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Original article

Synthesis and biological evaluation of new clofibrate analogues as potential PPARα agonists

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Abstract

Clofibrate is a lipid-profile modifying agent belonging to the fibrate class of drugs. Fibrates are known to exhibit their beneficial effects by activating peroxisome proliferator-activated receptor-α (PPARα) and used in the treatment of dyslipidemia and atherosclerosis and for the prevention of heart failure. Hereby, the preparation of two new sets of clofibrate analogues, ethyl 2-(4-chlorophenoxy)-3-oxoalkanoates and ethyl 2-(4-chlorophenoxy)-3-hydroxyalkanoates is described starting from commercially available 3-oxoalkanoates in fair to good yields. Treatment of 3-oxoalkanoates with SO_2Cl_2 yielded the corresponding 2-chloro-3-oxoalkanoates, that were then converted into 2-(4-chlorophenoxy)-3-oxoalkanoates by reacting with sodium or caesium 4-chlorophenate. Reduction of the keto group with $NaBH_4$ afforded the corresponding 2-(4-chlorophenoxy)-3-hydroxyalkanoates in very high yields and with variable diastereoselectivity. Biological evaluation of the compounds was performed by a transactivation assay in a transiently transfected monkey kidney fibroblast cell line. The newly synthesised clofibrate analogues failed to show noticeable levels of PPAR activation at concentrations where clofibrate showed an evident activity, suggesting that the structural modifications caused the loss of PPAR activity.

Keywords: Clofibrate analogues; Dyslipidemia; Ethyl 2-(4-chlorophenoxy)-3-oxoalkanoates; Ethyl 2-(4-chlorophenoxy)-3-hydroxyalkanoates

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors. To date, three isoforms (namely PPARα, PPARγ and PPARδ) have been identified. PPARs are ligand-activated transcription factors and control the gene expression by binding to specific response elements (PPREs) within the promoter regions of several target genes. Binding of a ligand to PPARs causes the heterodimerisation of PPARs with the 9-*cis* retinoic acid-activated receptor (RXR). The heterodimer then recruits a number of specific cofactors. This series of events ultimately results in the regulation of the transcription rate of the target genes. PPARs play a critical physiological role as lipid sensors in the regulation of lipid and energy metabolism. Fatty acids and eicosanoids have been identified as natural ligands for PPARs. More potent synthetic PPAR ligands, including fibrates and thiazolidinediones, have been proven to be effective in the treatment of dyslipidemia and type 2 diabetes through activation of the α- and γ-PPAR isoform, respectively. Use of such ligands has helped unveil many potential roles for PPARs in pathological states including atherosclerosis, inflammation, cancer, infertility and demyelination [1].

Fibrates, such as clofibrate (**1**, R = C_2H_5, Fig. 1), are generally effective in lowering elevated plasma triglycerides [2]. They are well tolerated in the clinic, and enjoy favourable safety profiles. A rare incidence of fibrate-associated toxicity has been reported in almost every organ system [3]. The most pronounced contraindication is the liver and renal insufficiency [4,5]. Fibrate toxicities include myalgia, myotonia, sporadic rhabdomyolysis (may be aggravated by combination with statins), elevated transaminases and gallstone formation [6,7]. The magnitude of lipid changes depends on the

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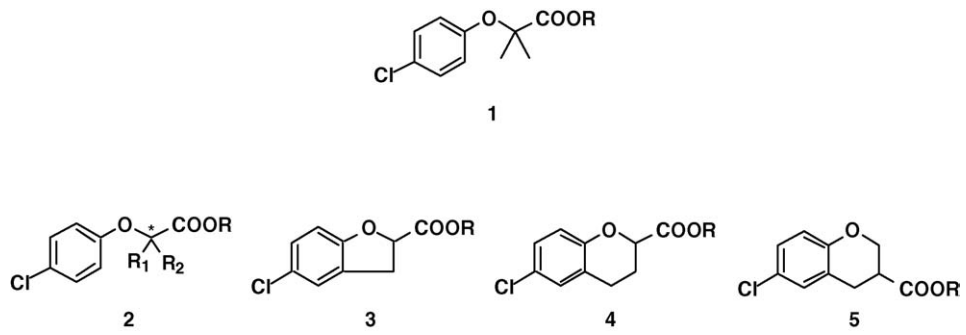


Fig. 1. Clofibrate (**1**, R= Et) and some of its acyclic (**2**) and cyclic (**3**–**5**) analogues.

patient's lipoprotein status [8] as well as the relative potency of the fibrate used in the treatment [9].

It has been reported that stereochemistry of clofibrate analogues bearing a stereogenic centre (**2**, Fig. 1) affects their pharmacological profile (therapeutic and adverse side effects) [10–14]. In fact, many clofibrate derivatives have been synthesised and tested both as racemates and in enantiomerically pure form [15].

In our previous investigation, a number of racemic and optically active acyclic (**2**, Fig. 1) [16] and cyclic clofibrate (**3**–**5**, Fig. 1) [15] analogues were synthesised and pharmacologically evaluated on PPAR [17] and muscle tissue chloride channels [18–20] for their therapeutic activity and toxic side effects, respectively, with the aim of discriminating the structural determinants responsible for the different activities.

Herein, synthesis of new and more highly functionalised clofibrate derivatives, together with the investigation of their potential activity as PPARα ligands, is described.

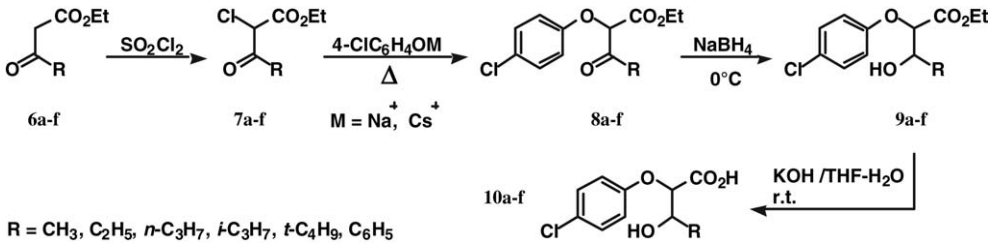
2. Chemistry

Clofibrate analogues **8a–f** and **9a–f** were synthesised as reported in the scheme of Table 1.

2-Chloro-3-oxoalkanoates **7a–f** were prepared in fair to very good yields (51–98%) by treating the corresponding commercially available 3-oxoalkanoates **6a–f** with SO_2Cl_2 (scheme of Table 1) [21].

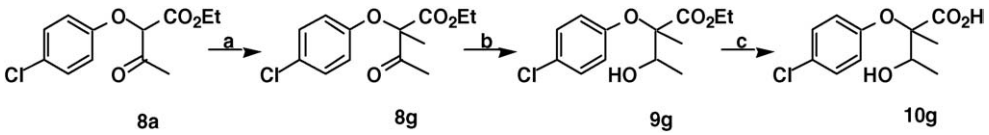
Then, 2-(4-chlorophenoxy)-3-oxoalkanoates **8a–f** were obtained by reacting **7a–f** with sodium or caesium 4-chlorophenate. Initially, the nucleophilic displacement of chloride was performed by using sodium 4-chlorophenate in toluene, DMF or without any solvent. In contrast, when caesium 4-chlorophenate was used instead of corresponding sodium salt, reaction at 50 °C in the absence of solvent gave the desired product in a similar yield but the work-up became easier. The keto group of **8a–f** was reduced to the corresponding alcohols **9a–f** by treating with $NaBH_4$ in methanol at 0 °C (Scheme in Table 1).

Table 1
Yields of each step of the synthetic route to 2-chloro-3-oxoalkanoates **7a–f**, 2-(4-chlorophenoxy)-3-oxoalkanoates **8a–f**, 2-(4-chlorophenoxy)-3-hydroxyalkanoates **9a–f** and 2-(4-chlorophenoxy)-3-hydroxyalkanoic acids **10a–f**



R	Yield (%)			
	7a–f [a]	**8a–f** [b]	**9a–f** [b] (d.e.) [c]	**10a–f** [d] (d.e.) [c]
a:**CH_3**	65	69	90 (11)	79 (15)
b:**C_2H_5**	60	76	88 (7)	78 (11)
c:***n*-C_3H_7**	64	70	73 (22)	71 (4)
d:***i*-C_3H_7**	51	68	93 (25)	44 (27)
e:***t*-C_4H_9**	98	65	94 (8)	14 (41)
f:**C_6H_5**	92	78	96 (34)	84 (32)

[a] Yields refer to the product isolated by chromatography.
[b] GC yield based on the starting **7a–f** (see Section 6).
[c] The d.e. was determined by ^{1}H NMR by comparing the integration value of the signals due to the C*H*OC_6H_4Cl of the two diastereomeric couples.
[d] Yields refer to the product crystallised.



Scheme 1. Reagents and conditions: (a) Cs_2CO_3, CH_3I, r.t., 90% yield; (b) $NaBH_4$, MeOH, 0 °C, 87% yield; (c) KOH, THF–H_2O, 64% yield.

The reduction proceeded in very high chemical yields. The diastereomeric excess (d.e.) depended on the group (R) bonded to carbonyl (Table 1). It was around 10% for R = CH_3 (**9a**) and C_2H_5 (**9b**). D.e was increased almost twofold for R = C_3H_7 (**9c** and **9d**) and was again around 10% for R = *t*-C_4H_9 (**9e**). It was increased threefold (d.e. = 34%) when the group bonded to the carbonyl is a phenyl (**9f**).

