmol, 52% yield, mp 77–83 °C. IR (KBr) 3076, 2953 (broad), 1707, 1513, 1261 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.18 (s, 6H, CH_3), 1.61–1.90 (m, 4H, CH_2CH_2), 3.32 (m, 2H, CH_2C_{Ar}), 3.83 (s, 3H, CH_3O), 4.11 (q, J = 4.9 Hz, 2H, CH_2O), 5.06 (t, J = 6.9 Hz, 2H, H_2C=CH), 5.90–5.96 (m, 1H, H_2C=C*H*), 6.70 (s, 2H, $CH_{Ar}CH_2$), 6.69 (d, J = 7.5 Hz, 1H, $CH_{Ar}CO$); ^{13}C NMR ($CDCl_3$) δ 24.49 (CH_3), 25.02 (CH_3), 28.90 (CH_2CH_2O), 36.87 (CH_2CH_2CO), 40.04 (CCO), 42.19 (CH_2C=C), 56.01 (CH_3O), 69.54 (CH_2O), 109.5 (C_{Ar}), 113.45 (C_{Ar}), 118.83 (CH_2=C), 123.18 (C_{Ar}), 130.79 (C_{Ar}), 131.8 (*C*H=CH_2), 147.76 (C_{Ar}), 149.75 (C_{Ar}), 180.65 (C=O). Anal. Calcd for $C_{17}H_{24}O_4$: C, 69.84; H, 8.27. Found: C, 69.80; H, 8.29.

*4.1.3.6. 2,2-Dimethyl-5-{4-[(E)-2-phenylvinyl]phenoxy}pentanoic acid (**34**).* White solid, 1.2 mg, 4.0 mmol, 80% yield, mp 151–155 °C. IR (KBr) 3023, 2955 (broad), 1690, 1604, 1510, 1267, 1170, 1021 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.21 (s, 6H, CH_3), 7.70–1.79 (m, 4H, CH_2CH_2), 3.96 (t, J = 6 Hz, 2H, CH_2O), 6.8 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.0 (q, J = 13.5 Hz, 2H, *H*C=C*H*), 7.24 (d, J = 8.1 Hz, 1H, CH_{Ar}), 7.33 (t, J = 7.5 Hz, 2H, CH_{Ar}), 7.43 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.48 (d, J = 7.2 Hz, 2H, CH_{Ar}); ^{13}C NMR (CD_3OD) δ 21.89 (CH_2CCO), 28.90 (CH_2CH_2O), 25.22 (CH_3), 36.97 (CCO), 68.31 (CH_2O), 114.89 (C_{Ar}), 126.46 (CH=CH), 126.76 (C_{Ar}), 127.42 (C_{Ar}), 127.93 (C_{Ar}), 128.45 (CH=CH), 128.87 (C_{Ar}), 130.29 (C_{Ar}), 137.88 (C_{Ar}), 158.91 (C_{Ar}), 173.70 (*C*=O). Anal. Calcd for $C_{21}H_{24}O_3$: C, 77.75; H, 7.46. Found: C, 77.79; H, 7.43.

*4.1.3.7. 5-(4-Benzoylphenoxy)-2,2-dimethylpentanoic acid (**36**).* White solid, 930 mg, 2.8 mmol, 57% yield, mp 103–105 °C; IR (KBr) 3040, 2960, 1690, 1630, 1550 cm^{-1}; ^1H NMR (CD_3OD) δ 1.22 (s, 6H, CH_3), 1.70–1.76 (m, 4H, CH_2CH_2), 3.84 (d, J = 6.3 Hz, 2H, CH_2CH), 6.92 (d, J = 8.7 Hz, 2H, CH_{Ar}), 7.43–7.49 (m, 2H, CH_{Ar}), 7.53–7.59 (m, 1H, CH_{Ar}), 7.76 (d, J = 8.4 Hz, 2H, CH_{Ar}), 7.81 (d, J = 9.0 Hz, 2H, CH_{Ar}); ^{13}C NMR ($CDCl_3$) δ 25.15 (CH_2), 25.45 (CH_3CCH_3), 37.14 (CH_2C), 42.39 (CCH_3), 68.56 (CH_2O), 114.18 (C_{Ar}), 128.40 (C_{Ar}), 129.95 (C_{Ar}), 130.20 ($C_{Ar}CO$), 132.09 (C_{Ar}), 132.80 (C_{Ar}), 138.53 (CArCO), 162.91 ($C_{Ar}O$), 177.90 (COOH), 196.0 (*C*=O). Anal. Calcd for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79. Found: C, 73.63; H, 6.78.

*4.1.3.8. 2,2-Dimethyl-5-(4-phenoxyphenoxy)pentanoic acid (**37**).* White solid, 864 mg, 2.7 mmol, 55% yield, mp 85–88 °C, IR (KBr) 2963, 2930, 2638, 1693, 1676, 1505, 1386, 1202 cm^{-1}; ^1H NMR (CD_3OD) δ 1.21 (s, 6H, CH_3), 1.64–1.82 (m, 4H, CH_2CH_2), 3.92 (t, J = 6.0 Hz, 2H, CH_2O), 6.90 (s, 6H, CH_{Ar}), 6.98–7.07 (m, 1H, CH_{Ar}), 7.27 (d, J = 7.5 Hz, 2H, $CH_{Ar}C$); ^{13}C NMR (CD_3OD) δ 25.41 (CH_3), 28.00 (CH_2), 37.23 (CH_2C), 42.41 (CCO), 70.72 (CH_2O), 116.20 (C_{Ar}-CO_{Al}), 118.9 ($C_{Ar}CO$), 120.3 ($C_{Ar}CO$), 123.7 (C_{Ar}), 150.6 ($C_{Ar}O$), 151.10 ($C_{Ar}O_{Al}$), 157.3 ($C_{Ar}O$), 178.04 (*C*=O). Anal. Calcd for $C_{19}H_{22}O_4$: C, 72.59; H, 7.05. Found: C, 72.56; H, 7.07.

