Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 2473–2488
Bioorganic & Medicinal Chemistry

# Stereospecific synthesis and bio-activity of novel $\beta_3$-adrenoceptor agonists and inverse agonists

Maria Grazia Perrone,[a] Ernesto Santandrea,[a] Laura Bleve,[a] Paola Vitale,[a] Nicola Antonio Colabufo,[a] Ralf Jockers,[b] Ferdinando Maria Milazzo,[c] Anna Floriana Sciarroni[c] and Antonio Scilimati[a,*]

[a]*Dipartimento Farmaco-Chimico, Università di Bari, Via E.Orabona 4, 70125 Bari, Italy*
[b]*Institut Cochin Inserm U567, CNRS 8104, Université Paris, Descartes 22 rue Méchain, 75014 Paris, France*
[c]*Dipartimento di Endocrinologia e Metabolismo, Sigma-Tau S.p.A, Via Pontina km 30.400, 00040 Pomezia, Italy*

Received 22 June 2007; revised 13 November 2007; accepted 21 November 2007
Available online 28 November 2007

**Abstract**—Since it is widely distributed into the body, $\beta_3$-adrenoceptor is becoming an attractive target for the treatment of several pathologies such as obesity, type 2 diabetes, metabolic syndrome, cachexia, overactive bladder, ulcero-inflammatory disorder of the gut, preterm labour, anxiety and depressive disorders, and heart failure. New compounds belonging to the class of arylethanolamines bearing one or two stereogenic centres were prepared in good yields as racemates and optically active forms. They were, then, evaluated for their intrinsic activity towards $\beta_3$-adrenoceptor and their affinity for $\beta_1$- and $\beta_2$-adrenergic receptors. Stereochemical features were found to play a crucial role in determining the behaviour of such compounds. In particular, α-racemic, (α*R*)- and (α*S*)-2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}-2- methylpropanoic acid, (α-rac, β-rac)-, (α*R*, β*S*)- and (α*R*, β*R*)- 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoic acid were found to be endowed with $\beta_3$-adrenoceptor agonistic activity. Whereas, (α*S*, β*S*)- and (α*S*, β*R*)-2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoic acid behaved as $\beta_3$-adrenoceptor inverse agonists. Such compounds showed no affinity for $\beta_1$- and $\beta_2$-adrenergic receptor, respectively. Thus, resulting highly selective $\beta_3$-adrenoceptor ligands.
© 2007 Elsevier Ltd. All rights reserved.

## 1. Introduction

β-Adrenergic receptors (β-ARs) have been classified as $\beta_1$- and $\beta_2$-AR since 1967.[1] This subdivision led to the discovery of selective drugs for the treatment of hypertension and asthma. At the beginning of 1980s, a new β-adrenergic receptor subtype,[2] later called $\beta_3$-AR, was found in several species including man, bovine, rat and mouse.[3]

The $\beta_3$-AR, like the other β-adrenergic receptors, is a seven-transmembrane domain G-protein coupled receptor. It is usually coupled to a Gs protein and its stimulation increases the production of cAMP.[4a] Recent evidences show that in human heart $\beta_3$-AR activity induces a reduction in the contractility through a Gi protein activation that leads to impaired production of cAMP and increase of nitric oxide levels.[5]

$\beta_3$-AR is expressed in several tissues including adipose tissue, heart, uterus, bladder and gut, modulating different physiological functions. Due to its wide distribution, it can be considered a reasonable drug target for the treatment of several pathologies (Table 1).

Early developments of $\beta_3$-AR agonists, for the treatment of obesity and type 2 diabetes, based on rodent $\beta_3$-AR activity, are represented by the first agonist generation, i.e. BRL 37344, CL 316243 and CGP 12177A (Fig. 1).[7]

Later, many other full and partial agonists like L-770,644[8,9] and BMS-196085[10] AJ-9677,[11] BMS 201620[12] were reported and very recently, a new agonist (GW427353, Fig. 2) endowed with a positive pharmaco-

*Keywords*: Agonist; Inverse agonist; $\beta_3$-adrenoceptor; (α*S*; β*S*)- and (α*S*; β*R*)-2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoic acid; Obesity; Type 2 diabetes; Heart failure; Cachexia; Metabolic syndrome; Overactive bladder; Preterm labour; Anxiety and depressive disorders; DELFIA.
* Corresponding author. Tel.: +39 080 544 2762; fax: +39 080 544 2231; e-mail: ascilimati@farmchim.uniba.it

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.11.060

**Table 1.** Possible clinical use of $\beta_3$-AR agents

| Indication | Type of drug |
|---|---|
| Obesity | Agonist |
| Type 2 diabetes | Agonist |
| Metabolic (insulin resistance) syndrome | Antagonist, Inverse agonist |
| Cachexia | Antagonist, Inverse agonist |
| Overactive bladder | Agonist |
| Ulcero-inflammatory disorder of the gut | Agonist |
| Preterm labour | Agonist |
| Anxiety and depressive disorders | Agonist |
| Heart failure | Agonist, Antagonist/Inverse Agonist[a] |

[a] Agonists should serve in the first stage of the heart failure, whereas antagonists and inverse agonists in the late phase of the disease.[6]

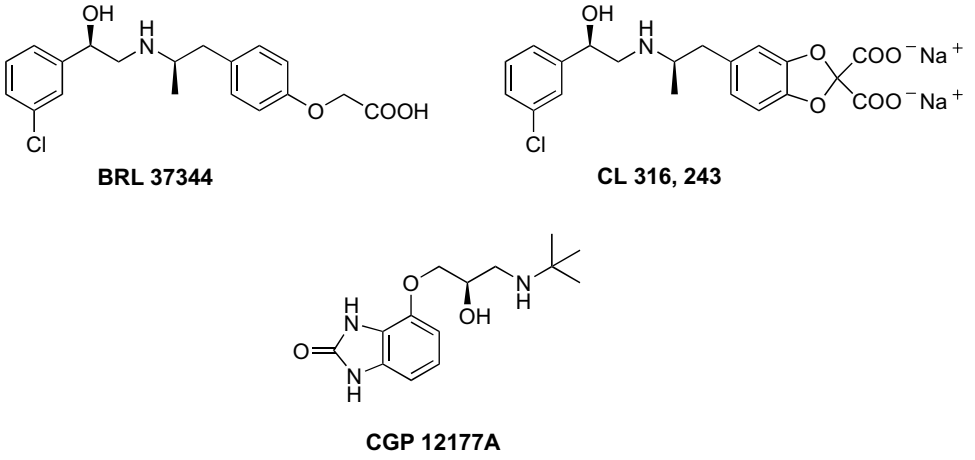



**Figure 1.** First generation of $\beta_3$-AR agonists.

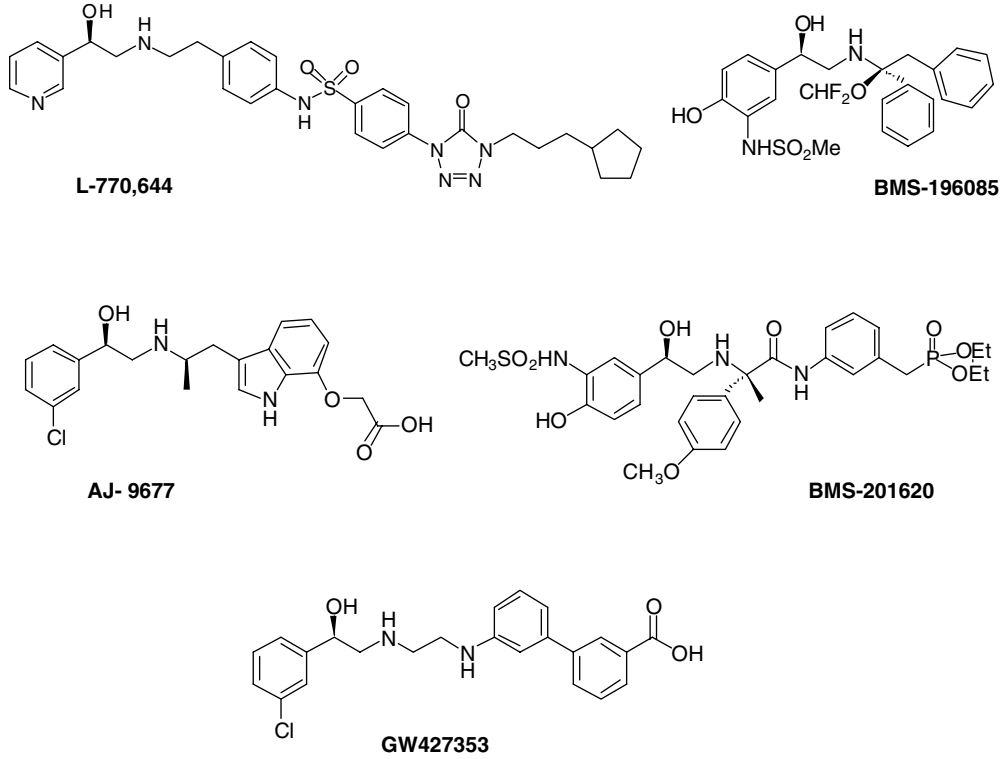



**Figure 2.** Second generation of $\beta_3$-AR agonists.[14]

logical profile has been chosen for clinical trials as drug candidate for the treatment of type 2 diabetes and overactive bladder.[13]

Few $\beta_3$-AR antagonists have been reported (Fig. 3),[15–17] but none of them has been chosen for clinical trials.

Very little is known about $\beta_3$-AR inverse agonists.[7]

Inverse agonism is not a new concept and it describes ligand behaviour displaying negative efficacy.[18] In particular, for G-protein-coupled receptors (GPCRs), it has been widely assumed that inverse agonists suppress the agonist-independent activity of the receptor by stabilizing it in its inactive state. Whereas receptor activation (receptor-G protein coupling) occurs in the absence of the agonist, the inverse agonist suppresses this activity in some way.[19]

Novel data suggest that some classical β-AR antagonists behave either as partial agonists, neutral antagonists or inverse agonists in cell systems expressing the wild type or a constitutively activated mutant of the human β-AR. For example, $\beta_1$-selective AR antagonists with significant inverse agonistic activity, such as metoprolol, have been proven to be safe in the treatment of heart failure patients. Clinical studies with inverse agonists of the $\beta_2$- and $\beta_3$-AR are not yet available.

Stimulation of $\beta_3$-AR subtype inhibits cardiac contractility, thus opposing to the response of $\beta_1$- and $\beta_2$-AR. In failing heart, $\beta_3$-ARs are up-regulated. They probably serve as a buffer, exerting a 'rescue' function from the effects of high plasma levels of catecholamines, as those observed in hyperadrenergic states including heart failure. Upon disease progression, $\beta_3$-adrenoceptor up-regulation may produce a depression in contractility, which exacerbates heart failure.[5,6]

Hence, selective $\beta_3$-AR agonists should serve in the early stage of heart failure, whereas highly selective antagonists/inverse agonists might be useful in the advanced stage of the disease.

Another possible therapeutic relevance of $\beta_3$-AR antagonists and inverse agonists appears clear by considering first, the increased lipolytic action of $\beta_3$-AR in visceral adipose tissue of obese subjects, and second, the association between increased $\beta_3$-AR function and metabolic syndrome.

In fact, in upper-body obese subjects with signs of the metabolic syndrome, $\beta_3$-AR blockade might preferentially inhibit fatty acid release from visceral adipose tissue and improve some of the metabolic abnormalities associated with the high 'portal' fatty acid flux.[4b]

As reported in Table 1, $\beta_3$-AR antagonists and inverse agonists would also be useful to prevent or control cachexia.

In cachectic cancer patients, the remarkable loss in adipose tissue (≈30% of pre-illness stable weight at diagnosis) seems to be mediated by a lipid mobilizing factor (LMF). Reported evidences demonstrate that, at least in part, LMF produces this effect by interaction with $\beta_3$-AR.[20]

Besides, very recently, the use of $\beta_3$-AR agonists in the treatment of preterm labour[21] and, anxiety and depressive disorders has been proven.[22]

Thus, based on these considerations the development of $\beta_3$-AR agonists, antagonists and inverse agonists is of great interest.