Ethyl 2-(4-chlorophenoxy)-2-methyl-3-hydroxybutanoate (**9g**) was prepared by treating **8a** with CH_3I in the presence of Cs_2CO_3 affording **8g** in 90% yield, that was reduced to **9g** with $NaBH_4$ in methanol at 0 °C (87% yield, and d.e. = 60%; Scheme 1).

Ethyl esters **9a–g** were hydrolysed to the corresponding acids **10a–g** by KOH in THF–H_2O (14–84% yield, Table 1 and Scheme 1).

3. Biology

Evaluation of the PPAR functional activities of newly synthesised compounds was performed by a transactivation assay in eukaryotic cells.

COS-7 cells were transiently transfected with an expression vector encoding a fusion protein between the DNA binding domain (DBD) of the yeast GAL4 transcription factor and the ligand-binding domain (LBD) of the mouse PPARα (GAL4DBD/PPARαLBD). The reporter vector, containing five copies of the high affinity binding site for GAL4 (named UAS, *upstream activating sequence*) upstream of a strong viral promoter linked to the reporter gene chloramphenicol-acetyl transferase (CAT), was co-transfected. Beside expression and reporter vectors, cells were transfected with a control vector pCH110 that encodes the β-galactosidase enzyme to correct for differences in transfection efficiency. Following transfection, cells were treated for 48 h with increasing concentrations (50, 150 and 300 μM) of the test compounds **8a**, **9a**, **10a–g**, and of clofibrate. CAT activity, normalised to β-galactosidase activity, was determined as "fold activation" relative to cells treated with vehicle (DMSO 0.1%) alone. Finally, results were expressed as percentage activation of the CAT reporter gene compared to that measured in the presence of positive control WY-14,643 (2 μM), conventionally taken as equal to 100%.

4. Results and discussion

All the new synthesised compounds were biologically evaluated to determine their ability to activate PPARα. Results of transactivation assays are shown in Table 2.

The loss of activity of **8a** and **9a** could be attributed to the presence of the ester group, that prevents interaction with the four amino acids (Ser280, Tyr314, His440, Tyr464) [22] present in the ligand-binding pocket of PPARα.

As far as the low activity of the other compounds is concerned, at least three aspects should be taken into consideration. First, it is well known that the differences in the pharmacological properties of the stereoisomers depend on their ability to interact with the receptor, which possesses its stereospecificity. All of the compounds were biologically evaluated as mixtures of four stereoisomers (**9a** and **10a–g**) with the exception of **8a** (existing only as a pair of enantiomers). Each stereoisomer could have a different behaviour with respect to PPARα and this could explain the observed activity of the racemates **8a**, **9a**, and **10a–g**.

Secondly, it could be that the hydroxy group, present in almost all the molecules, is responsible for the not suitable interaction between the compounds and the LBD of the receptor.

Thirdly, another reason for the loss of activity could reside in the higher size of the moiety linked to the C-2 of **8a**, **9a** and **10a–g** than the two hydrogens of WY-14,643 and the two methyls of the clofibrate [23].

5. Conclusion

In summary, new clofibrate analogues have been prepared in good yields. Their behaviour as PPARα ligands was evaluated by a transactivation assay in comparison with clofibrate. Unfortunately, they failed to show PPAR activity. The loss of activity might be attributed to the steric congestion near the carboxylic acid moiety, which presumably binds to the helix 12 of the LBD of the receptor.

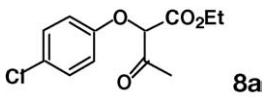
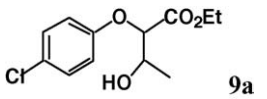
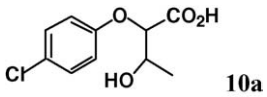
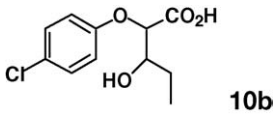
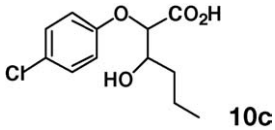
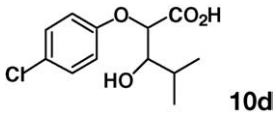
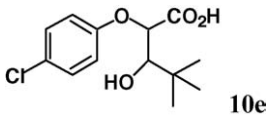
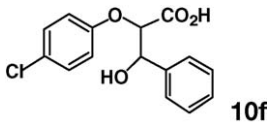
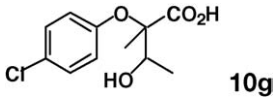
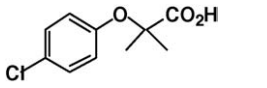
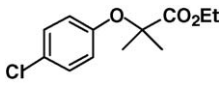
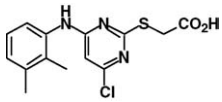
6. Experimental protocols

6.1. General

Melting points were taken on electrothermal apparatus and are uncorrected. Reaction progress was monitored by TLC or GC analysis. Thin-layer chromatography (TLC) was performed on silica gel sheets with a fluorescent indicator (Statocrom SIF, 60 F_{254} MERK); TLC spots were observed under ultraviolet light or visualised with I_2 vapour. Column chromatography was conducted using silica gel MERK 60 (0.063–0.200 μm).

GC analyses were performed by using a HP1 column (methyl silicone gum; 30 m × 0.25 mm × 250 μm film

Table 2
Percentage of activation of the CAT reporter gene by **8a**, **9a** and **10a–g** at the concentration of 50, 150 and 300 μM, compared to that measured in the presence of reference compound WY-14,643 (2 μM), conventionally taken as equal to 100%

Compound	50 μM (%)	150 μM (%)	300 μM (%)
 **8a**	5.2	4.2	4.2
 **9a**	2.7	1.5	1.5
 **10a**	4.0	5.6	7.3
 **10b**	2.7	3.4	9.0
 **10c**	3.4	4.6	5.9
 **10d**	3.2	3.7	5.9
 **10e**	3.7	10.8	13.0
 **10f**	3.2	4.3	5.1
 **10g**	5.1	8.8	17.2
 Clofibric acid	Not tested	Not tested	86.5
 Clofibrate	12.6	11	89.9
 WY-14,643	100 (2 μM)	–	–

thickness) on a Hewlett Packard 5890 model, SERIES II. GC–MS analyses were performed on a Hewlett Packard 6890-5793MSD, and microanalysis on an Elemental Analyser 1106-Carlo Erba-instrument.

^{1}H NMR spectra were recorded in $CDCl_3$ on VARIAN EM-390 or Mercury 300 MHz spectrometers and the chemical shifts were reported in parts per million (δ). Absolute values of the coupling constant (J) are reported. The extent of enolisation of **7a–f** and **8a–f** was measured by ^{1}H NMR. IR spectra were recorded on a Perkin–Elmer 681 spectrometer.

3-Oxoalkanoates, ethyl 2-chloro-3-oxobutanoate (also prepared by us, as depicted in the scheme of Table 1) and all other chemicals and solvents were purchased from Aldrich Chemical Co.

One hundred to 500 mg of biologically tested compounds (Table 1 and Scheme 1) were prepared.

*6.2. General procedure for the synthesis of ethyl 2-chloro-3-oxoalkanoates **7a–f** [21]*

Sulphuryl chloride (SO_2Cl_2) (139 mg, 5 mmol) was added dropwise to the oxoalkanoate (5 mmol) kept under stirring at 0 °C for 10 min. Then, the mixture was allowed to warm at room temperature. The reaction progress was monitored by TLC, and reactant/products visualised by exposure to iodine vapour. After the time indicated below (see each compound), the reaction mixture was washed with a saturated solution of Na_2CO_3. The aqueous layer was extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The product was isolated by chromatography.

In $CDCl_3$ solution, the ethyl 2-chloro-3-oxoalkanoates **7a–f** are in equilibrium with the corresponding enol forms (Scheme 2). The extent of enolisation is between 12% and 22% for **7a–d** (see each compound), and less than 3% for **7e**

R= CH_3, C_2H_5, n-C_3H_7, i-C_3H_7, t-C_4H_9, C_6H_5

Scheme 2.

and **7f**. Hence, IR and NMR spectral descriptions, whenever possible, are given for both keto and enol forms.

*6.2.1. Ethyl 2-chloro-3-oxobutanoate (**7a**) [21]*

The product was isolated from the crude reaction mixture as an oil (b.p. = 145 °C/22 mmHg) [21] by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 9.5:0.5; reaction time = 1 h; 65% yield; keto form/enol form = 80:20. IR (neat): 3449 (weak band due to the enol form), 2986, 2942, 2912, 2874, 1761, 1733, 1639, 1619, 1469, 1447, 1364, 1303, 1256, 1096, 1069, 1032, 882, 851, 771 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 12.29 (s, 1H, enol OH: exchanges with D_2O); 4.74 (s, 1H, *CH*Cl); 4.30–4.22 (q, J = 7.14 Hz, 4H, CH_2OCO, 2H of enol form and 2H of keto form); 2.35 (s, 3H, CH_3CO, keto form); 2.14 (s, 3H, CH_3COH, enol form); 1.34–1.29 (t, J = 7.14 Hz, 3H, CH_3CH_2O, enol form); 1.30–1.26 (t, J = 7.14 Hz, 3H, CH_3CH_2O, keto form). ^{13}C NMR ($CDCl_3$, δ): 196.78, 165.14, 63.33, 61.53, 26.41 14.11. GC–MS (70 eV) (*m*/*z*) (rel. int.) 166 [$M(^{37}Cl)^+$, 4], 164 [$M(^{35}Cl)^+$, 12], 136 (11), 124 (17), 122 (49), 119 (14), 118 (25), 96 (16), 94 (48), 76 (22), 69 (7), 43 (100). Anal. Calc. for $C_6H_9ClO_3$: C, 43.90; H, 5.49. Found: C, 43.89; H, 5.48.

