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Hit-to-lead optimization of mouse Trace Amine Associated Receptor 1 (mTAAR1) agonists with a diphenylmethane-scaffold: Design, Synthesis, and biological study

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Keywords: mTAAR1 agonist; diphenylmethane-scaffold; Thyronamine; T1AM; T0AM
Abstract.

The trace amine-associated receptor 1 (TAAR1) is a G-protein-coupled receptors (GPCR) potently activated by a variety of molecules besides trace amines (TAs), including thyroid hormone-derivatives like 3-iodothyronamine (T1AM), catechol-O-methyltransferase products like 3-methoxytyramine, and amphetamine-related compounds. Accordingly, TAAR1 is considered a promising target for medicinal development. To gain more insights into TAAR1 physiological functions and validation of its therapeutic potential we recently developed a new class of thyronamine-like derivatives. Among them compound SG2 showed high affinity and potent agonist activity at mouse TAAR1. In the present work we describe design, the synthesis and SAR study of a new series of compounds (1-16) obtained by introducing specific structural changes at key points of our lead-compound SG2 skeleton. Five of the newly synthesized compounds displayed mTAAR1 agonist activity higher than both SG2 and T1AM. Selected diphenylmethane analogs, namely 1 and 2, showed potent functional activity in *in vitro* and *in vivo* models.
Introduction.

Thyronamine (TAM) is generally used to indicate a class of endogenous compounds deriving from thyroid hormones through metabolic reactions such as deiodination and decarboxylation. Over the past decade, thyronamines have been recognized as ligands of trace amine-associated receptor 1 (TAAR1), and to date they represent a thriving research field. Currently, only two compounds that belong to the class of TAM have been identified in vivo: the 3-iodothyronamine (T1AM) and the thyronamine (T0AM). These compounds have been postulated to derive from thyroid hormone (T4) through deiodination and decarboxylation. T1AM is widely expressed in various tissues, such as brain, liver, heart and blood and it seems to be the most abundant thyronamine present in circulation.

Among several potential receptors, it has been proved that T1AM binds to TAAR1, a G-protein coupled receptor (GPCR) that has been identified in specific areas of the central nervous system and in some peripheral areas. The binding of T1AM to TAAR1 receptor leads to increase of cAMP levels by the activation of the adenylate cyclase. Further studies also highlighted that T1AM interact with other targets such as the biogenic amines transporters and the apoB100 protein. Thyronamines, and in particular T1AM, produce different functional effects, although their physiological role still remains unclear. Among the most significant effects attributed to T1AM are a hypothermic as well as negative inotropic and chronotropic cardiac effects. Moreover, endocrine and metabolic effects such as the modulation of insulin secretion, the inhibition of catecholamines resorption at the neuronal level and an increased metabolism of lipids at the expense of that of carbohydrates have been also described. The wide variety of functions attributed to T1AM makes this molecule a potentially useful tool for the treatment of many diseases such as obesity, neuropsychiatric disorders and cancer. Unfortunately, TAMs are rapidly metabolized by different enzyme systems, such as amino-oxidase (MAO, SSAO), deiodinase (DIO3), sulfotransferase (SULT1A1 and SULT1A3), N-acetyltransferase, and glucuronidase. The action of these enzymes is
a limit to their therapeutic use. Consequently, the great therapeutic potential as well as the lack of new useful tools to elucidate the physiological function of T1AM, have pushed medicinal chemists to develop new synthetic analogues of T1AM.

Recently, with the aim of increasing the number of selective ligands for TAAR1 receptor and provide new tools to facilitate the understanding of the physiological functions of this receptor, we reported the design and synthesis of a new class of thyronamine-like compounds with a diphenylmethane scaffold, which proved to be synthetically more accessible than endogenous T1AM and T0AM⁴,⁵. Within the small series of analogues previously synthesized, SG1 and SG2 were found to activate mouse TAAR1 receptor (mTAAR1) with a potency comparable to the corresponding endogenous ligands⁴ (Figure 1). In particular, SG2, the analogue of T1AM, showed an EC₅₀ = 240 nM comparable to T1AM (EC₅₀ = 189 nM). In order to expand the SAR study for this new class of compounds, in the present work we describe the design and synthesis of compounds 1-16 obtained by introducing specific structural changes at key points of our lead-compound SG2 skeleton. The structural optimization process of the new thyronamine-like compounds was based on molecular docking studies that highlighted the main differences in the interaction between the endogenous ligand T1AM or the synthetic analogue SG2 with the mTAAR1 receptor binding site. The newly designed compounds were first screened in vitro for mTAAR1 activation. Five compounds (1,2,4,7 and 8) showed a higher potency than the lead molecule SG2, as well as, the endogenous ligand T1AM, with 1 and 2 being the most potent of the new series. These compounds were then evaluated both in vitro and in vivo to characterize their ability to modulate plasma glucose levels.
Results.