Most of $\beta_3$-AR agents reported so far, share a similar overall structure, in which three molecular portions can be identified (Fig. 4): a left-hand side (LHS), a linker (LK) and a right-hand side (RHS). LHS is typically an arylethanolamine or aryloxypropanolamine, LK has various structures including both aromatic and aliphatic moieties, RHS typically contains polar and/or ionizable functionalities including ureas, acylamides, sulfonamides and sulfonic, phosphonic and carboxylic groups.[12,23–29]

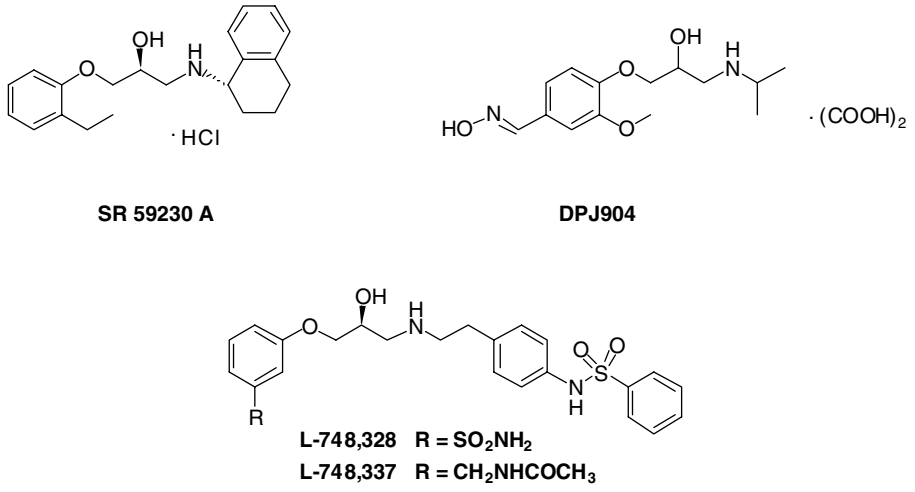
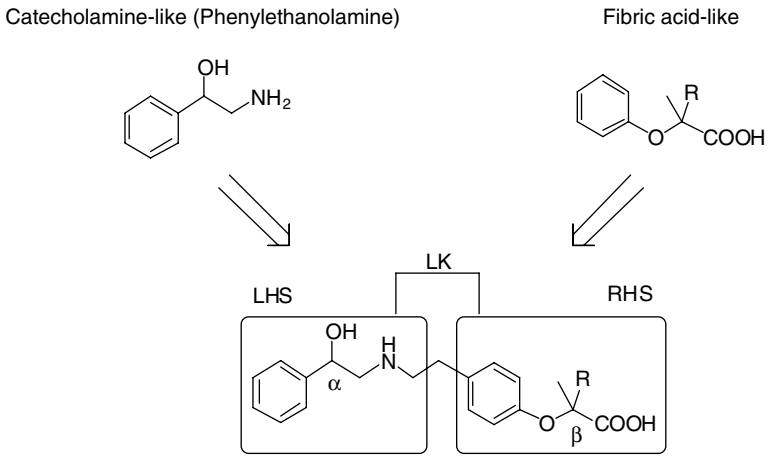



**Figure 3.** $\beta_3$-AR antagonists.




| Compound | R | Absolute Configuration |
|---|---|---|
| **1a** | $CH_3$ | *α-rac* |
| **1b** | $CH_3$ | *αR* |
| **1c** | $CH_3$ | *αS* |
| **1d** | H | *α-rac, β-rac* |
| **1e** | H | *αS, βS* |
| **1f** | H | *αR, βS* |
| **1g** | H | *αS, βR* |
| **1h** | H | *αR, βR* |

**Figure 4.** Structures and absolute configurations of compounds **1a**–**h.**

Up to now, several studies also dealing with the relationship between stereochemical demand and $\beta_3$-AR agonistic activity have been carried out using optically active compounds, proving that the activity towards the receptor is higher when the stereogenic centre bearing the hydroxy group in the LHS has (*R*)- absolute configuration in the series of arylethanolamines, (*S*) in that of aryloxypropanolamines. Besides, several research groups synthesized and evaluated new optically active $\beta_3$-AR agonists, bearing additional stereogenic centres. They were almost exclusively located on the carbon atoms adjacent to the aminic nitrogen, in both LHS and LK.[10,11,26,30–35] Such compounds resulted full or partial agonists and antagonists of the $\beta_3$-AR.

Herein, we present new $\beta_3$-AR agonists bearing stereogenic centres in both the left- and the right-hand sides. These compounds were found to be agonists or inverse agonists at the receptor. Unlike $\beta_1$-[36,37] and $\beta_2$-AR,[38] few data on $\beta_3$-AR inverse agonism[7,39] have ever been reported. The influence of the absolute configurations of two stereogenic centres α/β in LHS/RHS of a series of arylethanolamines (**1a**–**h**, Fig. 4) on $\beta_3$-AR inverse agonism is also described.

The new compounds were prepared in both racemic and optically active forms in order to evaluate the effect of the stereochemistry on $\beta_3$-AR activity, that in turn was measured using Chinese Hamster Ovary (CHO)-K1 cells stably expressing human cloned $\beta_3$-AR. Affinity for human cloned $\beta_1$- and $\beta_2$-ARs, expressed from the same CHO cells, was also determined.

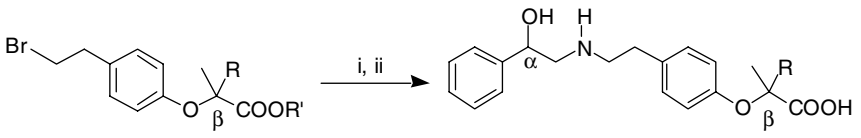
## 2. Chemistry

Compounds **1a**–**h** were prepared by *N*-alkylation of the corresponding racemic-, (*R*)- or (*S*)-2-amino-1-phenylethanol with phenethyl bromide derivatives **3a**–**d**, followed by hydrolysis of the ester intermediates **2a**–**h** (not shown), as depicted in Scheme 1.[40]

Compounds **3a**–**d** were obtained by $Et_3SiH$ reduction of their precursors **4a**–**d**, that in turn were prepared by Friedel–Crafts acylation of 2-methyl-2-phenoxypropanoates (**5a**, R = $CH_3$) and racemic-, (*R*)- or (*S*)-2-phenoxypropanoates (**5b**–**d**, R = H) with bromoacetyl bromide (Scheme 2).

Different routes were followed for **5a**–**d** preparation. **5a** was obtained in a two-step process, in which phenol was first reacted with 1,1,1-trichloro-2-methyl-2-propanol (Chloretone®)[41] in alkaline conditions and then treated with thionyl chloride in ethanol under reflux (Scheme 3).

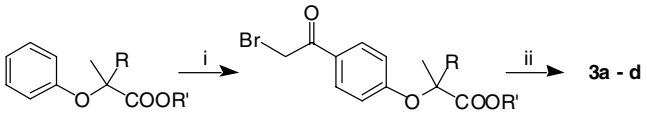
Compound **5b** was obtained from the reaction of phenol with racemic ethyl 2-bromopropanoate.[42] Its optically active analogues (*R*)-**5c** and (*S*)-**5d** were prepared by reacting the phenol with the *p*-toluenesulfonyl derivatives **6c**–**d** of commercially available ethyl (*S*)-lactate




**3a:** R = $CH_3$, R' = $C_2H_5$
**3b:** β-rac, R = H, R' = $C_2H_5$
**3c:** (*R*), R = H, R' = $C_2H_5$
**3d:** (*S*), R = H, R' = $CH_3$

**1a:** α-rac, R = $CH_3$
**1b:** (α*R*), R = $CH_3$
**1c:** (α*S*), R = $CH_3$
**1d:** α–rac, β–rac, R = H
**1e:** (α*S*, β*S*), R = H
**1f:** (α*R*, β*S*), R = H
**1g:** (α*S*, β*R*), R = H
**1h:** (α*R*, β*R*), R = H

**Scheme 1.** General procedure for the preparation of **1a**–**h**. Reagents and conditions: (i) racemic-, (*R*)- or (*S*)-2-amino-1-phenylethanol, DMF, 70 °C; (ii) NaOH, THF/$H_2O$, rt.




**Scheme 2.** Preparation of **3a**–**d**. Reagents and conditions: (i) bromoacetyl bromide, $AlCl_3$, $CH_2Cl_2$, reflux; (ii) $Et_3SiH$, TFA, 70 °C.

and methyl (*R*)-lactate, respectively, in the presence of CsF (Scheme 4).[43]

## 3. Biology, results and discussion

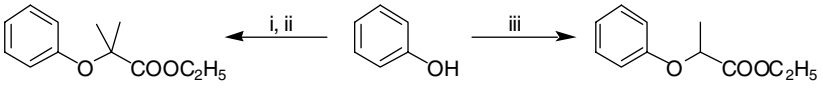
Novel compounds were assayed by measuring cAMP levels in CHO cells expressing human cloned $\beta_3$-AR to determine their $\beta_3$-AR activity (Table 2, Fig. 5). cAMP was quantified by DELFIA, that has the advantage of being a non radiolabelled ligand-based assay.[44]

DELFIA was found to be a suitable method to evaluate $\beta_3$-AR activity: isoproterenol, noradrenaline, adrenaline and BRL 37344 were used as reference compounds, and the found $\beta_3$-AR $EC_{50}$ values (Table 2) confirmed the rank order of potency and magnitude (BRL 37344 > noradrenaline ≈ isoproterenol > adrenaline) obtained by different assays, such as [α-$^{32}$P]- and [$^3$H]-cAMP.[7,45,46]

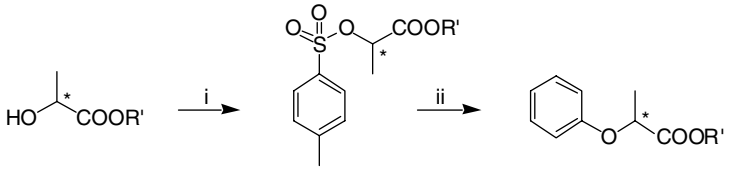
Compounds bearing two methyl groups at the β-position (**1a**–**c**) showed a similar $\beta_3$-AR agonistic activity. Racemic **1a** had an $EC_{50}$ = 4.9 nM, with 68% intrinsic activity with respect to maximal effect (100%) by isoproterenol. Surprisingly, its enantiomers **1b** and **1c** proved equally active as the racemic form, irrespective of the absolute configuration at Cα, which is generally (*R*) in $\beta_3$-AR agonists ($EC_{50}$ = 3.9 and 3.4 nM; IA = 72 and 76%, respectively).

A methyl group was then removed from Cβ leading to the racemic **1d**. In order to evaluate the effect of this structural modification and the corresponding stereochemical implication on $\beta_3$-AR agonistic activity, all the four possible stereoisomers of **1d** were separately tested.

The racemic form **1d** behaved as a strong agonist at the $\beta_3$-AR, with a potency ($EC_{50}$ = 3.8 nM, IA = 65%) comparable to that observed for compounds **1a**–**c**.




**Scheme 3.** Preparation of **5a**–**b**. Reagents and conditions: (i) Chloretone®, NaOH, acetone, reflux; (ii) $SOCl_2$, EtOH, reflux; (iii) ethyl 2-bromopropanoate, $K_2CO_3$, acetone, reflux.



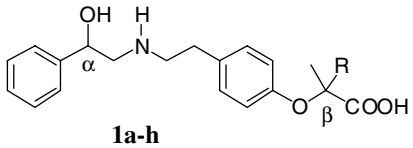

ethyl (*S*)-lactate, R' = $C_2H_5$
methyl (*R*)-lactate, R' = $CH_3$

**6c:** (*S*), R' = $C_2H_5$
**6d:** (*R*), R' = $CH_3$

**5c:** (*R*), R' = $C_2H_5$
**5d:** (*S*), R' = $CH_3$

**Scheme 4.** Preparation of **5c**–**d**. Reagents and conditions: (i) *p*-toluenesulfonyl chloride, $CH_2Cl_2$, pyridine, rt; (ii) phenol, CsF, DMF, rt.