*6.2.2. Ethyl 2-chloro-3-oxopentanoate (**7b**) [21]*

The product was isolated from the crude reaction mixture as an oil (b.p. = 125 °C/17 mmHg) [21] by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 9:1; reaction time = 1 h; 60% yield; keto form/enol form = 88:12. IR (neat): 3660–3400 (enol form), 2978, 2943, 2908, 1768, 1733, 1644, 1606, 1462, 1369, 1250, 1180, 1104, 1068, 1019, 947, 879, 822, 737 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 12.38 (s, 1H, enol OH: exchanges with D_2O); 4.77 (s, 1H, *CH*Cl); 4.29–4.22 (q, J = 7.14 Hz, 4H, CH_2OCO, 2H of keto form and 2H of enol form); 2.75–2.68 (q, J = 7.28 Hz, 2H, CH_2CO, keto form); 2.55–2.46 (q, J = 7.55 Hz, 2H, CH_2COH, enol form); 1.31–1.26 (t, J = 7.14 Hz, 6H, CH_3CH_2O, 3H of keto form and 3H of enol form); 1.18–1.13 (t, J = 7.55 Hz, 3H, CH_3CH_2, enol form); 1.11–1.07 (t, J = 7.28 Hz, 3H, CH_3CH_2, keto form). ^{13}C NMR ($CDCl_3$, δ): 199.89, 165.33, 63.28, 60.94, 32.62, 14.12, 7.84. GC–MS (70 eV) (*m*/*z*) (rel. int.) 180 [$M(^{37}Cl)^+$, 2], 178 [$M(^{35}Cl)^+$, 5], 132 (10), 94 (12), 69 (6), 57 (100), 41 (4). Anal. Calc. for $C_7H_{11}ClO_3$: C, 47.19; H, 6.18. Found: C, 47.20; H, 6.19.

*6.2.3. Ethyl 2-chloro-3-oxohexanoate (**7c**)*

The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 9:1; reaction time = 3 h; 64% yield; keto form/enol form = 78:22. IR (neat): 3442 (enol form), 2969, 2933, 2877, 1733, 1720, 1466, 1400, 1372, 1299, 1251, 1176, 1023, 851, 733 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 12.30 (s, 1H, enol OH: exchanges with D_2O); 4.72 (s, 1H, *CH*Cl); 4.21–4.14 (q, J = 7.14 Hz, 2H, CH_2OCO, enol form); 4.20–4.13 (q, J = 7.14 Hz, 2H, CH_2OCO, keto form); 2.60–2.55 (t, J = 7.28 Hz, 2H, CH_2CO, keto form); 2.40–2.35 (t, J = 7.28 Hz, 2H, CH_2COH, enol form); 1.64–1.51 (sextet, J = 7.28 Hz, 2H, CH_2CH_2, enol form); 1.60–1.47 (sextet, J = 7.28 Hz, 2H, CH_2CH_2, keto form); 1.26–1.23 (t, J = 7.14 Hz, 3H, CH_3CH_2O, enol form); 1.23–1.17 (t, J = 7.14 Hz, 3H, CH_3CH_2O, keto form); 0.89–0.84 (t, J = 7.28 Hz, 3H, $CH_3CH_2CH_2$, enol form); 0.89–0.84 (t, J = 7.28 Hz, 3H, $CH_3CH_2CH_2$, keto form). ^{13}C NMR ($CDCl_3$, δ): 199.02, 165.23, 63.22, 61.13, 40.94, 17.13, 14.09, 13.53. GC–MS (70 eV) (*m*/*z*) (rel. int.) 194 [$M(^{37}Cl)^+$, 1], 192 [$M(^{35}Cl)^+$, 3], 177 (0.2), 164 (2), 146 (4), 118 (5), 94 (12), 71 (100), 43 (58), 41 (16). Anal. Calc. for $C_8H_{13}ClO_3$: C, 50.00; H, 6.77. Found: C, 50.01; H, 6.78.

*6.2.4. Ethyl 2-chloro-3-oxo-4-methylpentanoate (**7d**)*

The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 9:1; reaction time = 3 h; 51% yield; keto form/enol form = 81:19. IR (neat): 3435 (enol form), 2979, 2937, 2873, 1763, 1735, 1632, 1595, 1467, 1448, 1386, 1367, 1295, 1255, 1177, 1096, 1022, 871, 809 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 12.49 (s, 1H, enol OH: exchanges with D_2O); 4.90 (s, 1H, *CH*Cl); 4.27–4.19 (q, J = 7.14 Hz, 4H, CH_2O, 2H of enol form and 2H keto form, two completely overlapped quartets); 3.12–2.98 (heptet, J = 6.87 Hz, 2H, $CH(CH_3)_2$, 1H of enol form and 1H of keto form, two completely overlapped heptets); 1.28–1.23 (t, J = 7.14 Hz, 6H, CH_3CH_2, 3H of enol form and 3H of keto form, two completely overlapped triplets); 1.14–1.10 (2d partially overlapped, J = 6.87 Hz, 6H, $(CH_3)_2CH$, keto form); 1.13–1.08 (2d partially overlapped, J = 6.87 Hz, 6H, $(CH_3)_2CH$, enol form). ^{13}C NMR ($CDCl_3$, δ): 202.80, 165.26, 63.22, 59.79, 38.17, 18.85, 18.66, 14.12. GC–MS (70 eV) (*m*/*z*) (rel. int.) 194 [$M(^{37}Cl)^+$, 2], 192 [$M(^{35}Cl)^+$, 6], 146 (8), 122 (6), 121 (8), 96 (11), 94 (35), 76 (9), 71 (100), 69 (10), 43 (96), 41 (24). Anal. Calc. for $C_8H_{13}ClO_3$: C, 50.00; H, 6.77. Found: C, 50.00; H, 6.75.

*6.2.5. Ethyl 2-chloro-3-oxo-4,4-dimethylpentanoate (**7e**)*

The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 9:1; reaction time = 1 h; 98% yield. IR (neat): 3427 (very weak band due to almost 3% of enol form as by NMR, see below), 2977, 2839, 1769, 1721, 1478, 1467, 1398, 1369, 1299, 1278, 1225, 1182, 1057, 1031, 1004, 877, 812 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 12.39 (s, 1H, enol OH: exchanges with D_2O); 5.17 (s, 1H, *CH*Cl); 4.16–4.09 (q, J = 7.14 Hz, 2H, CH_2CH_3); 1.24–1.21 (t, J = 7.14 Hz, 3H, CH_3CH_2); 1.18 (s, 9H, $(CH_3)_3C$). ^{13}C NMR ($CDCl_3$, δ): 203.90, 165.26, 63.01, 54.66, 45.24, 26.39, 13.99. GC–MS (70 eV) (*m*/*z*) (rel. int.) 208 [$M(^{37}Cl)^+$, 0.1], 206 [$M(^{35}Cl)^+$, 0.2], 122 (21), 96 (6), 94 (19), 85 (28), 69 (7), 57 (100), 41 (25). Anal. Calc. for $C_9H_{15}ClO_3$: C, 52.43; H, 7.28. Found: C, 52.40; H, 7.29.

*6.2.6. Ethyl 2-chloro-3-oxo-4-phenylpropanoate (**7f**) [21]*

The product was isolated from the crude reaction mixture as an oil (b.p. = 150 °C/22 mmHg) [21] by chromatography:

silica gel, mobile phase: petroleum ether/ethyl acetate = 9:1; reaction time = 2 h; 92% yield. IR (neat): 3467 (very weak band due to almost 3% of enol form as by NMR, see below), 3066, 2984, 1764, 1692, 1597, 1581, 1468, 1450, 1369, 1303, 1269, 1184, 1025, 1002, 950, 875, 824, 763, 735, 689 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 12.40 (s, 1H, enol OH: exchanges with D_2O); 7.95–7.92 (m, 2H, aromatic protons); 7.59–7.53 (m, 1H, aromatic proton); 7.45–7.40 (t, 2H, aromatic protons); 5.64 (s, 1H, *CH*Cl); 4.24–4.16 (q, J = 7.14 Hz, 2H, CH_2CH_3); 1.17–1.13 (t, J = 7.14 Hz, 3H, CH_3CH_2). ^{13}C NMR ($CDCl_3$, δ): 188.57, 165.47, 134.64, 133.51, 129.43, 129.14, 63.36, 58.11, 14.05. GC–MS (70 eV) (*m/z*) (rel. int.) 228 [$M(^{37}Cl)^+$, 0.5], 226 [$M(^{35}Cl)^+$, 1.5], 125 (3), 106 (13), 105 (100), 77 (44), 51 (11). Anal. Calc. for $C_{11}H_{11}ClO_3$: C, 58.41; H, 4.87. Found: C, 58.42; H, 4.87.

6.3. Synthesis of ethyl 2-(4-chlorophenoxy)-3-oxoalkanoates **8a–g**

6.3.1. Sodium 4-chlorophenate

To a solution of 4-chlorophenol (10.51 g, 8.17 mmol) in absolute ethanol (10 ml) was slowly added a solution of NaOEt (0.56 g, 8.17 mmol) in absolute EtOH (110 ml). The mixture protected from light and moisture was stirred at room temperature for 1.5 h. Then, the EtOH was removed under reduced pressure. A white, light and moisture sensitive solid was obtained.

6.3.2. Caesium 4-chlorophenate

A solution of 4-chlorophenol (10.51 g, 8.17 mmol) in absolute ethanol (15 ml) was slowly added to a suspension of Cs_2CO_3 (2.7 g, 8.17 mmol) in absolute EtOH (35 ml). The mixture was stirred for 3 h at room temperature. Then, the EtOH was removed under reduced pressure. The obtained white powder was more stable and less hygroscopic than sodium 4-chlorophenate.