*4.1.3.9. 2,2-Dimethyl-5-{4-[(E)-phenyldiazenyl]phenoxy}pentanoic acid (**38**).* Orange solid, 1.4 mg, 4.3 mmol, 87% yield, mp 85–87 °C; IR (KBr) 2946, 2873, 1693, 1602, 1503, 1255, 1145, 1030 cm^{-1}; ^1H NMR (CD_3OD) δ 1.21 (s, 6H, CH_3), 1.64–1.82 (m, 4H, CH_2CH_2), 4.05 (t, J = 6.0 Hz, 2H, CH_2O), 7.05 (d, J = 9.0 Hz, 2H, $CH_{Ar}CO$), 7.32–7.58 (m, 3H, CH_{Ar}), 7.83–7.90 (m, 4H, $CH_{Ar}N$); ^{13}C NMR (CD_3OD) δ 25.41 (CH_3), 28.00 (CH_2), 37.23 (CH_2C), 42.41 (CCO), 70.72 (CH_2O), 116.01 ($C_{Ar}CO$), 122.44 ($C_{Ar}CN$), 125.07 ($C_{Ar}CN$), 129.45 ($C_{Ar}C$), 130.58 (C_{Ar}), 145.53 ($C_{Ar}N$), 152.31 ($C_{Ar}N$), 161.03 ($C_{Ar}O$), 178.04 (*C*=O). Anal. Calcd for $C_{19}H_{22}N_2O_3$: C, 69.92; H, 6.79; N, 8.58. Found: C, 69.95; H, 6.78; N, 8.59.

4.2. Biology

4.2.1. Reporter plasmids and luciferase assays

Human embryonic kidney cells (HEK293) were grown in Dulbecco's Modified Eagles's Medium (DMEM) containing 10% of FCS, penicillin/streptomycin, sodium pyruvate and nonessential amino acids. HEK293 cells were incubated at 37 °C in 5% CO_2 incubator until they were 80% confluent. One day before the experiment, the cells were plated in 96-well plates. The next day, the culture medium was replaced by a fresh one without FCS and the transient transfection was conducted using the calcium phosphate method. Cells were transfected with expression plasmids encoding the fusion protein GAL4-PPARαLBD or GAL4-PPARγLBD (30 ng), reporter plasmid (50 ng), renilla luciferase normalization vector (20 ng), and pGEM carrier DNA (40 ng) to make a total of 140 ng of DNA per well. 8 h after transfection, cells were treated for 18 h with the indicated ligands. The two luciferase activities were measured using a dual luciferase assay kit (Promega) on a microplate luminometer (Labsystems Ascent LuminoskanReader). All transfection experiments were repeated at least twice.

4.2.2. Real-time quantitative PCR (RTqPCR)

HepG2 cells were obtained from ATCC (ATCC-LGC Promochem, London, U.K.). Cells were maintained in growth medium composed of DMEM supplemented with 10% FCS-GOLD and 1% penicillin/streptomycin. One day before the treatment, HepG2 cells were seeded in six-well plates at a density of 5×10^5 cells/well and incubated at 37 °C, 5% CO_2. The next day, the cells were treated for 24 h with the indicated ligands dissolved in dimethyl sulfoxide (DMSO) and diluted in the growth medium. After incubation, cells were washed with PBS and assayed for the gene expression analysis.

Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. To avoid possible DNA contamination, RNA was treated with DNAase-1 (Ambion, Foster City, CA). RNA purity was checked by spectrophotometer and RNA integrity by examination on agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized retrotranscribing 4 μg of total RNA in a total volume of 100 μL using the High Capacity DNA Archive Kit (Applied Biosystems, Foster City, CA) and following the manufacturer's instructions.

RTqPCR primers were designed using Primer Express Software. PCR assays were performed in 96 well optical reaction plates using

the ABI 7500HT machine (Applied Biosystems). PCR assays amplification plots were kept constant to obtain normalized cycle times and linear regression data. The following reaction mixture per well was used: 12.5 μL Power Syber Green (Qiagen, Quantitect), 4.8 μL primer at the final concentration of 300 nM, 4.7 μL RNAse free water, 3 μL cDNA. All reactions were carried out using the following protocol: 95 °C for 15 min; 40 cycles of 94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s. Each experimental measurement was normalized to the corresponding cyclophilin mRNA levels. Relative quantification was performed using the ΔΔCt method.

Validated primers for RTqPCR are listed below:

Human CPT1A: FW-5′TGCCATGGATCTGCTGTATATCC3′,
RV-5′GCGTTGCCGGCTCTTG3′

Human GAPDH: FW-5′CAACTTTGGTATCGTGGAAGGAC3′,
RV-5′ACAGTCTTCTGGGTGGCAGTG3′.

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