**Table 2.** Evaluation of cAMP accumulation in CHO cells expressing human $\beta_3$-AR by **1a–h**




**1a-h**

| Compound | R | Absolute configuration at Cα- and Cβ-stereocentre | $EC_{50}$[a] (nM ± SEM[b]) (IA%)[c] |
|---|---|---|---|
| **1a** | $CH_3$ | α-*rac* | 4.9 ± 0.25 (68) |
| **1b** | $CH_3$ | α*R* | 3.9 ± 2.1 (72) |
| **1c** | $CH_3$ | α*S* | 3.4 ± 0.8 (76) |
| **1d** | H | α-*rac*, β-*rac* | 3.8 ± 0.7 (65) |
| **1e** | H | α*S*, β*S* | 181 ± 19 (−64)[d] |
| **1f** | H | α*R*, β*S* | 2.7 ± 0.7 (50) |
| **1g** | H | α*S*, β*R* | 136 ± 20 (−73)[d] |
| **1h** | H | α*R*, β*R* | 235 ± 37 (34) |
| Isoproterenol[e] | | | 5.8 ± 1.2 (100) |
| Noradrenaline[e] | | | 5.5 ± 1.2 (96) |
| Adrenaline[e] | | | 31 ± 10 (99) |
| BRL 37344[e] | | | 1.1 ± 0.3 (76) |

[a] $EC_{50}$ = substance concentration which produces a cAMP response equal to 50% of its maximal response.
[b] SEM, standard error mean from at least three experiments ($n \geqslant 3$).
[c] IA is the fitted maximal value of the concentration-response curve, expressed as a percent of the maximal response to *R*-(−)-isoproterenol ($10^{-4}$ M).
[d] Some further experiments were conducted in the presence of different concentrations (ranging from $10^{-9}$ to $10^{-7}$ M) of SR 59230A (Fig. 3), a well-known neutral antagonist of $\beta_3$-AR expressed in rat and human colon.[15] Unfortunately, the curve did not shift to the right, as expected, because SR 59230A behaves as partial agonist in CHO expressing human cloned $\beta_3$-AR.[4c,47] On the other hand, very preliminary experiments conducted on rat proximal colon showed that **1e** and **1g** are endowed with antagonist activity (in functional test in which colon motility is evaluated, it is not possible to discriminate antagonism from inverse agonism).
[e] $EC_{50}$ = 3.9, 6.3, 49 and 5.9 nM for isoproterenol, noradrenaline, adrenaline and BRL 37344, respectively, obtained by using [$^3$H]-cyclic AMP assay system[46] instead of DELFIA see above. In both assay systems, the same intact cells expressing human $\beta_3$-AR were used.

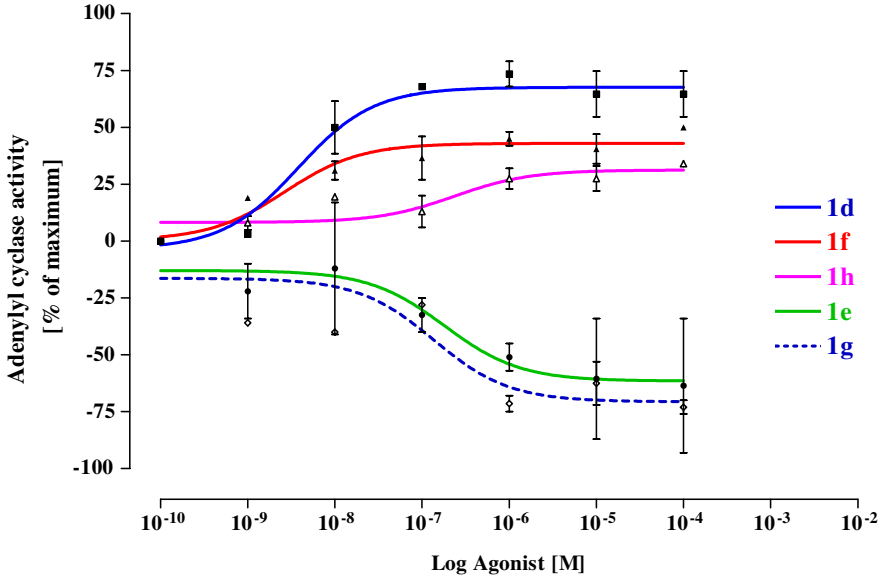



**Figure 5.** Representative curves of adenylyl cyclase activity evaluation in CHO cells stably expressing human $\beta_3$-AR subtype. Adenylyl cyclase stimulation was calculated as percentage (%) of the maximum effect by isoproterenol.

Among **1d** stereoisomers, the most potent one has absolute configuration (*R*) at Cα (**1f**, $EC_{50}$ = 2.7 nM, IA = 50%). This result is in agreement with data published for many other $\beta_3$-AR agonists. Opposite configuration at Cβ, as for **1h**, dramatically decreased $\beta_3$-AR agonistic potency ($EC_{50}$ = 235 nM, IA = 34%). In fact, **1h** was found 87-fold less active than **1f**.

Interesting results were found for **1e** and **1g**. These compounds differ from the previous two (**1f** and **1h**) in the stereochemistry at Cα. In this case, the compounds behaved as inverse agonists on $\beta_3$-AR. Such a finding seems crucial, since few $\beta_3$-AR inverse agonists have been identified to date.[7,48] The most potent inverse agonist was (α*S*, β*R*)-**1g** with an $EC_{50}$ = 136 nM and

IA = −73%. Its epimer (αS, βS)-**1e** had a comparable potency and intrinsic activity ($EC_{50}$ = 181 nM, IA = −64%).

To exclude that the compounds **1a**–**h** were active at the $\beta_1$- and $\beta_2$-ARs, we tested their ability to bind to these receptors using [$^3$H]-dihydroalprenolol on membranes of CHO cells expressing the receptors. At the concentrations of [$^3$H]-dihydroalprenolol used in the competition-binding experiments, specific binding was about 76% and 85% of the total binding, for the $\beta_1$- and $\beta_2$-ARs, respectively (Fig. 6A and B). The non specific binding was determined in the presence of 10 μM Alprenolol for both saturation binding experiments. As shown in Table 3, the percentage (%) of inhibition exerted by the compounds at the highest concentration used ($10^{-5}$ M) in competition binding experiments was never greater than 35%. This indicates that for all the compounds the affinity for both the $\beta_1$- and $\beta_2$-AR was very weak and therefore their activity at these receptors was not assessed.

Accurate determination of $\beta_3$-AR binding affinities for all the compounds using a [$^3$H]-dihydroalprenolol was not possible because of the low $\beta_3$-receptor expression level (about 100 fmol/mg of protein). On the other hand, the functional and activity data above-reported provide important informative about the behaviour of the novel compounds (**1a**–**h**).

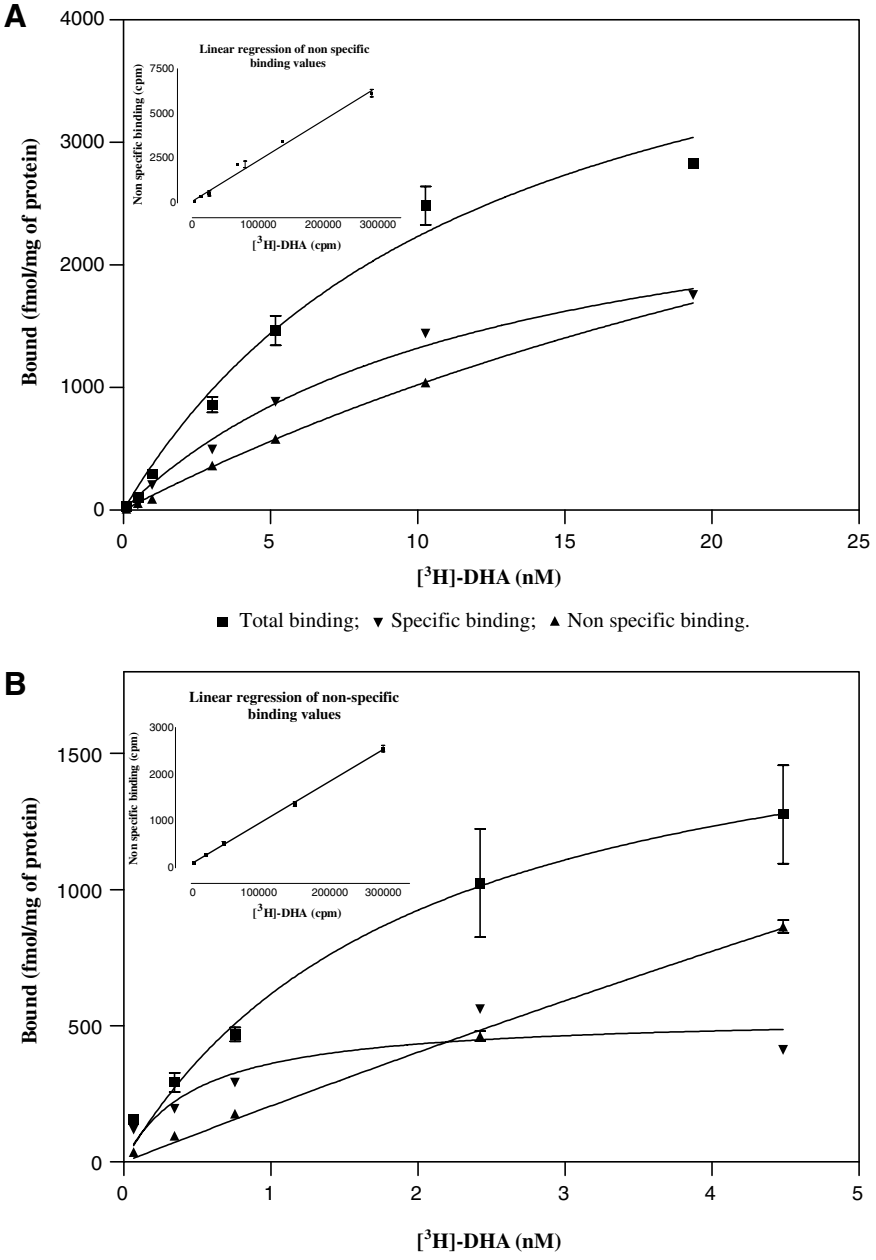



**Figure 6.** Saturation binding analysis with [$^3$H]-DHA towards $\beta_1$- (A) and $\beta_2$-AR (B) expressed in CHO cell line. In CHO-$\beta_1$ cells, $K_d$ value for Alprenolol was 12.49 nM and $B_{max}$ 2,970 fmol/mg of protein (A) while in CHO-$\beta_2$-AR cells $K_d$ value for Alprenolol was 0.50 nM and $B_{max}$ 540 fmol/mg of protein (B). The inserts represent the linear regression of non-specific binding values.

**Table 3.** Percentage of inhibition of **1a**–**h** for human $\beta_1$- and $\beta_2$-adrenoceptor subtypes

| Compound | Inhibition (%)[a] | |
|---|---|---|
| | $\beta_1$-AR | $\beta_2$-AR |
| **1a** | 18 | 24 |
| **1b** | 8 | 1 |
| **1c** | 13 | 5 |
| **1d** | 37 | 24 |
| **1e** | 14 | 9 |
| **1f** | 28 | 1 |
| **1g** | 10 | 1 |
| **1h** | 6 | 2 |

[a] Percentage of inhibition of **1a**–**h** ($10^{-5}$ M) for human $\beta_1$- and $\beta_2$-AR.

Since the compounds prepared share, in their RHS, a structural similarity with fibrates,[49] PPARα agonists, the evaluation of the PPARα functional activities of **1a**–**h** by a transactivation assay in eukaryotic COS-7 cells was performed. The results, summarized in Table 4, were expressed as a percent activation of the CAT reporter gene compared to that measured in the presence of positive control Wy,14-643 (2 μM, Table 4), conventionally taken as equal to 100%.

Though they present the molecular determinants for PPARα agonistic activity, the compounds tested resulted less active as PPARα activators (<4% activation) than the reference compounds (clofibrate, clofibric acid and Wy,14-643), thus proving to be highly selective on $\beta_3$-AR.