6.3.3. Procedure A

A mixture of sodium 4-chlorophenate (Table 3) in anhydrous toluene or anhydrous DMF (32 ml) was stirred at 80 °C for 30 min. Then, the reaction was cooled to 60 °C and a solution of ethyl 2-chloro-3-oxoalkanoate (2.968 g, 18.1 mmol) in anhydrous toluene or anhydrous DMF (5.5 ml) was added dropwise. The reaction mixture was stirred at 80 °C. The reaction was monitored by GC and stopped at the time indicated in Table 3. The mixture was treated with ethyl acetate and then washed with a saturated solution of NaCl. The organic extract was dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The product was isolated by chromatography.

6.3.4. Procedure B

Ethyl 2-chloro-3-oxoalkanoate and caesium 4-chlorophenate, in the ratio 1:1.4, were stirred at 50 °C. The reaction was monitored by GC and stopped at the indicated time (Table 3). The mixture was treated with ethyl acetate and then washed with a saturated solution of NaCl. The organic extract was dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The product was isolated by chromatography.

Table 3
4-Chlorophenate/ethyl 2-chloro-3-oxoalkanoates ratios and reaction times for the preparation of **8a–f**

Compound	4-Chlorophenate/substrate	Reaction time [a] (h)
8a	1.1	5 [a]
8b	1.1	3 [b]
8c	1.5	24 [c]
8d	1.4	18 [c] (30 h, 62% yield) [d]
8e	1.1	3 [c]
8f	1.5	24 [c] (6) [d]

[a] Unless otherwise indicated, reaction time was almost the same in the several conditions: reactants solubilised in toluene or DMF, the use of either sodium or caesium chlorophenate (see Sections 6.3.3. and 6.3.4.).
[b] It refers to the reaction accomplished with the ethyl alkanoate and sodium chlorophenate solubilised in toluene or DMF.
[c] It refers to the reaction accomplished with the ethyl alkanoate and sodium chlorophenate solubilised in toluene.
[d] The reaction was performed by using caesium chlorophenate in the absence of any solvent (Section 6.3.4.).

*6.3.4.1. Ethyl 2-(4-chlorophenoxy)-3-oxobutanoate (**8a**) [24].* The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 9:1; 69% yield; keto form/enol form = 68:32. IR (neat): 3600–3150, 3100, 3073, 2985, 2940, 2875, 1758, 1735, 1661, 1629, 1587, 1489, 1446, 1414, 1371, 1268, 1216, 1167, 1089, 1013, 827, 665 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 11.32 (s, 1H, OH enol: exchanges with D_2O); 7.26–7.20 (m, 4H, aromatic protons, 2H of keto form and 2H of enol form); 6.87–6.81 (m, 4H, aromatic protons, 2H of keto form and 2H of enol form); 5.03 (s, 1H, *CH*); 4.30–4.23 (q, J = 7.14 Hz, 2H, CH_2CH_3, keto form); 4.22–4.15 (q, J = 7.14 Hz, 2H, CH_2CH_3, enol form); 2.35 (s, 3H, COCH_3, keto form); 1.95 (s, 3H, COCH_3, enol form); 1.29-1.24 (t, J = 7.14 Hz, 3H, CH_3CH_2, keto form); 1.17–1.13 (t, J = 7.14 Hz, 3H, CH_3CH_2, enol form). ^{13}C NMR ($CDCl_3$, δ): 200.66, 165.88, 155.44, 129.95, 129.63, 127.87, 116.71, 116.05, 83.16, 62.79, 26.56, 14.22. GC–MS (70 eV) (*m/z*) (rel. int.) 258 [$M(^{37}Cl)^+$, 10], 256 [$M(^{35}Cl)^+$, 29], 216 (10), 214 (30), 147 (15), 143 (32), 141 (100), 139 (29), 129 (8), 128 (9), 113 (11), 111 (22), 75 (15), 43 (37). Anal. Calc. for $C_{12}H_{13}ClO_4$: C, 56.25; H, 5.08. Found: C, 56.21; H, 5.06.

*6.3.4.2. Ethyl 2-(4-chlorophenoxy)-3-oxopentanoate (**8b**).* The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 9:1; 76% yield; keto form/enol form = 69:31. IR (neat): 3418 (enol form), 3065, 2983, 2935, 1732, 1590, 1493, 1433, 1217, 1162, 1091, 1017, 827, 757, 643 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 11.39 (s, 1H, OH enol: exchanges with D_2O); 7.30–7.18 (m, 4H, aromatic protons, 2H of keto form and 2H of enol form); 6.88–6.79 (m, 4H,

aromatic protons, 2H of keto form and 2H of enol form); 5.04 (s, 1H, *CH*OC_6H_4Cl); 4.30–4.23 (q, *J* = 7.14 Hz, 2H, CH_2OCO of keto form); 4.22–4.15 (q, *J* = 7.14 Hz, 2H, CH_2OCO of enol form); 2.79–2.71 (dq, *J* = 18.95 and 7.28 Hz, 2H, CH_2CO of keto form); 2.34–2.26 (q, *J* = 7.28 Hz, 2H, CH_2COH of enol form); 1.29–1.24 (t, *J* = 7.14 Hz, 6H, CH_3CH_2OCO, 3H of keto form and 3H of enol form); 1.17–1.25 (t, *J* = 7.28 Hz, 3H, CH_3CH_2COH, enol form); 1.10–1.06 (t, *J* = 7.28 Hz, 3H, CH_3CH_2CO, keto form). ^{13}C NMR ($CDCl_3$, *δ*): 203.32, 166.10, 155.56, 129.94, 129.60, 127.80, 116.69, 116.05, 82.85, 62.68, 32.41, 14.25, 7.30. GC–MS (70 eV) (*m*/*z*) (rel. int.) 272 [M(^{37}Cl)$^+$, 8], 270 [M(^{35}Cl)$^+$, 25], 216 (15), 214 (48), 197 (6), 168 (4), 161 (9), 143 (29), 141 (100), 139 (22), 128 (10), 111 (19), 75 (15), 57 (90). Anal. Calc. for $C_{13}H_{15}ClO_4$: C, 57.78; H, 5.56. Found: C, 57.75; H, 5.58.

*6.3.4.3. Ethyl 2-(4-chlorophenoxy)-3-oxohexanoate (**8c**).* The product was isolated from the crude reaction mixture as an oil by chromatography: mobile phase: petroleum ether/ethyl ether = 7:3; 70% yield; keto form/enol form = 60:40. IR (neat): 3441 (enol form), 2967, 2933, 2876, 1752, 1728, 1658, 1622, 1490, 1266, 1216, 1090, 1025, 826, 677 cm^{-1}. ^{1}H NMR ($CDCl_3$, *δ*): 11.39 (s, 1H, OH enol: exchanges with D_2O); 7.28–7.21 (m, 4H, aromatic protons, 2H of enol form and 2H of keto form); 6.88–6.82 (m, 4H, aromatic protons, 2H of enol form and 2H of keto form); 5.03 (s, 1H, *CH*OC_6H_4Cl); 4.31–4.24 (q, *J* = 7.14 Hz, 2H, CH_2O of keto form); 4.23–4.16 (q, *J* = 7.14 Hz, 2H, CH_2O of enol form); 2.79–2.68 (dt, 1H, *J* = 7.14 and 18.13 Hz, *CH*HCO of keto form); 2.70–2.59 (dt, 1H, *J* = 7.14 and 18.13 Hz, *C*H*H*CO of keto form); 2.29–2.24 (t, *J* = 7.49 Hz, 2H, CH_2COH of enol form); 1.70–1.53 (m, 4H, CH_2CH_2, 2H of enol form and 2H of keto form); 1.30–1.25 (t, *J* = 7.14 Hz, 3H, CH_3CH_2O of keto form); 1.18–1.13 (t, *J* = 7.14 Hz, 3H, CH_3CH_2O of enol form); 0.94–0.89 (t, *J* = 7.28 Hz, 3H, $CH_3CH_2CH_2$ of keto form); 0.90–0.85 (t, *J* = 7.28 Hz, 3H, $CH_3CH_2CH_2$ of enol form). ^{13}C NMR ($CDCl_3$, *δ*): 202.64, 166.03, 155.53, 129.91, 129.55, 127.75, 116.68, 116.09, 83.00, 62.66, 40.76, 31.78, 16.64, 14.02. GC–MS (70 eV) (*m*/*z*) (rel. int.) 286 [M(^{37}Cl)$^+$, 9], 284 [M(^{35}Cl)$^+$, 26], 216 (18), 214 (53), 175 (11), 143 (28), 141 (97), 139 (22), 128 (15), 113 (10), 111 (23), 75 (16), 71 (100), 43 (77), 41 (13). Anal. CH for $C_{14}H_{17}ClO_4$.