Design of new thyronamine-like analogues.

In our previous work, we explored the pharmacological profile of a new series of T1AM diphenylmethane analogues, and among these compounds SG1 and SG2 were found to be a good mimic of the corresponding endogenous ligands T0AM and T1AM\(^4\), respectively. In addition, a docking study was performed to investigate the main differences between T1AM and the lead diphenylmethane-analogue SG2 on mTAAR1 receptor binding. According to this study, T1AM appears to be highly stabilized in the binding site through the formation of two H-bonds between the protonated amino group of the ethylamine side chain and aspartic acid 102 (D102) and tyrosine 291 (Y291). Furthermore, T1AM established an additional H-bond between the hydroxyl-group on the outer ring and arginine 82 (R82), while no significant amino acid interactions were observed for the biaril-ethereal oxygen of T1AM. Notably, the SG2 derivative appeared to share most of the main mTAAR1/T1AM interactions \(^4\). Interestingly, the replacement of the hydroxyl group on the T1AM outer ring with an amino group, as in SG2, allowed the formation of a cation-\(\pi\) interaction, while no H-bonds to the R82 backbone oxygen atom were observed. Conceivably, the oxygen atom in the SG2-inner ring side chain may induce a shift of the molecule within the binding site, thus preventing the H-bond formation with R82. In order to expand the SAR study for this new class of
thyronamine analogues, we performed specific structural changes at key points of the SG2-molecular scaffold. Initially, to restore the H-bond with R82, the oxo-ethylamino side chain of SG2 was replaced with an ethylamine one (1-6). Among these new compounds, analogue 2 was synthesized to evaluate the effects induced by the recovery of the phenolic function as in T1AM. Additionally, to gain further insights on how the pattern of substitutions modulate the SG-analogues potency as mTAAR1 agonists, we investigated the following structure modifications: (a) the introduction of small alkyl substituents (Me, i-Pr) on both outer- and inner-rings (4,6-10); (b) the removal of the methylene-bridge, producing the diaryl-analogues 5 and 6; (c) the introduction of a biguanide-moiety at the oxy-alkyl side chain (15,16) and finally (d) mono- and bis-N-acetylation (3, 9-15). This last chemical modification has been suggested by a recent study conducted by Hoefig et al, showing that O-acetyl and N-acetyl derivatives of T1AM may be found in white adipose tissue and liver endogenous cells. Notably, N-Ac-T1AM was also found to be an endogenous cardioactive metabolite. On the basis of these findings, the corresponding mono- and bis-N-acetylated analogues of SG1 and SG2 lead compounds (i.e. 11,12 and 13,14, respectively) as well as the new acetylated products namely 3, 9,10 and 15, were synthetised.

Synthesis.

The derivatives 1-6 in which the oxo-ethylamino side chain of SG2 has been replaced with the ethylamine one have been synthesized as reported in Scheme 1. Briefly, the products 17-20 obtained by the palladium(0)-catalyzed Suzuki–Miyaura cross-coupling reaction of the trifluoroborate salt or boronic acid with the appropriate benzylbromide, reacted with SOCl₂ to give benzylchloride 21-24. Subsequently, the nucleophilic substitution with NaCN afforded the compound 25-28 with high yields. As previously described, reduction of nitrile derivative with LiAlH₄ in the presence of a Lewis’s acid afforded the diamine derivative 1,4-6. Then, the treatment of 1 with a mixture of Ac₂O/NaHCO₃ provided the acetylanilide 3 (Scheme 1a).
Compound 2 was obtained starting from the acetonitrile derivative 25. Reduction of the nitro-group with H₂ in the presence of 10% Pd-C afforded the aniline 29. The subsequent hydrolysis of the diazonium salt obtained by reaction of 29 with NaNO₂/H₂SO₄ afforded the final product 2 (Scheme 1b).