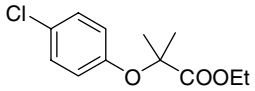
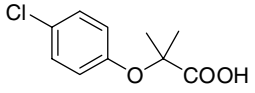
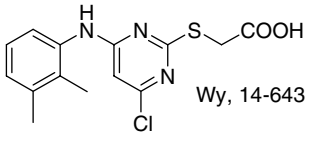
## 4. Conclusions

A set of new compounds (**1a**–**h**) selective for the $\beta_3$-AR were prepared in good yields and evaluated for their functional effect on the receptor. **1a**–**c** resulted almost equally active as noradrenaline and isoproterenol on this receptor subtype, and their intrinsic activity was comparable with that of BRL 37344. Compounds **1a**–**c** showed no significant correlation between stereochemistry and potency as $\beta_3$-AR agonists. Among **1d**–**h**, isomer (α*R*, β*S*)-**1f** was the most potent $\beta_3$-AR agonist in **1a**–**h** series, though endowed with modest intrinsic activity (50%). Some inverse agonists for $\beta_1$- and $\beta_2$-AR have already been identified:[48] Interestingly, the structural modifications introduced in this series of molecules, allowed the identification of the first potent and selective $\beta_3$-AR inverse agonists.[7,50] (*S*)-configuration at Cα seems to be required for inverse agonistic activity, at least for **1e** and **1g**.

The recently developed DELFIA[44] was efficiently used to evaluate the activity of **1a**–**h**; the observed order of potency for reference compounds is in agreement with data obtained by using other assays, such as [α-$^{32}$P]- and [$^3$H]-cAMP.[7,45,46]

In binding experiments, **1a**–**h** did not bind either $\beta_1$- or $\beta_2$-ARs with high affinity, thus confirming that they are selective $\beta_3$-AR agonists or inverse agonists, being resulted also less active than clofibric acid as PPARα activators, although they present structural similarities with fibrates.[49]

**Table 4.** Percentage of activation of the CAT reporter gene by **1a**–**h** 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 (%) |
|---|---|---|---|
| **1a** | 2 | 1.7 | 1.8 |
| **1b** | 1.3 | 1.5 | 1.5 |
| **1c** | 1.7 | 1.5 | 2.1 |
| **1d** | 1.5 | 1.9 | 1.8 |
| **1e** | 1 | 1 | 1.8 |
| **1f** | 1.6 | 1.8 | 1.5 |
| **1g** | 1.3 | 2 | 3.6 |
| **1h** | 1.1 | 1.5 | 2.6 |
|  Clofibrate | 12.6 | 11 | 89.9 |
|  Clofibric acid | Not tested | Not tested | 86.5 |
|  Wy, 14-643 | 100 (2 μM) | — | — |

Further studies are in progress aimed both at identifying mechanism of action of **1e** and **1g**, and correlating molecular structure modifications with their $\beta_3$-AR inverse agonistic activity and pharmacological features by model study of metabolic syndrome, heart failure and cachexia.

## 5. Experimental

### 5.1. General methods

Melting points taken on an Electrothermal apparatus are uncorrected. $^1$H NMR spectra were recorded on a Varian Inova 400 MHz Mercury 300 MHz spectrometer or on a Bruker Aspect 500 MHz, and chemical shifts are reported in parts per million (δ). Absolute values of the coupling constant (J) are reported. IR spectra were recorded on a Perkin-Elmer 681 spectrometer. GC analyses were performed by using an HP-5 MS column (5% phenyl methyl siloxane; 30 m × 0.25 mm × 250 μm film thickness) on a HP 6890 model, series II. Thin-layer chromatography (TLC) was performed on silica gel sheets with fluorescent indicator (Stratochrom SIF, 60 F254 Merk), the spots on the TLC were observed under ultraviolet light or were visualized with $I_2$ vapour. Chromatography was conducted by using silica gel 60 with a particle size distribution of 40–63 μm and 230–400 ASTM. GC–MS analyses were performed on an HP 5995C model, and microanalyses were performed on an elemental analyzer 1106 (Carlo Erba Instrument). ESI-MS analyses were performed on an Agilent 1100 LC/MSD trap system VL. Optical rotations were measured at 20 °C on a Perkin-Elmer model 341 polarimeter. Stereoisomeric purity of the final targets **1b**–**c** and **1e**–**h** was determined by using OD-R HPLC column (Daicel Chemical Industries Ltd.); acetonitrile—(0.5N percloric acid–sodium perclorate, pH 2.0) = 60:40 and 65:35 for **1b**–**c** and **1e**–**h**, respectively, as mobile phase; detection at $\lambda$ = 254 nm; flow rate = 0.4 mL/min. Capacity factors ($k'$) were calculated by using as $t_R^\circ$ the retention time of $D_2O$.

DMF and $CH_2Cl_2$ from a commercial source were purified by distillation from $CaH_2$. Commercially available acetone was distilled from anhydrous $K_2CO_3$ prior to use. All other chemicals and solvents purchased from Aldrich & Co. were used as supplied without further purification.

### 5.2. Ethyl 2-methyl-2-phenoxypropanoate (5a)[41]

Phenol (5 g, 53 mmol) and 1,1,1-trichloro-2-methyl-2-propanol (19.8 g, 106 mmol) were dissolved in acetone (250 mL). NaOH (17 g, 425 mmol) was carefully added. The reaction mixture was stirred overnight at room temperature. Then, the solvent was removed under reduced pressure. Water was added to the crude residue and washed three times with ethyl ether. The organic phase was discarded and the aqueous layer was acidified with 37% HCl and extracted several times with ethyl ether. The extracts were washed three times with a saturated solution of NaCl, dried over anhydrous $Na_2SO_4$ and the solvent was removed under reduced pressure. $SOCl_2$ (3.9 ml, 2.39 g, 20 mmol) was dropwise added to the obtained residue dissolved in ethanol (250 mL). The reaction mixture was refluxed for 6 h. The solvent was then removed under reduced pressure. The residue was dissolved in water and extracted four times with ethyl ether. The combined extracts were washed three times with a saturated solution of $NaHCO_3$, three times with a saturated solution of NaCl and dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure. The product was isolated as colourless oil (65% yield) by chromatography (petroleum ether/ethyl acetate = 9.5:0.5). FT-IR (neat): 3063, 2996, 2945, 1760, 1594, 1499, 1458, 1381, 1303, 1255, 1200, 1112, 1019, 964, 695 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$, $\delta$): 1.24 (t, $J$ = 7.2 Hz, 3H, $OCH_2CH_3$), 1.59 (s, 6H, $C(CH_3)_2$), 4.23 (q, $J$ = 7.2 Hz, 2H, $OCH_2CH_3$), 6.83–6.86 (m, 2H, aromatic protons), 6.95–7.00 (m, 1H, aromatic proton), 7.20–7.26 (m, 2H, aromatic protons). $^{13}$C NMR (75 MHz, $CDCl_3$, $\delta$): 14.27, 25.60, 61.60, 79.26, 119.36, 122.31, 129.35, 155.66, 174.56. GC–MS (70 eV) *m/z* (rel. int.): 208 ($M^+$, 16), 136 (5), 135 (52), 115 (5), 107 (7), 95 (15), 94 (100), 87 (11), 77 (17), 65 (6). Anal. Calcd for $C_{12}H_{16}O_3$: C, 69.19; H, 7.75. Found: C, 69.15; H, 7.72.

### 5.3. (±)-Ethyl 2-phenoxypropanoate (5b)[42]

(±)-Ethyl 2-bromopropanoate (0.71 mL, 5.5 mmol) and anhydrous $K_2CO_3$ (1.520 g, 11 mmol) were added to a solution of phenol (471 mg, 5 mmol) in anhydrous acetone (125 mL). The reaction mixture was refluxed for 2.5 days. Solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate and washed three times with water and once with 2N NaOH. The organic layer was dried over anhydrous $Na_2SO_4$ and the solvent was removed under reduced pressure to afford a colourless oil (95% yield). FT-IR (neat): 3065, 3042, 2987, 2939, 2902, 2876, 1763, 1588, 1496, 1457, 1375, 1343, 1272 1050, 1020, 946, 885, 860, 802, 758, 691 cm$^{-1}$. $^1$H NMR (300 MHz, $CDCl_3$, $\delta$): 1.24 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 1.62 (d, $J$ = 6.7 Hz, 3H, $CHCH_3$), 4.21 (q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 4.75 (q, $J$ = 6.7 Hz, 1H, $CHCH_3$), 6.87–6.90 (m, 2H, aromatic protons), 6.94–6.99 (m, 1H, aromatic proton), 7.23–7.30 (m, 2H, aromatic protons). $^{13}$C NMR (75 MHz, $CDCl_3$, $\delta$): 14.33, 18.78, 61.44, 72.82, 115.33, 121.76, 129.74, 157.85, 172.44. GC–MS (70 eV) *m/z* (rel. int.): 194 ($M^+$, 49), 122 (13), 121 (100), 103 (4), 94 (27), 93 (18), 77 (39), 65 (7), 51 (11). Anal. Calcd for $C_{11}H_{14}O_3$: C, 68.00; H, 7.27. Found: C, 67.98; H, 7.24.

### 5.4. 1-(Alkyloxycarbonyl)ethyl 4-methylbenzenesulfonates (6c–d): General procedure[43]

Alkyl lactate [(*S*)-(−)-ethyl lactate or (*R*)-(+)-methyl lactate] (25 mmol) was dissolved in $CH_2Cl_2$ (10 mL) and, *p*-toluenesulfonyl chloride (50 mmol) and pyridine (5 mL) were added. The reaction mixture was stirred at rt for 6 h. The solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate and washed three times with 1N HCl and three times with water. The product was isolated by chromatography (silica gel; mobile phase: petroleum ether/ethyl acetate = 8:2).

**5.4.1. (*S*)-(−)-1-(Ethoxycarbonyl)ethyl 4-methylbenzenesulfonate (6c)[43].** Mp: 33.5–34.6 °C, white solid (85% yield). $[\alpha]_D$ −36.6 (*c* 1.45, $CHCl_3$). FT-IR (KBr): 3075, 2988, 2935, 2877, 1756, 1598, 1495, 1449, 1370, 1307, 1191, 1179, 1122, 1083, 1029, 944, 888, 818, 785, 665 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.18 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 1.48 (d, $J$ = 6.9 Hz, 3H, $CHCH_3$), 2.42 (s, 3H, $CH_3C_6H_4SO_3$), 4.09 (q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 4.90 (q, $J$ = 6.9 Hz, 1H, $CHCH_3$), 7.31–7.34 (m, 2H, aromatic protons), 7.78–7.82 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 14.15, 18.62, 21.88, 62.04, 74.39, 128.23, 130.02, 133.56, 145.31, 169.29. GC–MS (70 eV) *m*/*z* (rel. int.): 274 [($^{34}S$)$M^+$, 1], 272 [($^{32}S$)$M^+$, 11], 201 (3), 199 (48), 157 (9), 156 (18), 155 (100), 139 (5), 92 (22), 91 (71), 65 (21). Anal. Calcd for $C_{12}H_{16}O_5S$: C, 52.91; H, 5.93. Found: C, 52.88; H, 5.89.

**5.4.2. (*R*)-(+)-1-(Methoxycarbonyl)ethyl 4-methylbenzenesulfonate (6d)[43].** Colourless oil (quantitative yield). $[\alpha]_D$ +35.1 (*c* 1.05, $CHCl_3$). FT-IR (neat): 3101, 2993, 2957, 2928, 1762, 1598, 1458, 1433, 1369, 1311, 1221, 1193, 1179, 1083, 1027, 975, 945, 824 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.46 (d, $J$ = 6.9 Hz, 3H, $CHCH_3$), 2.41 (s, 3H, $CH_3C_6H_4$), 3.62 (s, 3H, $OCH_3$), 4.90 (q, $J$ = 6.9 Hz, 1H, $CHCH_3$), 7.30–7.33 (m, 2H, aromatic protons), 7.75–7.78 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 18.56, 21.84, 52.77, 74.26, 128.18, 130.04, 133.51, 145.36, 169.70. GC–MS (70 eV) *m*/*z* (rel. int.): 260 [($^{34}S$)$M^+$, 1], 258 [($^{32}S$)$M^+$, 13], 199 (45), 157 (7), 156 (12), 155 (100), 139 (10), 91 (79), 65 (23). Anal. Calcd for $C_{11}H_{14}O_5S$: C, 51.13; H, 5.47. Found: C, 51.11; H, 5.46.