*6.3.4.4. Ethyl 2-(4-chlorophenoxy)-3-oxo-4-methylpentanoate (**8d**).* The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl ether = 7:3; 68% yield; keto form/enol form = 46:54. IR (neat): 3440 (enol form), 3060, 2979, 2927, 2869, 1755, 1725, 1657, 1621, 1592, 1489, 1469, 1411, 1376, 1342, 1263, 1230, 1169, 1091, 1028, 1014, 826, 692 cm^{-1}. ^{1}H NMR ($CDCl_3$, *δ*): 11.45 (s, 1H, OH enol: exchanges with D_2O); 7.28–7.20 (m, 4H, aromatic protons, 2H of enol form and 2H of keto form); 6.88–6.81 (m, 4H, aromatic protons, 2H of enol form and 2H of keto form); 5.13 (s, 1H, *CH*OC_6H_4Cl); 4.31–4.23 (q, *J* = 7.14 Hz, 2H, CH_2CH_3 of keto form); 4.22–4.15 (q, *J* = 7.14 Hz, 2H, CH_2CH_3 of enol form); 3.24–3.13 (heptet, *J* = 6.87 Hz, 1H, $CH(CH_3)_2$ of enol form); 2.91–2.81 (heptet, *J* = 6.87 Hz, 1H, $CH(CH_3)_2$ of keto form); 1.29–1.24 (t, *J* = 7.14 Hz, 3H, CH_3CH_2 of keto form); 1.17–1.15 (d, *J* = 6.87 Hz, 6H, $(CH_3)_2$CH of enol form); 1.11–1.07 (2d, *J* = 6.87 Hz, 6H, $(CH_3)_2$CH of keto form); 1.12–1.07 (t, *J* = 7.14 Hz, 3H, CH_3CH_2 of enol form). ^{13}C NMR ($CDCl_3$, *δ*): 206.26, 166.31, 155.68, 129.94, 129.58, 127.83, 116.73, 116.01, 81.99, 62.63, 37.32, 19.20, 18.38, 14.25. GC–MS (70 eV) (*m*/*z*) (rel. int.) 286 [M(^{37}Cl)$^+$, 7], 284 [M(^{35}Cl)$^+$, 20], 216 (12), 214 (37), 143 (12), 141 (43), 139 (17), 128 (13), 111 (17), 75 (13), 71 (84), 43 (100), 41 (11). Anal. CH for $C_{14}H_{17}ClO_4$.

*6.3.4.5. Ethyl 2-(4-chlorophenoxy)-3-oxo-4,4-dimethylpentanoate (**8e**).* The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl acetate = 10:1; 65% yield; enol form content in $CDCl_3$ solution measured by NMR was less than 3%. IR (neat): 3429 (very weak band, enol form), 3099, 2975, 2873, 1756, 1719, 1646, 1586, 1491, 1396, 1369, 1339, 1215, 1086, 1009, 825, 646 cm^{-1}. ^{1}H NMR ($CDCl_3$, *δ*): 12.01 (bs, 1H, OH enol: exchanges with D_2O; the extent of enolisation found was approximately 3%); 7.22–7.18 (m, 2H, aromatic protons); 6.85–6.80 (m, 2H, aromatic protons); 5.35 (s, 1H, *CH*OC_6H_4Cl); 4.26–4.19 (q, *J* = 7.14 Hz, 2H, CH_2CH_3); 1.25–1.20 (t, *J* = 7.14 Hz, 3H, CH_2CH_3); 1.21 (s, 9H, $(CH_3)_3CH$). ^{13}C NMR ($CDCl_3$, *δ*): 205.77, 166.83, 155.92, 129.83, 127.72, 116.75, 78.99, 62.35, 44.94, 26.52, 14.21. GC–MS (70 eV) (*m*/*z*) (rel. int.) 300 [M(^{37}Cl)$^+$, 4], 298 [M(^{35}Cl)$^+$, 13], 214 (14), 143 (18), 141 (53), 139 (8), 128 (10), 111 (11), 85 (23), 57 (100), 41 (13). Anal. CH for $C_{15}H_{19}ClO_4$.

*6.3.4.6. Ethyl 2-(4-chlorophenoxy)-3-oxo-3-phenylpropanoate (**8f**).* The product was isolated from the crude reaction mixture as an oil by chromatography: silica gel, mobile phase: petroleum ether/ethyl ether = 9.5:0.5; 78% yield; enol form content in $CDCl_3$ solution measured by NMR was less than 3%. M.p. = 60.2–61.3 °C (hexane), white crystals. IR ($CHCl_3$): 3449 (very weak band, enol form), 3072, 2978, 2942, 2913, 1735, 1697, 1596, 1581, 1493, 1447, 1372, 1223, 1209, 1075, 1010, 820, 706, 685 cm^{-1}. ^{1}H NMR ($CDCl_3$, *δ*): 11.99 (bs, 1H, OH enol: exchanges with D_2O; the extent of enolisation found was approximately 3%); 8.09–8.05 (m, 2H, aromatic protons); 7.64–7.58 (m, 1H, aromatic proton); 7.51–7.45 (m, 2H, aromatic protons); 7.26–7.21 (m, 2H, aromatic protons); 6.92–6.87 (m, 2H, aromatic protons); 5.70 (s, 1H, *CH*OC_6H_4Cl); 4.32–4.25 (q, *J* = 7.14 Hz, 2H, CH_2CH_3); 1.25–1.20 (t, *J* = 7.14 Hz, 3H, CH_3CH_2). ^{13}C NMR ($CDCl_3$, *δ*): 191.16, 166.49, 155.68, 134.53, 134.00, 129.88, 129.72, 129.00, 127.84, 117.03, 81.38, 62.74, 14.19. GC–MS (70 eV) (*m*/*z*) (rel. int.) 320 [M(^{37}Cl)$^+$, 2], 318 [M(^{35}Cl)$^+$, 6], 245 (1), 209 (3), 128 (3), 105 (100), 77 (22), 51 (4). Anal. CH for $C_{17}H_{15}ClO_4$.

*6.3.5. Synthesis of ethyl 2-(4-chlorophenoxy)-2-methyl-3-oxobutanoate (**8g**)*

To a solution of **8a** (700 mg) in anhydrous DMF (2.8 ml) kept in an ice bath, Cs_2CO_3 (891 mg) was added. The mixture was stirred at 0 °C for 15 min, then CH_3I (1.7 ml) in DMF (5.7 ml) was added dropwise. The reaction mixture was brought to room temperature and stirred for further 15 h. Then, brine was added and the product was extracted three times with ethyl ether. The organic phase was washed three times with brine, then with aqueous saturated $Na_2S_2O_3$ and aqueous saturated $NaHCO_3$. The organic layer was dried with anhydrous Na_2SO_4 and then the solvent was removed under reduced pressure. The product was isolated as colourless oil (90% yield) by chromatography (silica gel, petroleum ether/ethyl acetate = 10:1). IR (neat): 3064, 2984, 2933, 1752, 1728, 1593, 1490, 1456, 1374, 1356, 1263, 1232, 1130, 1098, 1012, 828, 669 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 7.25–7.19 (m, 2H, aromatic protons); 6.85–6.80 (m, 2H, aromatic protons); 4.27–4.20 (q, J = 7.14 Hz, 2H, CH_2CH_3); 2.40 (s, 3H, $COCH_3$); 1.61 (s, 3H, CH_3C_q); 1.25–1.21 (t, J = 7.14 Hz, 3H, CH_3CH_2). ^{13}C NMR ($CDCl_3$, δ): 204.25, 169.13, 153.32, 129.64, 128.46, 120.61, 87.84, 62.54, 25.45, 18.68, 14.12. GC–MS (70 eV) (*m/z*) (rel. int.) 272 [$M(^{37}Cl)^+$, 11], 270 [$M(^{35}Cl)^+$, 31], 230 (32), 229 (27), 228 (91), 227 (42), 199 (33), 197 (29), 183 (14), 181 (42), 157 (33), 155 (100), 153 (40), 147 (16), 139 (15), 130 (13), 129 (30), 128 (33), 113 (11), 112 (10), 111 (30), 99 (11), 75 (21), 43 (83). Anal. CH for $C_{13}H_{15}ClO_4$.

*6.3.6. General procedure for the reduction of ethyl 2-(4-chlorophenoxy)-3-oxoalkanoates **8a–g** by $NaBH_4$/MeOH*

To a solution of 2-(4-chlorophenoxy)-3-oxoalkanoate (0.406 mmol) in methyl alcohol (5 ml) kept in an ice bath was added $NaBH_4$ (see each compound). The reaction mixture was stirred at 0 °C for 10 min, then at room temperature and stopped at the indicated time (see each compound). Water (3 ml) was added and the methyl alcohol was evaporated under vacuum. The residue was extracted three times with EtOAc (30 ml). The extracts were dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The product (**9a–g**) was obtained in very high yield (Table 1).

*6.3.6.1. Ethyl 2-(4-chlorophenoxy)-3-hydroxybutanoate (**9a**).* Substrate/$NaBH_4$ used in the reaction was 1:1.3; reaction time = 20 min; 90% yield; d.e. = 11%. Oil. IR (neat): 3500–3100, 3061, 2982, 2933, 1738, 1596, 1584, 1492, 1375, 1283, 1238, 1200, 1094, 1023, 825 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 7.26–7.18 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.88–6.78 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 4.56–4.53 (d, J = 4.29 Hz, 1H, $CHOC_6H_4Cl$ of a stereoisomer couple); 4.43–4.41 (d, J = 4.94 Hz, 1H, $CHOC_6H_4Cl$ of the other stereoisomer couple); 4.30–4.17 (m, 6H, 3H for each couple of stereoisomers: 1H of $CHCH_3$ and 2H of CH_2CH_3); 2.90–2.50 (bs, 1H for each couple of stereoisomers, OH: exchange with D_2O); 1.34–1.32 (d, J = 6.45 Hz, 3H, $CHCH_3$ of one stereoisomer couple); 1.33–1.31 (d, J = 6.45 Hz, 3H, $CHCH_3$ of the other stereoisomer couple); 1.25–1.21 (t, J = 7.14 Hz, 3H, CH_3CH_2 of one stereoisomer couple); 1.24–1.20 (t, J = 7.14 Hz, 3H, CH_3CH_2 of the other stereoisomer couple). ^{13}C NMR ($CDCl_3$, δ): 169.76, 156.45, 129.76, 127.29, 116.88, 81.52, 81.06, 68.65, 68.60, 61.95, 19.10, 14.37. GC–MS (70 eV) (*m/z*) (rel. int.) 260 [$M(^{37}Cl)^+$, 5], 258 [$M(^{35}Cl)^+$, 17], 214 (21), 143 (30), 141 (100), 139 (16), 130 (17), 128 (52), 111 (13), 75 (12), 43 (13). Anal. CH for $C_{12}H_{15}ClO_4$.