### 5.5. Alkyl 2-phenoxypropanoates (5c–d): General procedure[43]

CsF (11 mmol) was dried at 200 °C in vacuum for 20 min, and it was then cooled at room temperature under a nitrogen stream. Anhydrous DMF (34 mL) was then added to CsF and the resulting suspension was stirred at room temperature under nitrogen atmosphere. Phenol (11 mmol) was added and the resulting reaction mixture was stirred at room temperature for 20 min. 1-(Alkoxycarbonyl)ethyl 4-methylbenzenesulfonate (**6c–d**, 3.67 mmol) in anhydrous DMF (8.5 mL) was added dropwise. The reaction mixture was stirred at room temperature for 32 h. The mixture was then diluted with ethyl acetate, washed three times with a saturated solution of $NaHCO_3$ and with a saturated solution of NaCl. The organic layer was dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure. The product was isolated as a colourless oil by chromatography (petroleum ether/ethyl acetate = 10:1).

**5.5.1. (*R*)-(+)-Ethyl 2-phenoxypropanoate (5c)[43].** Yield 77%. $[\alpha]_D$ +27.9 (*c* 1.4, $CHCl_3$). Spectroscopic data are identical to those ones reported for **5b**.

**5.5.2. (*S*)-(−)-Methyl 2-phenoxypropanoate (5d).** Yield 77%. $[\alpha]_D$ −22.5 (*c* 1.15, $CHCl_3$). FT-IR (neat): 3065, 3042, 2993, 2955, 2848, 1760, 1601, 1589, 1495, 1456, 1376, 1287, 1244, 1205, 1136, 1100, 1053, 979, 754, 692 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.62 (d, $J$ = 6.9 Hz, 3H, $CHCH_3$), 3.74 (s, 3H, $OCH_3$), 4.77 (q, $J$ = 6.9 Hz, 1H, $CHCH_3$), 6.87–6.90 (m, 2H, aromatic protons), 6.95–7.00 (m, 1H, aromatic proton), 7.25–7.30 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 18.81, 52.49, 77.73, 115.29, 121.84, 129.80, 157.79, 172.93. GC–MS (70 eV) *m*/*z* (rel. int.): 180 ($M^+$, 59), 122 (13), 121 (100), 94 (37), 93 (17), 91 (9), 77 (41), 65 (11), 59 (7), 51 (11). Anal. Calcd for $C_{10}H_{12}O_3$: C, 66.63; H, 6.72. Found: C, 66.61; H, 6.69.

### 5.6. Ethyl 2-[4-(2-bromoacetyl)phenoxy]-2-methylpropanoate (4a) and alkyl 2-[4-(bromoacetyl)phenoxy]propanoates (4b–d): General procedure[41]

Anhydrous $AlCl_3$ (11.54 g, 86.5 mmol) was suspended in anhydrous $CH_2Cl_2$ (65 mL) under nitrogen atmosphere. Bromoacetylbromide (7.5 mL, 86.5 mmol) was carefully added and the mixture was stirred at 0 °C for 1 h. Then, ethyl 2-methyl-2-phenoxypropanoate (**5a**, 6 g, 28.8 mmol) or alkyl 2-phenoxypropanoate (**5b–d**, 28.8 mmol) in anhydrous $CH_2Cl_2$ (25 mL) was added. The resulting solution was stirred overnight at room temperature under nitrogen atmosphere, and then refluxed for 6 h. The mixture was then poured into crushed ice and extracted three times with ethyl acetate. The combined extracts were washed three times with a saturated solution of $NaHCO_3$, three times with water and dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure. The product was isolated as a yellow oil by chromatography (silica gel; mobile phase: petroleum ether/ethyl acetate = 8:2 for **4a** and 1:1 for **4b–d**).

**5.6.1. Ethyl 2-[4-(2-bromoacetyl)phenoxy]-2-methylpropanoate (4a)[41].** Yield 21%. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.19 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 1.64 (s, 6H, $C(CH_3)_2$), 4.20 (q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 4.37 (s, 2H, $CH_2Br$), 6.80–6.84 (m, 2H, aromatic protons), 7.87–7.90 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 14.24, 25.59, 31.00, 61.97, 79.69, 117.65, 127.54, 131.14, 160.70, 173.70, 190.13. GC–MS (70 eV) *m*/*z* (rel. int.): 330 [($^{81}Br$)$M^+$, 8], 328 [($^{79}Br$)$M^+$, 8], 257 (26), 255 (26), 216 (7), 214 (7), 177 (5), 122 (9), 121 (100), 115 (25), 107 (19), 87 (21), 59 (10). Anal. Calcd for $C_{14}H_{17}BrO_4$: C, 51.06; H, 5.21. Found: C, 51.08; H, 5.22.

**5.6.2. (±)-Ethyl 2-[4-(bromoacetyl)phenoxy]propanoate (4b).** Yield 71%. FT-IR (neat): 3073, 3048, 2987, 2941, 2875, 1755, 1682, 1594, 1575, 1509, 1446, 1431, 1393, 1378, 1285, 1190, 1136, 1097, 1050, 1017, 949, 843, 763, 606 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.22 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 1.62 (d, $J$ = 6.7 Hz, 3H, $CH_3CH$), 4.19 (q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 4.36 (s, 2H, $CH_2Br$), 4.80 (q, $J$ = 6.7 Hz, 1H, $CH_3CH$), 6.86–6.91 (m, 2H, aromatic protons), 7.89–7.93 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 14.32, 18.59, 31.01, 61.78, 72.77, 115.13, 127.77, 131.54, 162.26, 171.48, 190.04. GC–MS (70 eV) *m*/*z* (rel. int.): 316 [($^{81}Br$)$M^+$, 10], 314 [($^{79}Br$)$M^+$, 10], 243 (15), 241 (15), 222 (19), 221 (100), 163 (8), 121 (46),

107 (17), 104 (8), 90 (7), 77 (4), 76 (7). Anal. Calcd for $C_{13}H_{15}BrO_4$: C, 49.51; H, 4.80. Found: C, 49.53; H, 4.84.

**5.6.3. (*R*)-(+)-Ethyl 2-[4-(bromoacetyl)phenoxy]propanoate (4c).** Yield 79%. $[\alpha]_D$ +29.8 (*c* 1.1, $CHCl_3$). Spectroscopic data are identical to those ones reported for **4b**.

**5.6.4. (*S*)-(−)-Methyl 2-[4-(bromoacetyl)phenoxy]propanoate (4d).** Yield 84%. $[\alpha]_D$ −32.7 (*c* 1.65, $CHCl_3$). FT-IR (neat): 3101, 3043, 2995, 2953, 2848, 1754, 1676, 1600, 1509, 1434, 1375, 1285, 1256, 1202, 1176, 1135, 1100, 1051, 978, 843, 606 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.63 (d, $J$ = 6.9 Hz, 3H, $CH_3CH$), 3.74 (s, 3H, $OCH_3$), 4.37 (s, 2H, $CH_2Br$), 4.84 (q, $J$ = 6.9 Hz, 1H, $CH_3CH$), 6.87–6.90 (m, 2H, aromatic protons), 7.91–7.94 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 18.63, 30.96, 52.75, 72.69, 115.10, 127.84, 131.59, 162.19, 171.97, 190.04. GC–MS (70 eV) *m*/*z* (rel. int.): 302 [($^{81}Br$)$M^+$, 6], 300 [($^{79}Br$)$M^+$, 6], 243 (7), 241 (8), 222 (6), 208 (14), 207 (100), 163 (8), 121 (46), 107 (15), 93 (6), 76 (6). Anal. Calcd for $C_{12}H_{13}BrO_4$: C, 47.84; H, 4.35. Found: C, 47.86; H, 4.38.

### 5.7. Ethyl 2-[4-(2-bromoethyl)phenoxy]-2-methylpropanoate (3a) and alkyl 2-[4-(2-bromoethyl)phenoxy]propanoates (3b–d): General procedure.[41]

Ethyl 2-[4-(2-bromoacetyl)phenoxy]-2-methylpropanoate (**4a**, 66 mg, 0.2 mmol) {or (±)- or (*R*)-(+)- or (*S*)-(−)-alkyl 2-[4-(bromoacetyl)phenoxy]propanoate (**4b–d**, 1 g, 3.18 mmol)} was dissolved in trifluoroacetic acid (1 mL or 15 mL). Triethylsilane (0.07 mL, 0.44 mmol for **4a** and 1.12 mL, 7 mmol for **4b–d**) was added and the reaction mixture was stirred at 70 °C for 4 h. It was, then, cooled at room temperature and ethyl acetate was added. The organic phase was washed several times with a saturated solution of $NaHCO_3$, three times with water and then dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure and the product was isolated by chromatography (petroleum ether/ethyl acetate = 9:1).

**5.7.1. Ethyl 2-[4-(2-bromoethyl)phenoxy]-2-methylpropanoate (3a).[41]** Colourless oil (93% yield). FT-IR (neat): 2988, 2935, 2855, 1734, 1611, 1583, 1509, 1467, 1383, 1365, 1283, 1237, 1178, 1141, 1023, 971, 839, 738, 642 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.24 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 1.58 (s, 6H, $C(CH_3)_2$), 3.08 (t, $J$ = 7.8 Hz, 2H, $CH_2CH_2Br$), 3.51 (t, $J$ = 7.8 Hz, 2H, $CH_2Br$), 4.23 (q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 6.77–6.80 (m, 2H, aromatic protons), 7.05–7.08 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 14.30, 25.59, 33.31, 38.89, 61.64, 79.32, 119.45, 129.57, 132.75, 154.57, 174.50. GC–MS (70 eV) *m*/*z* (rel. int.): 316 [($^{81}Br$)$M^+$, 14], 314 [($^{79}Br$)$M^+$, 14], 243 (29), 241 (30), 235 (9), 202 (46), 200 (47), 121 (32), 120 (10), 108 (9), 107 (100), 103 (10), 91 (15), 77 (13), 59 (7). Anal. Calcd for $C_{14}H_{19}BrO_3$: C, 53.32; H, 6.08. Found: C, 53.36; H, 6.11.

**5.7.2. (±)-Ethyl 2-[4-(2-bromoethyl)phenoxy]propanoate (3b).** Colourless oil (83% yield). FT-IR (neat): 3033, 2986, 2938, 2874, 1752, 1612, 1585, 1512, 1448, 1376, 1297, 1265, 1243, 1199, 1135, 1098, 1052, 1016, 971, 823, 734 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.24 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 1.60 (d, $J$ = 6.9 Hz, 3H, $CHCH_3$), 3.08 (t, $J$ = 7.7 Hz, 2H, $CH_2CH_2Br$), 3.50 (t, $J$ = 7.7 Hz, 2H, $CH_2Br$), 4.21 (q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 4.71 (d, $J$ = 6.9 Hz, 1H, $CHCH_3$), 6.80–6.84 (m, 2H, aromatic protons), 7.08–7.11 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 14.36, 18.78, 33.41, 38.80, 61.48, 72.91, 115.45, 129.95, 132.18, 156.82, 172.40. GC–MS (70 eV) *m*/*z* (rel. int.): 302 [($^{81}Br$)$M^+$, 61], 300 [($^{79}Br$)$M^+$, 60], 230 (12), 229 (100), 228 (13), 227 (99), 221 (24), 207 (38), 185 (4), 183 (5), 147 (20), 121 (23), 120 (14), 119 (26), 107 (70), 104 (21), 103 (21), 91 (22), 78 (11), 77 (22), 65 (7). Anal. Calcd for $C_{13}H_{17}BrO_3$: C, 51.82; H, 5.69. Found: C, 51.84; H, 5.70.

**5.7.3. (*R*)-(+)-Ethyl 2-[4-(2-bromoethyl)phenoxy]propanoate (3c).** Yellow oil (81% yield). $[\alpha]_D$ +22.0 (*c* 1.1, $CHCl_3$). Spectroscopic data are identical to those ones reported for **3b**.

**5.7.4. (*S*)-(−)-Methyl 2-[4-(2-bromoethyl)phenoxy]propanoate (3d).** Yellow oil (75% yield). $[\alpha]_D$ −22.0 (*c* 1.1, $CHCl_3$). FT-IR (neat): 3033, 2993, 2953, 1761, 1613, 1586, 1507, 1448, 1376, 1231, 1134, 1100, 1053, 979, 823, 638 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.60 (d, $J$ = 6.9 Hz, 3H, $CHCH_3$), 3.08 (t, $J$ = 7.7 Hz, 2H, $CH_2CH_2Br$), 3.51 (t, $J$ = 7.7 Hz, 2H, $CH_2Br$), 3.75 (s, 3H, $OCH_3$), 4.73 (q, $J$ = 6.9 Hz, 1H, $CHCH_3$), 6.80–6.84 (m, 2H, aromatic protons), 7.09–7.13 (m, 2H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 18.81, 33.36, 38.80, 52.54, 72.84, 115.41, 130.01, 132.26, 156.75, 172.92. GC–MS (70 eV) *m*/*z* (rel. int.): 288 [($^{81}Br$)$M^+$, 70], 286 [($^{79}Br$)$M^+$, 70], 229 (94), 227 (93), 207 (33), 193 (74), 147 (21), 121 (31), 120 (18), 119 (28), 107 (100), 104 (21), 103 (23), 91 (32), 90 (9), 89 (8), 78 (14), 77 (26), 65 (11), 59 (13). Anal. Calcd for $C_{12}H_{15}BrO_3$: C, 50.15; H, 5.27. Found: C, 50.14; H, 5.31.

### 5.8. Ethyl 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]-phenoxy}-2-methylpropanoate (2a–c) and alkyl 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoates (2d–h): General procedure

A mixture of ethyl 2-[4-(2-bromoethyl)phenoxy]-2-methylpropanoate (**3a**) or alkyl 2-[4-(2-bromoethyl)phenoxy]propanoate (**3b–d**) and racemic, (*R*)- or (*S*)-2-amino-1-phenylethanol (ratio indicated in table below) in anhydrous DMF was stirred under nitrogen atmosphere at 70 °C for 70 h. The reaction mixture was then diluted with ethyl acetate, washed with a saturated solution of NaCl and dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure and the product was isolated as yellow oil by chromatography (silica gel; mobile phase: petroleum ether/ethyl acetate = 8:2 for **2a–c** and dichloromethane/ethanol = 40:1 for **2d–h**).

| Alkyl propanoate | Alkyl propanoate/ 2-amino-1-phenylethanol | Alkyl propanoate concentration (mol/L) |
|---|---|---|
| **3a** | 1:1.2 | 0.85 |
| **3b, 3c** | 1:1.3 | 1 |
| **3d** | 1:1.1 | 0.85 |

**5.8.1. (±)-Ethyl 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}-2-methylpropanoate (2a).** Yield 80%. FT-IR (neat): 3600–3100, 3060, 3032, 2989, 2935, 2854, 1733, 1611, 1509, 1454, 1383, 1266, 1235, 1179, 1144, 1026, 914, 850, 736, 702 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.24 (t, $J = 7.1$ Hz, 3H, $OCH_2CH_3$), 1.56 (s, 6H, $C(CH_3)_2$), 2.70–2.96 (m, 6H, $CH_2CHOH$, $CH_2NH$, $CH_2CH_2NH$), 3.90–4.10 (b s, 2H, O*H* and N*H*: exchange with $D_2O$), 4.22 (q, $J = 7.1$ Hz, 2H, $OCH_2CH_3$), 4.75 (dd, $J = 9.2$ and 3.4 Hz, 1H, C*H*OH), 6.75–6.78 (m, 2H, aromatic protons), 7.00–7.03 (m, 2H, aromatic protons), 7.23–7.33 (m, 5H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 14.31, 25.58, 35.54, 50.88, 57.12, 61.62, 71.82, 79.31, 119.67, 126.03, 127.73, 128.60, 129.54, 133.46, 142.84, 154.03, 174.59. GC–MS (70 eV) *m*/*z* (rel. int.): 353 [(M-18)$^+$, 4], 298 (11), 264 (100), 238 (9), 235 (7), 150 (25), 132 (68), 121 (44), 107 (24), 91 (11), 77 (12), 43 (8). Anal. Calcd for $C_{22}H_{29}NO_4$: C, 71.11; H, 7.88; N, 3.77. Found: C, 71.01; H, 7.85; N, 3.74.

**5.8.2. Ethyl (−)-2-{4-[2-((*R*)-2-hydroxy-2-phenylethylamino)ethyl]phenoxy}-2-methylpropanoate (2b).** Yield 64%. $[\alpha]_D$ −32.1 (*c* 1.0, $CHCl_3$). Spectroscopic data are identical to those ones reported for **2a**.

**5.8.3. Ethyl (+)-2-{4-[2-((*S*)-2-hydroxy-2-phenylethylamino)ethyl]phenoxy}-2-methylpropanoate (2c).** Yield 61%. $[\alpha]_D$ +32.0 (*c* 1.0, $CHCl_3$). Spectroscopic data are identical to those ones reported for **2a**.

**5.8.4. (±)-Ethyl 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoate (2d).** Yield 49%. FT-IR (neat): 3600–3100, 3058, 3022, 2986, 2935, 2855, 1750, 1669, 1612, 1581, 1511, 1449, 1375, 1292, 1239, 1135, 1050, 1014, 826, 733, 702 $cm^{-1}$. $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$): 1.23 (t, $J = 7.1$ Hz, 3H, $OCH_2CH_3$), 1.58 (d, $J = 6.8$ Hz, 3H, $CHCH_3$), 2.66–3.00 (m, 6H, $CH_2CHOH$, $CH_2NH$, $CH_2CH_2NH$), 4.20 (q, $J = 7.1$ Hz, 2H, $OCH_2CH_3$), 4.70 (q, $J = 6.8$ Hz, 2H, $CHCH_3$), 4.86 (dd, $J = 9.3$ and 3.3 Hz, 1H, C*H*OH), 5.10–5.30 (b s, 2H, O*H* and N*H*: exchange with $D_2O$), 6.74–6.82 (m, 2H, aromatic protons), 6.97–7.12 (m, 2H, aromatic protons), 7.24–7.35 (m, 5H, aromatic protons). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$): 14.35, 34.29, 50.50, 56.52, 61.47, 66.05, 72.90, 115.51, 126.03, 127.86, 128.66, 129.94, 131.84, 142.28, 156.52, 172.47. GC–MS (70 eV) *m*/*z* (rel. int.): 339 [(M-18)$^+$, 1], 284 (4), 250 (100), 221 (24), 207 (7), 176 (10), 150 (14), 147 (14), 132 (69), 121 (19), 107 (18), 105 (14), 104 (10), 103 (11), 91 (10), 77 (14), 43 (9). Anal. Calcd for $C_{21}H_{27}NO_4$: C, 70.55; H, 7.72; N, 3.92. Found: C, 70.58; H, 7.74; N, 3.90.

**5.8.5. (2*S*)-Methyl 2-{4-[2-((2*S*)-2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoate (2e).** Yield 31%. $[\alpha]_D$ +11.8 (*c* 0.95, $CHCl_3$). FT-IR (neat): 3600–3200, 3029, 2987, 2937, 2885, 1752, 1612, 1585, 1512, 1451, 1426, 1377, 1348, 1299, 1250, 1207, 1181,1137, 1100, 1071, 1047, 825, 758, 700 $cm^{-1}$. $^1H$ NMR (400 MHz, $CDCl_3$, $\delta$): 1.57 (d, $J = 6.7$ Hz, 3H, $CHCH_3$), 2.69–2.93 (m, 6H, $CH_2CHOH$, $CH_2NH$, $CH_2CH_2NH$), 3.45–3.64 (b s, 2H, O*H* and N*H*: exchange with $D_2O$), 3.71 (s, 3H, $OCH_3$), 4.70 (q, $J = 6.7$ Hz, 2H, $CHCH_3$), 4.74 (dd, $J = 9.3$ and 3.2 Hz, 1H, C*H*OH), 6.75–6.77 (m, 2H, aromatic protons), 7.03–7.05 (m, 2H, aromatic protons), 7.21–7.24 (m, 1H, aromatic proton), 7.27–7.32 (m, 4H, aromatic protons). $^{13}C$ NMR (100 MHz, $CDCl_3$, $\delta$) 18.83, 35.05, 50.79, 52.56, 56.91, 71.53, 72.81, 115.36, 126.03, 127.80, 128.63, 129.99, 132.50, 142.51, 154.31, 173.01. GC–MS (70 eV) *m*/*z* (rel. int.): 325 [(M-18)$^+$, 9], 325 (41), 324 (25), 238 (31), 206 (24), 193 (40), 147 (30), 134 (30), 132 (100), 130 (21), 120 (20), 107 (44), 106 (21), 105 (50), 104 (45), 103 (30), 91 (69), 90 (20), 78 (20), 77 (38), 70 (25), 51 (19). MS-ESI *m*/*z* (%): 344 $[M+H]^+$ (100%). Anal. Calcd for $C_{20}H_{25}NO_4$: C, 69.93; H, 7.34; N, 4.08. Found: C, 69.95; H, 7.31; N, 4.06.

**5.8.6. (2*S*)-Methyl 2-{4-[2-((2*R*)-2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoate (2f).** Yield 47%. $[\alpha]_D$ −58.5 (*c* 1.05, $CHCl_3$). Spectroscopic data are identical to those ones reported for **2e**.

**5.8.7. (2*R*)-(+)-Ethyl 2-{4-[2-((2*S*)-2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoate (2g).** Yield 66%. $[\alpha]_D$ +55.6 (*c* 1.01, $CHCl_3$). Spectroscopic data are identical to those ones reported for **2d**.

**5.8.8. (2*R*)-(−)-Ethyl 2-{4-[2-((2*R*)-2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoate (2h).** Yield 54%. $[\alpha]_D$ −6.9 (*c* 0.96, $CHCl_3$). Spectroscopic data are identical to those ones reported for **2d**.

**5.9. 2-{4-[2-(2-Hydroxy-2-phenylethylamino)ethyl]phenoxy}-2-methylpropanoic acid (1a–c) and 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}propanoic acid (1d–h): General procedure.**

1N NaOH (8.4 mL, 8.4 mmol) was added to a solution of ethyl 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]-phenoxy}-2-methylpropanoate (**2a–c**, 4.2 mmol) or alkyl 2-{4-[2-(2-hydroxy-2-phenylethylamino)ethyl]phenoxy}-propanoate (**2d–h**, 4.2 mmol) in THF (10 mL). The reaction mixture was stirred at rt for 1 h. Then, THF was removed under reduced pressure and 2 N HCl was then added to pH 6. A precipitate formed that was filtered and washed with water. The residue was treated with hot acetone to afford a crystalline product.

**5.9.1. (±)-2-{4-[2-(2-Hydroxy-2-phenylethylamino)ethyl]-phenoxy}-2-methylpropanoic acid (1a).** Mp: 223 °C (dec), white solid (35% yield). HPLC data: $k'_{(R)} = 1.62$, $k'_{(S)} = 1.85$, $\alpha = 1.14$; elution time: $t_{(R)} = 20.96$ min, $t_{(S)} = 22.77$ min. FT-IR (KBr): 3650–3200, 3000, 2987, 2935, 2792, 1613, 1560, 1512, 1462, 1402, 1362, 1243, 1199, 1151, 837, 703 $cm^{-1}$. $^1H$ NMR (300 MHz, DMSO-$d_6$, $\delta$): 1.38 (s, 6H, $C(CH_3)_2$), 2.57–2.82 (m,

6H, C$H_2$CHOH, C$H_2$NH, C$H_2$C$H_2$NH), 3.00–4.60 (b s, 3H, O*H*, N*H* and COO*H*: exchange with $D_2O$), 4.72 (dd, $J = 5.8$ and 2.6 Hz, 1H, C*H*OH), 6.69–6.72 (m, 2H, aromatic protons), 6.86–6.88 (m, 2H, aromatic protons), 7.19–7.34 (m, 5H, aromatic protons). MS-ESI *m*/*z* (%): 344 $[M+H]^+$ (100%). MS-ESI *m*/*z* (%): 342 $[M-H]^-$ (100%). Anal. Calcd for $C_{20}H_{25}NO_4$: C, 69.93; H, 7.34; N, 4.08. Found: C, 69.97; H, 7.38; N, 4.04.

**5.9.2. (*R*)-(−)-2-{4-[2-(2-Hydroxy-2-phenylethylamino)-ethyl]phenoxy}-2-methylpropanoic acid (1b).** Mp: 232–233 °C (dec.), white solid (61% yield); $ee_{HPLC} = 97\%$. $[\alpha]_D$ −21.3 (*c* 0.34, $CH_3COOH$). Spectroscopic data are identical to those ones reported for **1a**.

**5.9.3. (*S*)-(+)-2-{4-[2-(2-Hydroxy-2-phenylethylamino)-ethyl]phenoxy}-2-methylpropanoic acid (1c).** Mp: 232–233 °C (dec.), white solid (45% yield); $ee_{HPLC} = 98\%$. $[\alpha]_D$ +23.6 (*c* 0.86, $CH_3COOH$). Spectroscopic data are identical to those ones reported for **1a**.