*6.3.6.2. Ethyl 2-(4-chlorophenoxy)-3-hydroxypentanoate (**9b**).* Substrate/$NaBH_4$ used in the reaction was 1:1.3; reaction time = 30 min; 88% yield; d.e. = 7%. Oil. IR (neat): 3432–3237, 3050, 2964, 2920, 2872, 1730, 1642, 1490, 1458, 1262, 1216, 1092, 1017, 800, 760 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 7.18–7.14 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.80–6.71 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 4.51–4.49 (d, J = 4.54 Hz, 1H, $CHOC_6H_4Cl$ of one stereoisomer couple); 4.43–4.41 (d, J = 3.98 Hz, 1H, $CHOC_6H_4Cl$ of the other stereoisomer couple); 4.20–4.13 (q, J = 7.14 Hz, 4H, CH_2OCO, 2H for each stereoisomer couple); 3.98–3.89 (m, 2H, *CH*OH, 1H for each stereoisomer couple); 2.40–2.00 (bs, 1H for each couple of stereoisomers, OH: exchange with D_2O); 1.62–1.57 (m, 4H, CH_2CHOH, 2H for each stereoisomer couple); 1.20–1.15 (t, J = 7.14 Hz, 6H, CH_3CH_2O, 3H for each stereoisomer couple); 0.99–0.92 (m, 6H, CH_3CH_2CHOH, 3H for each stereoisomer couple). ^{13}C NMR ($CDCl_3$, δ): 170.00, 156.60, 129.72, 127.30, 116.84, 80.29, 79.88, 73.92, 73.92, 61.86, 61.78, 26.46, 25.78, 14.32. GC–MS (70 eV) (*m/z*) (rel. int.) 274 [$M(^{37}Cl)^+$, 4], 272 [$M(^{35}Cl)^+$, 13], 216 (8), 214 (24), 168 (7), 143 (33), 142 (9), 141 (100), 139 (11), 130 (13), 129 (9), 128 (38), 111 (10), 99 (9), 75 (8), 57 (8), 43 (6). Anal. CH for $C_{13}H_{17}ClO_4$.

*6.3.6.3. Ethyl 2-(4-chlorophenoxy)-3-hydroxyhexanoate (**9c**).* Substrate/$NaBH_4$ used in the reaction was 1:1.5; reaction time = 3 h; 73% yield; d.e. = 22%. Oil. IR (neat): 3500–3150, 3065, 2961, 2934, 2872, 1737, 1595, 1492, 1465, 1379, 1282, 1238, 1199, 1094, 1075, 1030, 825, 668 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 7.18–7.13 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.77–6.73 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 4.50–4.49 (d, J = 4.40 Hz, 1H, $CHOC_6H_4Cl$ of one stereoisomer couple); 4.42–4.40 (d, J = 3.98 Hz, 1H, $CHOC_6H_4Cl$ of the other stereoisomer couple); 4.19–4.10 (2q, J = 7.14 Hz, 4H, CH_2O, 2H for each stereoisomer couple); 4.09–3.95 (m, 2H, *CH*OH, 1H for each stereoisomer couple); 2.28–2.10 (bs, 1H for each couple of stereoisomers, OH: exchange with D_2O); 1.65–1.42 (m, 8H, CH_2CH_2CHOH, 4H for each stereoisomer couple); 1.27–1.22 (2t, J = 7.14 Hz, 6H, CH_3CH_2O, 3H for each stereoisomer couple); 0.94–0.82 (m, 6H, $CH_3CH_2CH_2$, 3H for each stereoisomer couple). ^{13}C NMR ($CDCl_3$, δ): 169.98, 156.53, 129.73, 116.89, 116.84, 80.61, 80.22, 72.35, 72.23, 61.93, 35.44, 19.00, 14.33, 14.08.

GC–MS (70 eV) (*m/z*) (rel. int.) 288 [M(^{37}Cl)$^+$, 4], 286 [M(^{35}Cl)$^+$, 12], 216 (11), 214 (32), 168 (8), 143 (37), 142 (13), 141 (100), 139 (11), 130 (14), 128 (38), 113 (10), 111 (11), 75 (8), 71 (1), 43 (11). Anal. CH for $C_{14}H_{19}ClO_4$.

*6.3.6.4. Ethyl 2-(4-chlorophenoxy)-3-hydroxy-4-methylpentanoate (**9d**).* Substrate/$NaBH_4$ used in the reaction was 1:1.5; reaction time = 3 h; 93% yield; d.e. = 25%. Oil. IR (neat): 3500–3100, 2926, 2848, 1739, 1491, 1462, 1375, 1237, 1162, 1130, 1097, 953, 874, 800, 860 cm^{-1}. ^{1}H NMR ($CDCl_3$, δ): 7.27–7.20 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.86–6.80 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 4.67–4.66 (d, J = 3.29 Hz, 1H, *CH*OC_6H_4Cl of one stereoisomer couple); 4.63–4.61 (d, J = 5.36 Hz, 1H, *CH*OC_6H_4Cl of the other stereoisomer couple); 4.28–4.21 (2q, J = 7.14 Hz, 4H, CH_2CH_3, 2H for each stereoisomer couple); 3.86–3.82 (t, J = 5.36 Hz, 1H, *CH*OH of one stereoisomer couple); 3.75–3.72 (dd, J = 3.29 and 7.62 Hz, 1H, *CH*OH of the other stereoisomer couple); 2.36–2.28 (m, 1H, $CH(CH_3)_2$ of one stereoisomer couple); 2.05–1.97 (m, 1H, $CH(CH_3)_2$ of the other stereoisomer couple); 1.70–1.50 (bs, 1H for each couple of stereoisomers, OH: exchange with D_2O); 1.29–1.22 (t, J = 7.14 Hz, 6H, CH_3CH_2, 3H for each stereoisomer couple); 1.09–1.03 (2d, J = 6.73 Hz, 6H, $(CH_3)_2$CH of one stereoisomer couple); 1.00–0.92 (2d, J = 6.73 Hz, 6H, $(CH_3)_2$CH of the other stereoisomer couple). ^{13}C NMR ($CDCl_3$, δ): 170.25, 156.00, 129.79, 127.15, 116.67, 78.54, 78.14, 70.78, 61.94, 31.21, 29.38, 27.44, 14.34. GC–MS (70 eV) (*m/z*) (rel. int.) 288 [M(^{37}Cl)$^+$, 3], 286 [M(^{35}Cl)$^+$, 9], 216 (10), 214 (31), 168(6), 143 (32), 141 (100), 130 (12), 128 (34), 113 (10), 111 (12), 75 (8), 71 (9), 43 (17), 41 (9). Anal. CH for $C_{14}H_{19}ClO_4$.

*6.3.6.5. Ethyl 2-(4-chlorophenoxy)-3-hydroxy-4,4-dimethylpentanoate (**9e**).* Substrate/$NaBH_4$ used in the reaction was 1:1.2; ethanol replaced methanol as reaction solvent; reaction time = 1.5 h; 94% yield; d.e. = 8%. Oil. IR (neat): 3550–3100, 3062, 2960, 2872, 1739, 1595, 1584, 1491, 1397, 1370, 1337, 1283, 1237, 1094, 1064, 1021, 825, 667 cm^{-1}. ^{1}H NMR ($CDCl_3$, δ): 7.25–7.21 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.86–6.77 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 4.76–4.75 (d, J = 1.65 Hz, 1H, *CH*OC_6H_4Cl of one stereoisomer couple); 4.62–4.60 (d, J = 5.91 Hz, 1H, *CH*OC_6H_4Cl of the other stereoisomer couple); 4.27–4.20 (q, J = 7.14 Hz, 4H, CH_2CH_3, 2H for each stereoisomer couple); 3.81–3.79 (d, J = 5.91 Hz, 1H, *CH*OH of one stereoisomer couple); 3.75–3.74 (d, J = 1.65 Hz, 1H, *CH*OH of the other stereoisomer couple); 2.60–2.20 (bs, 1H for each couple of stereoisomers, OH: exchange with D_2O); 1.27–1.22 (t, J = 7.14 Hz, 6H, CH_3CH_2, 3H for each stereoisomer couple); 1.01 (s, 9H, $(CH_3)_3$CH of one stereoisomer couple); 1.00 (s, 9H, $(CH_3)_3$CH of the other stereoisomer couple).

^{13}C NMR ($CDCl_3$, δ): 170.66, 155.89, 129.91, 127.06, 116.66, 79.37, 76.75, 62.02, 61.78, 35.71, 35.24, 26.74, 26.18, 14.33. GC–MS (70 eV) (*m/z*) (rel. int.) 302 [M(^{37}Cl)$^+$, 3], 300 [M(^{35}Cl)$^+$, 9], 227 (2), 216 (13), 214 (39), 168 (6), 143 (35), 141 (100), 139 (8), 130 (15), 128 (33), 111 (11), 57 (27), 41 (10). Anal. CH for $C_{15}H_{21}ClO_4$.