**5.9.4. (±)-2-{4-[2-(2-Hydroxy-2-phenylethylamino)ethyl]-phenoxy}propanoic acid (1d).** Mp: 165–166 °C, white solid (42% yield). HPLC elution time: $t_{(S,S)} = 14.66$ min, $t_{(R,S)} = 15.18$ min, $t_{(R,R)} = 19.12$ min and $t_{(S,R)} = 22.40$ min. FT-IR (KBr): 3600–3200, 2998, 2807, 1613, 1585, 1511, 1453, 1423, 1227, 1138, 1099, 1037, 932, 816, 747, 698 $cm^{-1}$. $^1H$ NMR (500 MHz, acetic acid-$d_4$, $\delta$): 1.61 (d, $J = 6.8$ Hz, 3H, CHC$H_3$), 3.06–3.08 (m, 2H, C$H_2$C$H_2$NH), 3.27 (dd, $J = 12.5$ and 10.7 Hz, 1H, C$H_2$CHOH), 3.31–3.37 (m, 2H, C$H_2$NH), 3.41 (dd, $J = 12.5$ and 2.5 Hz, 1H, C$H_2$CHOH), 4.84 (q, $J = 6.8$ Hz, 1H, C*H*$CH_3$), 5.26 (dd, $J = 10.7$ and 2.5 Hz, 1H, C*H*OH), 6.86–6.88 (m, 2H, aromatic protons), 7.18–7.19 (m, 2H, aromatic protons), 7.28–7.31 (m, 1H, aromatic proton), 7.34–7.37 (m, 2H, aromatic protons), 7.39–7.41 (m, 2H, aromatic protons). $^{13}C$ NMR (125 MHz, $CDCl_3$, $\delta$): 17.84, 31.24, 49.56, 54.15, 69.45, 72.18, 115.50, 126.04, 128.43, 128.78, 129.76, 130.12, 140.26, 156.91, 176.99. MS-ESI *m*/*z* (%): 330 $[M+H]^+$ (100%). MS-ESI *m*/*z* (%): 328 $[M-H]^-$ (100%). Anal. Calcd for $C_{19}H_{23}NO_4$: C, 69.26; H, 7.04; N, 4.25. Found: C, 69.28; H, 7.06, N, 4.22.

**5.9.5. (2*S*)-(+)-2-{4-[2-((2*S*)-2-Hydroxy-2-phenylethyl-amino)ethyl]phenoxy}propanoic acid (1e).** Mp: 202 °C (dec.), white solid (45% yield), which was judged by HPLC peak integrations to be a 90:10 mixture of (*S*,*S*)/minor diastereoisomers.[51] $[\alpha]_D$ +0.68 (*c* 1.19, $CH_3COOH$). Spectroscopic data are identical to those ones reported for **1d**.

**5.9.6. (2*R*)-(+)-2-{4-[2-((2*S*)-2-Hydroxy-2-phenylethyl-amino)ethyl]phenoxy}propanoic acid (1f).** Mp: 193–194.5 °C, white solid (65% yield), which was judged by HPLC peak integrations to be a 87:13 mixture of (*R*,*S*)/minor diastereoisomers.[51] $[\alpha]_D$ +44.9 (*c* 1.00, $CH_3COOH$). Spectroscopic data are identical to those ones reported for **1d**.

**5.9.7. (2*S*)-(−)-2-{4-[2-((2*R*)-2-Hydroxy-2-phenylethyl-amino)ethyl]phenoxy}propanoic acid (1g).** Mp: 193–194 °C, white solid (41% yield); which was judged by HPLC peak integrations to be a 87:13 mixture of (*S*,*R*)/minor diastereoisomers.[51] $[\alpha]_D$ −48.2 (*c* 1.04, $CH_3COOH$). Spectroscopic data are identical to those ones reported for **1d**.

**5.9.8. (2*R*)-(−)-2-{4-[2-((2*R*)-2-Hydroxy-2-phenylethyl-amino)ethyl]phenoxy}propanoic acid (1h).** Mp: 202 °C (dec.), white solid (48% yield), which was judged by HPLC peak integrations to be a 90:10 mixture of (*R*,*R*)/minor diastereoisomers.[51] $[\alpha]_D$ −0.41 (*c* 0.92, $CH_3COOH$). Spectroscopic data are identical to those ones reported for **1d**.

## 6. Biological methods

### 6.1. General

CHO cell lines transfected with human cloned $\beta_3$- and $\beta_2$-ARs were kindly provided by the Institute Cochin de Génétique Moléculaire, Paris, France. The CHO cell line transfected with human cloned $\beta_1$-ARs was kindly provided by Institut für Pharmakologie und Toxikologie, Universität Würzburg, Germany. For receptor binding and activity studies, compounds were dissolved in absolute ethanol. Forskoline was purchased from Tocris Cookson Ltd., UK; [$^3$H]-dihydroalprenolol (3,59TBq) was obtained from Amersham Biosciences (Milan, Italy). Protein concentration was determined by commercial protein determination kit based on the Lowry method,[52] using a Perkin-Elmer UV/vis LAMBDA BIO 20 spectrophotometer. All binding and activity data obtained were analyzed by Graph-Pad Prism program.[53]

**6.1.1. Cell culture and membrane preparation.** Chinese hamster ovary (CHO) cells expressing each subtype of human cloned $\beta_1$-, $\beta_2$- or $\beta_3$-ARs were grown in an atmosphere of 5% $CO_2$ in air at 37 °C in Dulbecco's modified Eagle's medium with nutrient mixture F12 (DMEM/F12) supplemented with 10% Foetal calf serum, 2 mM L-glutamine, 100 U/mL of penicillin G and 100 μg/mL of streptomycin.

Preconfluent cells were washed with ice-cold PBS, scraped from the plate surface, collected in ice-cold lysis buffer ($10 \times 10^6$/mL; 5 mM Tris/HCl, 2 mM EDTA, pH 7.4 at 4 °C) and homogenised with a Brinkman politron (5 for $3 \times 10$ s). The cell membrane suspension was centrifuged for 10 min at 4 °C at 1000*g*. Supernatant was centrifuged at 10,000*g* for 30 min at 4 °C. The resultant membrane pellet was resuspended in ice cold incubation buffer (50 mM Tris/HCl, 10 mM $MgCl_2$, pH 7.4, for $\beta_1$-AR binding experiments; 50 mM Tris/HCl, pH 7.4, for $\beta_2$-AR binding experiments) and protein content was measured. The membrane suspension was used immediately or stored frozen at −80 °C, for radioligand binding experiments.

**6.1.2. $\beta_1$- and $\beta_2$-AR binding experiments.** Saturation binding experiments were performed by incubating cell membranes (50 μg of protein) in a total volume of 500 μL incubation buffer, containing increasing

concentrations of [$^3$H]-dihydroalprenolol (0.1 nM, 0.5 nM, 1 nM, 3 nM, 5 nM, 10 nM) (Fig. 6A). Incubations were carried out at 30 °C for 30 min for $\beta_1$- or 90 min for $\beta_2$-AR binding assay. Non specific binding was determined in the presence of 10 μM Alprenolol. Reactions were terminated by rapid filtration through Whatman GF/C glass fibre filters that had been soaked for 60 min in 0.5% polyethyleneamine for $\beta_1$- or 0.3% polyethyleneamine for $\beta_2$-AR binding assay. The filters were washed with 3 × 1 mL of ice-cold incubation buffer. The radioactivity bound to the filters was measured using LS6500 Multi-Purpose scintillation Counter, Beckman. Competition experiments were performed by incubating 50 μg of protein with increasing amounts of test compound (from $10^{-9}$M to $10^{-5}$ M) and 4 nM [$^3$H]-dihydroalprenolol for $\beta_1$- or 0.4 nM for $\beta_2$-AR binding assay (Fig. 6B), in a final incubation volume of 500 μL incubation buffer. Non specific binding was determined in the presence of 10 μM Alprenolol. Reactions were terminated and radioactivity quantified as previously described.

In CHO-$\beta_1$ cells, $K_d$ value for Alprenolol was 12.49 nM and $B_{max}$ 2970 fmol/mg of protein (Fig. 6A) while in CHO-$\beta_2$-AR cells $K_d$ value for Alprenolol was 0.50 nM and $B_{max}$ 540 fmol/mg of protein (Fig. 6B). $K_d$ and $B_{max}$ values have been obtained as a mean of two experiments with samples in duplicate.

**6.1.3. $\beta_3$-Adrenoceptor activity by DELFIA cAMP-Eu assay[44].** The DELFIA (Dissociation Enhanced Lanthanide Fluoro Immuno Assay) cAMP-binding assay was performed according to technical data sheet by Perkin-Elmer Life Science. The optimization of experimental conditions (amount of cell, incubation times and other parameters) is reported below.

One confluent plate of cells was trypsinised and resuspended in the above medium and cultured overnight at a concentration of 50,000 cells/200 μL per well into 96-well flat-bottomed plates. The medium was aspirated from each well and replaced with 100 μL of preheated (37 °C) medium without serum. The plate was then placed back in the $CO_2$-incubator for 30 min at 37 °C. About 50 μL of 1 mM IBMX (3-isobutyl-1-methylxanthine), phosphodiesterase inhibitor was added to each well. 50 μL of test compounds at different concentrations (100 nM, 500 nM, 1 μM, 10 μM, 100 μM) were then added to the wells and the plate was incubated for 30 min at 37 °C. Cells were then lysed and incubated at room temperature for 5 min. The plate was immediately used for measuring cAMP levels or stored at 4 °C until the assay was performed. Measure of the samples was carried out in Time Resolved Fluorometer using 1420 Multilabel Counter *Victor3*, Perkin-Elmer. The wavelengths were 340 nm and 615 nm in excitation and emission, respectively.

### 6.2. PPARα transactivation assay

**6.2.1. 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).

**6.2.2. Transfection assay.** Monkey kidney fibroblasts (COS-7) were seeded at $1.2 \times 10^5$ cells per well, in 12-well plates, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal 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 compounds **1a**–**h**, at 37 °C for 48 h in the complete culture medium. About 2 μM Wy,14-643, a known PPARα ligand, was used as a 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 centrifuging 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.

**6.2.3. 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*-butyryl-CoA, 5 μl (0.25 μCi) of [$^{14}$C]-chloramphenicol and 65 μl of distilled water and incubated for 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 was 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 (A420) was spectrophotometrically measured.

Finally, the CAT activity was normalised to the β-galactosidase activity.[49]

## Acknowledgments

Work carried out under the framework of the National Projects 'Progettazione, Sintesi e Valutazione Biologica di Nuovi Farmaci Cardiovascolari' supported by the Ministero dell'Università e della Ricerca (MiUR, Rome), shared also with the University of Bari and Istituto di Chimica dei Composti Organo-Metallici (ICCOM-CNR, Bari). Thanks are also due to Prof. Karl-Norbert Klotz (Institut für Pharmakologie und Toxikologie, Universität Würzburg, Germany) for his suggestions and supplying us CHO cells expressing human cloned $\beta_1$-adrenoceptor, and to Prof. Susanna Cotecchia for her suggestions and helpful text discussion.