*6.3.6.6. Ethyl 2-(4-chlorophenoxy)-3-hydroxy-3-phenylpropanoate (**9f**).* Substrate/$NaBH_4$ used in the reaction was 1:1.3; reaction time = 3 h; 96% yield; d.e. = 34%. M.p. = 82.3–83.3 °C (hexane). IR (KBr): 3550–3250, 3225, 2955, 2926, 2845, 1733, 1596, 1582, 1490, 1450, 1375, 1261, 1235, 1216, 1192, 1093, 1050, 1026, 824, 760 cm^{-1}. ^{1}H NMR ($CDCl_3$, δ): 7.40–7.16 (m, 10H, aromatic protons, 5H for each stereoisomer couple); 7.13–7.02 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.74–6.67 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 5.13–5.11 (d, J = 5.84 Hz, 1H, *CH*OH of one stereoisomer couple); 5.09–5.08 (d, J = 5.42 Hz, 1H, *CH*OH of the other stereoisomer couple); 4.65–4.63 (d, J = 5.84 Hz, 1H, *CH*OC_6H_4Cl of one stereoisomer couple); 4.61–4.59 (d, J = 5.42 Hz, 1H, *CH*OC_6H_4Cl of the other stereoisomer couple); 4.11–4.04 (q, J = 7.14 Hz, 2H, CH_2CH_3 of one stereoisomer couple); 4.01–3.96 (q, J = 7.14 Hz, 2H, CH_2CH_3 of the other stereoisomer couple); 3.10–2.70 (bs, 1H for each couple of stereoisomers, OH: exchange with D_2O); 1.08–1.03 (t, J = 7.14 Hz, 3H, CH_3CH_2 of one stereoisomer couple); 1.02–0.97 (t, J = 7.14 Hz, 3H, CH_3CH_2 of the other stereoisomer couple). ^{13}C NMR ($CDCl_3$, δ): 169.47, 139.12, 129.73, 129.66, 128.73, 128.61, 126.93, 126.89, 117.19, 82.34, 81.45, 75.03, 74.38, 61.82, 14.17. GC–MS (70 eV) (*m/z*) (rel. int.) 322 [M(^{37}Cl)$^+$, 0.1], 320 [M(^{35}Cl)$^+$, 0.2], 302 [M(^{35}Cl)$^+$ – 18, 0.3], 247 (2), 216 (22), 214 (68), 143 (34), 141 (100), 128 (20), 113 (16), 111 (25), 107 (19), 106 (27), 105 (36), 91 (14), 79 (16), 77 (49), 75 (15), 51 (16), 50 (10). Anal. CH for $C_{17}H_{17}ClO_4$.

*6.3.6.7. Ethyl 2-(4-chlorophenoxy)-2-methyl-3-hydroxybutanoate (**9g**).* Substrate/$NaBH_4$ used in the reaction was 1:1.3; reaction time = 30 min; 87% yield; d.e. = 60%. Oil. IR (neat): 3500–3100, 3062, 2983, 2936, 1736, 1593, 1490, 1443, 1376, 1239, 1094, 1047, 1012, 850, 823 cm^{-1}. ^{1}H NMR ($CDCl_3$, δ): 7.21–7.18 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.85–6.82 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 4.28–4.21 (q, J = 7.14 Hz, 4H, CH_2OCO, 2H for each stereoisomer couple); 4.18–4.12 (q, J = 6.41 Hz, 2H, *CH*OH, 1H for each stereoisomer couple); 2.80–2.40 (bs, 1H for each stereoisomer couple, OH: exchange with D_2O); 1.46 (s, 3H, CH_3C_q of one stereoisomer couple); 1.40 (s, 3H, CH_3C_q of the other stereoisomer couple); 1.28–1.17 (m, 12 H, 3H of CH_3CH_2 and 3H of CH_3CH for each stereoisomer couple, respectively). ^{13}C NMR ($CDCl_3$, δ): 172.55, 153.82, 129.48, 128.13, 121.27, 121.18, 85.80, 72.74, 72.47, 61.91, 16.85, 15.47, 14.27. GC–MS (70 eV) (*m/z*) (rel. int.) 274 [M(^{37}Cl)$^+$, 3], 272 [M(^{35}Cl)$^+$, 8], 228 (23), 199 (13), 157 (11), 155 (34), 130 (34), 129 (13), 128 (100), 111 (9), 99 (19), 43 (36). Anal. CH for $C_{13}H_{17}ClO_4$.

*6.3.7. General procedure for the preparation of 2-(4-chlorophenoxy)-3-hydroxyalkanoic acids **10a–g***

0.45 M aqueous KOH (substrate/KOH = 1/2) was added to a 0.08 M solution of ethyl 2-(4-chlorophenoxy)-3-hydroxyalkanoate in THF. The reaction mixture was stirred at room temperature and when no more substrate was observed by TLC (mobile phase: petroleum ether/ethyl acetate = 8:2), the solvent was removed under reduced pressure. The alkaline aqueous layer was washed three times with ethyl ether, then acidified with 2 N HCl and extracted three times with ethyl ether. The combined organic extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure to give a colourless oil which was crystallised from $CHCl_3$/hexane.

*6.3.7.1. 2-(4-Chlorophenoxy)-3-hydroxybutanoic acid (**10a**).* Reaction time = 1 h; 79% yield; d.e. = 15%. White solid. M.p. = 105.9–107 °C ($CHCl_3$/hexane). IR (KBr): 3650–3200, 3030, 2985, 2940, 1718, 1594, 1586, 1495, 1455, 1409, 1384, 1230, 1171, 1157, 1080, 1009, 878, 826, 799 cm^{-1}. ^1H NMR (CD_3OD, δ): 7.27–7.22 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.94–6.89 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 5.10–4.90 (bs, 4H, OH and COOH, 2H for each stereoisomer couple: exchange with D_2O); 4.61–4.59 (d, J = 4.26 Hz, 1H, *CH*OC_6H_4Cl of one stereoisomer couple); 4.54–4.52 (d, J = 4.12 Hz, 1H, *CH*OC_6H_4Cl of the other stereoisomer couple); 4.28–4.19 (m, 2H, *CH*OH, 1H for each stereoisomer couple); 1.34–1.32 (d, J = 6.46 Hz, 3H, CH_3CHOH of one stereoisomer couple); 1.33–1.30 (d, J = 6.59 Hz, 3H, CH_3CHOH of the other stereoisomer couple). ^{13}C NMR (CD_3OD, δ): 171.88, 171.74, 157.31, 157.14, 129.15, 126.26, 126.19, 116.71, 116.57, 81.21, 81.09, 67.90, 18.19, 17.28. Anal. CH for $C_{10}H_{11}ClO_4$.

*6.3.7.2. 2-(4-Chlorophenoxy)-3-hydroxypentanoic acid (**10b**).* Reaction time = 1.5 h; 78% yield; d.e. = 11%. White solid. M.p. = 103–104.2 °C ($CHCl_3$/hexane). IR (KBr): 3650–3200, 3060, 2971, 2884, 1723, 1595, 1584, 1491, 1411, 1341, 1233, 1174, 1146, 1070, 1006, 875, 827, 800, 741 cm^{-1}. ^1H NMR (CD_3OD, δ): 7.27–7.20 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.94–6.85 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 5.10–4.75 (bs, 4H, OH and COOH, 2H for each stereoisomer couple: exchange with D_2O); 4.63–4.55 (m, 2H, *CH*OC_6H_4Cl, 1H of each stereoisomer couple); 4.02–3.88 (m, 2H, *CH*OH, 1H of each stereoisomer couple); 1.74–1.60 (m, 4H, CH_3CH_2, 2H for each stereoisomer couple); 1.05–0.95 (m, 6H, CH_3CH_2, 3H for each stereoisomer couple). ^{13}C NMR (CD_3OD, δ): 176.02, 175.83, 161.29, 161.03, 133.07, 130.18, 130.04, 120.59, 120.42, 84.58, 83.44, 77.33, 77.24, 29.90, 29.16, 13.38. Anal. CH for $C_{11}H_{13}ClO_4$.

*6.3.7.3. 2-(4-Chlorophenoxy)-3-hydroxyhexanoic acid (**10c**).* Reaction time = 3 h; 71% yield; d.e. = 4%. White solid. M.p. = 101.5–103 °C ($CHCl_3$/hexane). IR (KBr): 3650–3200, 3035, 2964, 2876, 1723, 1595, 1584, 1491, 1415, 1344, 1227, 1175, 1133, 1066, 1008, 826 cm^{-1}. ^1H NMR (CD_3OD, δ): 7.28–7.20 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.94–6.87 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 5.00–4.80 (bs, 4H, OH and COOH, 2H for each stereoisomer couple: exchange with D_2O); 4.61–4.59 (2d partially overlapped, J = 4.67 and 3.57 Hz, 2H, *CH*OC_6H_4Cl, 1H of each stereoisomer couple); 4.12–3.99 (m, 2H, *CH*OH, 1H of each stereoisomer couple); 1.73–1.33 (m, 8H, CH_2CHOH and CH_3CH_2, 4H for each stereoisomer couple); 0.97–0.94 (m, 6H, CH_3CH_2, 3H for each stereoisomer couple). ^{13}C NMR (CD_3OD, δ): 172.08, 171.86, 157.36, 157.14, 129.14, 125.25, 126.12, 116.68, 116.51, 80.97, 79.95, 71.59, 71.50, 35.09, 34.33, 18.82, 13.08. Anal. CH for $C_{12}H_{15}ClO_4$.