## References and notes

1. Lands, A. M.; Arnold, A.; McAuliff, J. P.; Ludaena, F. P.; Brown, T. G., Jr. *Nature* **1967**, *214*, 597.
2. Arch, J. R. S. *Proc. Nutr. Soc.* **1989**, *48*, 215.
3. Strosberg, A. D.; Pietri-Rouxel, F. *Trends Pharmacol. Sci.* **1996**, *17*, 373.
4. (a) Strosberg, A. D. *The* $\beta_3$*-Adrenoreceptor*; Taylor & Francis: New York, 2000, pp. 11; (b) Strosberg, A. D. *The* $\beta_3$*-Adrenoreceptor*; Taylor & Francis: New York, 2000, pp. 84; (c) Strosberg, A. D. *The* $\beta_3$*-Adrenoreceptor*; Taylor & Francis: New York, 2000, pp. 54.
5. Pott, C.; Brixius, K.; Bloch, W.; Ziskoven, C.; Napp, A.; Schwinger, R. H. G. *Pharmazie* **2006**, *61*, 255.
6. Rozec, B.; Gauthier, C. *Pharmcol. Ther.* **2006**, *111*, 652.
7. Hoffmann, C.; Leitz, M. R.; Obendorf-Maass, S.; Lohse, M. J.; Klotz, K.-N. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2004**, *369*, 151.
8. Shih, T. L.; Candelore, M. R.; Cascieri, M. A.; Chiu, S-H. L.; Colwell, L. F.; Deng, L. D., Jr.; Feeney, W. P.; Forrest, M. J.; Hom, G. J.; MacIntyre, D. E.; Miller, R. R.; Stearns, R. A.; Strader, C. D.; Tota, L.; Wyvratt, M. J.; Fisher, M. H.; Weber, A. E. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1251.
9. Perrone, M. G.; Santandrea, E.; Giorgio, E.; Bleve, L.; Scilimati, A.; Tortorella, P. *Bioorg. Med. Chem.* **2006**, *14*, 1207; Scilimati, A.; Perrone, M. G.; Santandrea, E. Beta-3 adrenoceptors ligands and their use in therapy. Italian Patent MI2006A000820, **2006**.
10. Gavai, A. V.; Sher, P. M.; Mikkilineni, A. B.; Poss, K. M.; McCann, P. J.; Girotra, R. N.; Fisher, L. G.; Wu, G.; Bednarz, M. S.; Mathur, A.; Wang, T. C.; Sun, C. Q.; Slusarchyk, D. A.; Skwish, S.; Allen, G. T.; Hillyer, D. E.; Frohlich, B. H.; Abboa-Offei, B. E.; Cap, M.; Waldron, T. L.; George, R. J.; Tesfamarian, B.; Harper, T. W.; Ciosek, C. P., Jr.; Young, D. A.; Dickinson, K. E.; Seymour, A. A.; Arbeeny, C. M.; Washburn, W. N. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3041.
11. Harada, H.; Hirokawa, Y.; Suzuki, K.; Hiyama, Y.; Oue, M.; Kawashima, H.; Yoshida, N.; Furutani, Y.; Kato, S. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1301.
12. Dow, R. L.; Paight, E. S.; Schneider, S. R.; Hadcock, J. R.; Hargrove, D. M.; Martin, K. A.; Maurer, T. S.; Nardone, N. A.; Tess, D. A.; DaSilva-Jardine, P. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3235.
13. Uehling, D. E.; Shearer, B. G.; Donaldson, K. H.; Chao, E. Y.; Deaton, D. N.; Adkison, K. K.; Brown, K. K.; Cariello, N. F.; Faison, W. L.; Lancaster, M. E.; Lin, J.; Hart, R.; Milliken, T. O.; Paulik, M. A.; Sherman, B. W.; Sugg, E. E.; Cowan, C. *J. Med. Chem.* **2006**, *49*, 2758.
14. Sawa, M.; Harada, H. *Curr. Med. Chem.* **2006**, *13*, 25; Takasu, T.; Ukai, M.; Sato, S.; Matsui, T.; Nagase, I.; Maruyama, T.; Sasamata, M.; Miyata, K.; Uchida, H.; Yamaguchi, O. *J. Pharmacol. Exp. Ther.* **2007**, *32*, 642.
15. Manara, L.; Badone, D.; Baroni, M.; Boccardi, G.; Cecchi, R.; Croci, T.; Giudice, A.; Guzzi, U.; Landi, M.; Le Fur, G. *Br. J. Pharmacol.* **1996**, *117*, 435.
16. Nandakumar, K.; Bansal, S. K.; Singh, R.; Mohite, A. J.; Bodhankar, S. L.; Jindal, D. P.; Coumar, M. S.; Balaraman, R.; Bhardwaj, S. H. *Pharmacology* **2005**, *74*, 1.
17. Candelore, M. R.; Deng, L.; Tota, L.; Guan, X.-M.; Amend, A.; Liu, Y.; Newbold, R.; Cascieri, M. A.; Weber, A. E. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 649.
18. Costa, T.; Cotecchia, S. *Trends Pharmacol. Sci.* **2005**, *26*, 618.
19. Strange, P. G. *Trends Pharmacol. Sci.* **2002**, *23*, 89.
20. Tisdale, M. J. *J. Supp. Oncol.* **2003**, *1*, 159.
21. Croci, T.; Cecchi, R.; Marini, P.; Gouget, C.; Viviani, N.; Germain, G.; Guagnini, F.; Fradin, Y.; Descamps, L.; Pascal, M.; Advenier, C.; Breuiller-Fouch, M.; Leroy, M.-J.; Bardou, M. *J. Pharmacol. Exp. Ther.* **2007**, *321*, 1118.
22. Stemmelin, J.; Cohen, C.; Terranova, J. P.; Lopez-Grancha, M.; Pichat, P.; Bergis, O.; Decobert, M.; Santucci, V.; Francon, D.; Alonso, R.; Stahl, S. M.; Keane, P.; Avenet, P.; Scatton, B.; Griebel, G. *Neuropsychopharmacology* **2007**, *1*, 1.
23. Nakajima, Y.; Hamashima, H.; Washizuka, K.-I.; Tomishima, Y.; Ohtake, H.; Imamura, E.; Miura, T.; Kayakiri, H.; Kato, M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 251.
24. Mizuno, K.; Sawa, M.; Harada, H.; Taoka, I.; Yamashita, H.; Oue, M.; Tsujiuchi, H.; Arai, Y.; Suzuki, S.; Furutani, Y.; Kato, S. *Bioorg. Med. Chem.* **2005**, *13*, 855.
25. Mizuno, K.; Sawa, M.; Harada, H.; Tateishi, H.; Oue, M.; Tsujiuchi, H.; Furutani, Y.; Kato, S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5959.
26. Tanaka, N.; Tamai, T.; Mukaiyama, H.; Hirabayashi, A.; Muranaka, H.; Ishikawa, T.; Kobayashi, J.; Akahane, S.; Akahane, M. *J. Med. Chem.* **2003**, *46*, 105.
27. Kashaw, S. K.; Rathi, L.; Mishra, P.; Saxena, A. K. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2481.
28. Steffan, R.; Ashwell, M. A.; Solvibile, W. R.; Matelan, E.; Largis, E.; Han, S.; Tillet, J.; Mulvey, R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2957.
29. Brockunier, L. L.; Candelore, M. R.; Cascieri, M. A.; Liu, Y.; Tota, L.; Wyvratt, M. J.; Fisher, M. H.; Weber, A. E.; Parmee, E. R. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 379.
30. Fisher, L. G.; Sher, P. M.; Skwish, S.; Michel, I. M.; Seiler, S. M.; Dickinson, K. E. J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2253.
31. Sher, P. M.; Mathur, A.; Fisher, L. G.; Wu, G.; Skwish, S.; Michel, I. M.; Seiler, S. M.; Dickinson, K. E. J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1583.
32. Sher, P. M.; Fisher, L. G.; Skwish, S.; Michel, I. M.; Seiler, S. M.; Washburn, W. N.; Dickinson, K. E. J. *Med. Chem. Res.* **1997**, *7*, 109.
33. Weber, A. E.; Mathvink, R. J.; Perkins, L.; Hutchins, J. E.; Candelore, M. R.; Tota, L.; Strader, C. D.; Wyvratt, M. J.; Fisher, M. H. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1101.
34. Uehling, D. E.; Donaldson, K. H.; Deaton, D. N.; Hyman, C. E.; Sugg, E. E.; Barrett, D. G.; Hughes, R. G.; Reitter, B.; Adkison, K. K.; Lancaster, M. E.; Lee, F.; Hart, R.; Paulik, M. A.; Sherman, B. W.; True, T.; Cowan, C. *J. Med. Chem.* **2002**, *45*, 567.

35. Dallanoce, C.; Frigerio, F.; De Amici, M.; Dorsch, S.; Klotz, K.-N.; De Micheli, C. *Bioorg. Med. Chem* **2007**, *15*, 2533.
36. Maack, C.; Tyroller, S.; Schnabel, P.; Cremers, B.; Dabew, E.; Südkamp, M.; Böhm, M. *Br. J. Pharmacol.* **2001**, *132*, 1817.
37. Lohse, M. J.; Hoffmann, C.; Engelhardt, S. *International Congress Series* **2003**, *1249*, 55, Inverse Agonism.
38. Baker, J. G.; Hall, I. P.; Hill, S. J. *Mol. Pharmacol.* **2003**, *64*, 1357.
39. Baker, J. G. *Mol. Pharmacol.* **2005**, *68*, 1645.
40. Tanaka, N.; Tamai, T.; Mukaiyama, H.; Hirabayashi, A.; Muranaka, H.; Akahane, S.; Miyata, H.; Akahane, M. *J. Med. Chem.* **2001**, *44*, 1436.
41. Tanaka, N.; Tamai, T.; Mukaiyama, H.; Hirabayashi, A.; Muranaka, H.; Ishikawa, T.; Akahane, S.; Akahane, M. *Bioorg. Med. Chem.* **2001**, *9*, 3265.
42. Berglund, P.; Vallikivi, I.; Fransson, L.; Dannacher, H.; Holmquist, M.; Martinelle, M.; Bjorkling, F.; Parve, O.; Hult, K. *Tetrahedron: Asymmetry* **1999**, *10*, 4191.
43. Otera, J.; Nakazawa, K.; Sekoguchi, K.; Orita, A. *Tetrahedron* **1997**, *53*, 13633.
44. Gabriel, D.; Vernier, M.; Pfeifer, M. J.; Dasen, B.; Tenaillon, L.; Bouhelal, R. *Assay Drug Dev. Technol.* **2003**, *1*, 291.
45. $B_{max}$ for CHO–$\beta_3$ was 140 fmol/$10^6$ cell that normalized is 90,000 receptors/cell; $B_{max}$ for CHO–$\beta_2$ 400 fmol/$10^6$ cell that normalized is 240,000 receptors/cell; $B_{max}$ for CHO–$\beta_1$ 250 fmol/$10^6$ cell that normalized is 150,000 receptors/cell. See also: Tate, K. M.; Briend-Sutren, M. M.; Emorine, L. J.; Delavier-Klutchko, C.; Marullo, S.; Strosberg, A. D. *Eur. J. Biochem.* **1991**, *196*, 357.
46. Emorine, L. J.; Marullo, S.; Briend-Sutren, M.-M.; Patey, G.; Tate, K.; Delavier-Klutchko, C.; Strosberg, A. D. *Science* **1989**, *245*, 1118.
47. This finding was already found by other authors (A.D. Strosberg, et al. *Trends Pharmacol. Sci.* **1997**, *18*, 52). SR59230A behaves as a partial $\beta_3$-AR agonist in cells and an antagonist in tissue such as isolated rat colon (see Ref. 14 and A. J. Kaumann et al., *Br. J. Pharmacol.* **1996**, *118*, 2085-2098). We also tried DPJ 904 (and its enantiomers, too; their synthesis is not published yet) another $\beta_3$-AR antagonist, which activity has been measured only in isolated rat colon (K. Nandakumar, et al.*Pharmacology* **2005**, *74*, 1–5). Unfortunately, racemic and both DPJ 904 enantiomers behaved like SR59230A: they are partial $\beta_3$-AR agonist in CHO cells expressing human cloned $\beta_3$-AR. More tests on a variety of tissues are ongoing, in order to definitively characterize those compounds.
48. Soudijn, W.; van Wijngaarden, I.; Ijzerman, A. P. *Med. Res. Rev.* **2005**, *25*, 398.
49. Perrone, M. G.; Santandrea, E.; Dell'Uomo, N.; Giannessi, F.; Milazzo, F. M.; Sciarroni, A. F.; Scilimati, A.; Tortorella, V. *Eur. J. Med. Chem.* **2005**, *40*, 143.
50. Hieble, J. P. *Curr. Top. Med. Chem.* **2007**, *7*, 207.
51. The presence of minor diastereoisomers (≅10%) in the final targets could be due to a two main contributions: a very low extent of racemization at Cα and/or Cβ, and to the fact that the used chiral reagents such as (*R*)- and (*S*)-2-amino-1-phenylethanol and (*R*)- and (*S*)-lactates (see Aldrich and Fluka catalogues) were not enantiomerically pure.
52. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 309.
53. *GraphPad Prism Software* (version for Windows); GraphPad Software, Inc.: San Diego, CA.