*6.3.7.4. 2-(4-Chlorophenoxy)-3-hydroxy-4-methylpentanoic (**10d**).* Reaction time = 1.5 h; 44% yield; d.e. = 27%. White solid. M.p. = 144.4–145.7 °C ($CHCl_3$/hexane). IR (KBr): 3600–3200, 3035, 2961, 2872, 1715, 1598, 1583, 1490, 1410, 1388, 1226, 1175, 1136, 1064, 1007, 900, 965, 823, 799, 708 cm^{-1}. ^1H NMR (CD_3OD, δ): 7.27–7.23 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.93–6.89 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 5.10–4.83 (bs, 4H, OH and COOH, 2H for each stereoisomer couple: exchange with D_2O); 4.77–4.76 (d, J = 2.74 Hz, 1H, *CH*OC_6H_4Cl of one stereoisomer couple); 4.62–4.60 (d, J = 6.04 Hz, 1H, *CH*OC_6H_4Cl of the other stereoisomer couple); 3.79–3.75 (t, J = 5.77 Hz, 1H, C*H*OH of one stereoisomer couple); 3.72–3.68 (dd, J = 8.10 Hz, J = 2.74 Hz, 1H, C*H*OH of the other stereoisomer couple); 2.08–1.92 (m, 2H, *CH*$(CH_3)_2$, 1H for each stereoisomer couple); 1.07–1.05 (d, J = 6.59 Hz, 3H, CH_3 of one stereoisomer couple); 1.04–1.01 (d, J = 7.14 Hz, 3H, CH_3 of the other stereoisomer couple); 0.97–0.94 (d, J = 6.59 Hz, 3H, CH_3 of one stereoisomer couple); 0.89–0.87 (d, J = 7.14 Hz, 3H, CH_3 of the other stereoisomer couple). ^{13}C NMR (CD_3OD, δ): 172.44, 172.34, 157.31, 156.81, 129.22, 129.15, 126.32, 126.04, 116.54, 116.30, 78.91, 77.93, 77.51, 76.23, 30.65, 30.00, 18.71; 18.44; 18.12; 16.16. Anal. CH for $C_{12}H_{15}ClO_4$.

*6.3.7.5. 2-(4-Chlorophenoxy)-4,4-dimethyl-3-hydroxypentanoic acid (**10e**).* Reaction time = 48 h; 14% yield; d.e. = 41%. White solid. M.p. = 151.8–152.3 °C ($CHCl_3$/hexane). ^1H NMR (CD_3OD, δ): 7.28–7.23 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.93–6.87 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 4.95–4.80 (m, 5H, OH and COOH for each stereoisomer couple (4H, exchange with D_2O), completely overlapped to *CH*OC_6H_4Cl of one stereoisomer couple); 4.62–4.59 (d, J = 6.59 Hz, 1H, *CH*OC_6H_4Cl of the other stereoisomer couple); 3.81–3.80 (d, J = 1.38 Hz, 1H, C*H*OH of one stereoisomer couple); 3.70–3.68 (d, J = 6.59 Hz, 1H, C*H*OH of the other stereoisomer couple); 1.00 (s, 9H, 3 CH_3 of one stereoisomer couple); 0.99 (s, 9H, 3 CH_3 of the other stereoisomer couple). Anal. CH for $C_{13}H_{17}ClO_4$.

*6.3.7.6. 2-(4-Chorophenoxy)-3-hydroxy-3-phenylpropanoic acid (**10f**).* Reaction time = 1 h; 84% yield; d.e. = 32%. White solid. M.p. = 110.3–111.4 °C ($CHCl_3$/hexane). IR (KBr): 3600–3200, 3032, 2980, 1729, 1595, 1584, 1489, 1454, 1399, 1278, 1235, 1172, 1121, 1079, 1058, 1006, 821, 769, 743, 705, 672 cm^{-1}. ^1H NMR (CD_3OD, δ): 7.49–7.46 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 7.35–7.22 (m, 6H, aromatic protons, 3H for each stereoisomer couple); 7.21–7.14 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.86–6.78 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 5.22–5.21 (d, J = 4.12 Hz, 1H, *CH*OH of one stereoisomer couple); 5.14–5.09 (d, J = 6.32 Hz, 1H, *CH*OH of the other stereoisomer couple); 5.00–4.80 (bs, 4H, OH and COOH, 2H for each stereoisomer couple: exchange with D_2O); 4.80–4.79 (d, J = 4.12 Hz, 1H, C*H*OC_6H_4Cl of one stereoisomer couple); 4.78–4.76 (d, J = 6.32 Hz, 1H, C*H*OC_6H_4Cl of the other stereoisomer couple). ^{13}C NMR (CD_3OD, δ): 171.81, 171.44, 157.25; 156.91; 140.58; 140.36; 129.11; 129.03, 128.02, 127.91, 127.82, 127.71, 127.19, 126.70, 126.41, 126.28, 116.83, 81.80, 81.46, 74.24, 73.93. Anal. CH for $C_{15}H_{13}ClO_4$.

*6.3.7.7. 2-(4-Chlorophenoxy)-3-hydroxy-2-methylbutanoic acid (**10g**).* Reaction time = 21 h; 64% yield; d.e. = 46%. White solid. M.p. = 125.6–126.8 °C ($CHCl_3$/hexane). IR (KBr): 3600–3200, 3030, 2999, 2943, 2818, 1745, 1593, 1581, 1491, 1459, 1408, 1285, 1240, 1182, 1149, 1129, 1091, 1027, 927, 825, 761 cm^{-1}. ^1H NMR (CD_3OD, δ): 7.25–7.19 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 6.97–6.92 (m, 4H, aromatic protons, 2H for each stereoisomer couple); 5.10–4.75 (bs, 4H, OH and COOH, 2H for each stereoisomer couple: exchange with D_2O); 4.13–4.05 (m, 2H, *CH*OH, 1H of each stereoisomer couple); 1.40 (s, 3H, *CH*$_3$COC_6H_4Cl of one stereoisomer couple); 1.38 (s, 3H, *CH*$_3$COC_6H_4Cl of the other stereoisomer couple); 1.31–1.29 (d, J = 6.32 Hz, 3H, *CH*$_3$CHOH of one stereoisomer couple); 1.24–1.22 (d, J = 6.32 Hz, 3H, *CH*$_3$CHOH of the other stereoisomer couple). ^{13}C NMR (CD_3OD, δ): 175.07, 174.69, 154.56, 128.84; 127.36, 127.17; 121.45, 121.14, 85.40, 84.53, 72.24, 71.66, 16.17; 15.36; 14.83; 14.27. Anal. CH for $C_{11}H_{13}ClO_4$.

6.4. PPARα transactivation assay

6.4.1. Plasmids

The reporter construct pG5-CAT, containing five copies of the high affinity binding site for GAL4 (UAS) and used for CAT assay, was purchased from BD Biosciences Clontech (Palo Alto, CA). GAL4/mousePPARαLBD receptor plasmid was prepared by fusing the mouse PPARα LBD to the DBD of yeast GAL4, and then subcloning the fusion into the pSG5 expression vector. pCH110, that encodes the β-galactosidase enzyme to correct for differences in transfection efficiency, was purchased from Pharmacia Biotech (Piscataway, NJ).

6.4.2. Transfection assay

Monkey kidney fibroblasts (COS-7) were seeded at 1.2×10^5 cells per well, in 12-well plates, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, overnight at 37 °C. Two hours before transfection, the culture medium was replaced by fresh serum-free medium and then transfection was performed with the multicomponent lipid-based FuGENE6 Transfection Reagent (F. Hoffmann-La Roche; Basel, Switzerland) according to the instructions of the manufacturer.

The transfection mixture containing (for each well) 0.8 μg of the expression vector, 1.6 μg of the reporter vector, 0.8 μg of the control vector and 9 μl of FuGENE6 Transfection Reagent was added directly to the cells in the presence of serum-free medium. After 5 h the transfection medium was removed and transfected cells were treated with three different concentrations (50, 150 and 300 μM) of the test molecules **8a**, **9a** and **10a**–**g**, at 37 °C for 48 h in the complete culture medium. 2 μM WY-14,643, a known PPARα ligand, was used as positive control.

Cells were then washed twice with phosphate-buffered saline (PBS) and then dissolved in lysis buffer (0.25 M Tris–HCl, pH 8) and lysed by three rapid freeze/thaw cycles (three 5-min cycles). Cell debris was then removed by centrifugating at 4 °C, for 15 min at 15,000 rpm. Glycerol (final 10% v/v) and β-mercaptoethanol (final 5 mM) were then added (final volume 75 μl) and the cell extracts were stored at –80 °C until assayed.

6.4.3. Assays to determine CAT and β-galactosidase activity

The CAT activity assay was performed as follows: 20 μl of cell lysate (prewarmed at 65 °C for 10 min, to deactivate internal deacetylase enzymatic activity) were added to 10 μl of 3.5 mg ml^{-1} *n*-butirryl-CoA, 5 μl (0.25 μCi) of [^{14}C]-chloramphenicol and 65 μl of distilled water and incubated 2 h at 37 °C. Reaction was blocked by adding 200 μl of the solution xylene/2,6,10,14-tetramethylpentadecane (1:2 v/v). After a vigorous vortexing and centrifugation for 5 min at top speed, 150 μl of supernatant were transferred to scintillation vial in the presence of 5 ml of scintillation liquid, and the relative radioactivity was measured by a β-counter.

The β-galactosidase activity was measured as follows: 20 μl of cellular extracts were added to 750 μl of reaction buffer consisting of 1 vol. of 2 mg ml^{-1} ONPG (*o*-nitrophenyl-β-galactopyranoside) and 3 vol. of "Z buffer" (10 mM potassium chloride, 1 mM magnesium chloride, and 50 mM β-mercaptoethanol in phosphate buffer). Reaction was performed at 37 °C and blocked by adding 200 μl of 1 M Na_2CO_3 when a typical yellow colour became appreciable. Samples were incubated for 10 min at room temperature and then the absorbance at 420 nm (A_{420}) was spectrophotometrically measured.

Finally, the CAT activity was normalised to the β-galactosidase activity.

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