The Suzuki Miyaura cross-coupling reaction of the 2,6-dimethyl-4-methoxy-boronic acid with the 4-nitrobenzyl bromide gave 31 which was demethylated with BBr₃ at 0°C affording to phenol 32. The subsequent reaction of α-bromide-acetonitrile with 32 followed by reduction to the corresponding diamine-derivative gave the superior homolog of SG2 (7) (Scheme 2).
Scheme 2

Reagents and conditions: a: 4-Nitrobenzyl bromide, K$_2$CO$_3$, PdCl$_2$, Acetone/H$_2$O, rt, 72 h; b: BBr$_3$, DCM, 0°C, 1 h; c: BrCH$_2$CN, DMF, Cs$_2$CO$_3$, rt, 30'; d: LiAlH$_4$, AlCl$_3$, THF, reflux, 12 h.

Derivative 8-10 substituted with an i-Pr group in the outer ring and with a small N-alkyl (8) or N-acetyl-group (9,10) were synthesized starting from the cross coupling reaction of N-(4-(bromomethyl)-2-isopropylphenyl)acetamide (34) (see SI, Scheme S1) and the (4-(hydroxymethyl)-2-methylphenyl)fluoroboronate salt (Scheme 3). The tandem reactions with SOCl$_2$ and NaCN gave the nitrile derivative 37 with high yields. Subsequent reduction with LiAlH$_4$/AlCl$_3$ afforded the product 8, which was then reacted with Ac$_2$O in the presence of NaHCO$_3$ in a ratio of 1:1 or 1:2 to give 9 and 10, respectively.

As concerns the synthesis of the mono- and N-diacetylated derivatives 11-14 and the biguanide analogue of SG2 (15 and 16), the synthetic pathway as well as the procedures are reported in SI (Scheme S2).
SCHEME 3

![Chemical Reaction Diagram]

**Reagents and conditions:** a) PdCl$_2$ dppf, Cs$_2$CO$_3$, H$_2$O/Diossono, 95°C, 24h; b) SOCl$_2$, CHCl$_3$, rt, 2h; c) NaCN, H$_2$O/CH$_3$CN, mw; d) LiAlH$_4$, AlCl$_3$, THF, reflux, 12 h; e) Ac$_2$O/NaHCO$_3$, rt, 1h.

**Receptor Activation.**

mTAAR1 is coupled to stimulatory G protein and thus induces cAMP production in HEK293 upon agonist exposure. We measured the activity of the new compounds using a BRET-based assay as previously reported by us$^{4,7}$. The standard TAAR1 agonist β-PEA was used as reference compound (EC$_{50}$ = 138 nM).

Initially, all 1-16 compounds were tested at a screening concentration of 10µM. Then, for the compounds that were found to be active, a dose-response curve was performed to calculate their corresponding EC$_{50}$ values (Figure 2). As shown in Table 1, five compounds, namely 1, 2, 4, 7 and 8, appeared to be more potent than the lead compound SG2, as well as T1AM. All of them showed to be full agonists (Efficacy 100%). In particular, the more effective structure modifications resulted from the replacement of oxy-ethylamino side chain of SG2 with the ethylamino one (1) and the concomitant replacement of the amino group of the outer ring with the hydroxyl moiety (2).
Moreover, the addition of small alkyl groups in the inner ring of SG2 (7), as well as in the outer ring of the N-ethyl analogue of 1 (8), turned out to be successful, thus obtaining new compounds with a potency 2-fold higher than the lead compound SG2.

Table 1. Activity of the new diphenylmethane derivatives 1-6, 8-10 using BRET-based assay in mTAAR1 transfected HEK 293 cells. The EC$_{50}$ values are expressed in nM.

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Table 2. Activity of the new diphenylmethane derivatives 7, 11-16 using BRET-based assay in mTAAR1 transfected HEK 293 cells. The EC$_{50}$ values are expressed in nM.

![Chemical Structure](image)

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Figure 2. Dose-response curves of the most active SG2-analogues. All the compounds are full agonists (Efficacy =100%)

Docking study.

In our previous work, we investigated T<sub>1</sub>AM and a series of T<sub>1</sub>AM analogues whose potency profile proved to be highly related to the presence of a proper basic feature linked to an aromatic core<sup>4</sup>. Indeed, our molecular modelling studies pointed out the relevance of a key salt bridge between the protonated amine group of SG1 and SG2 lead compounds and the highly conserved residue D102, together with a number of π–π stacking and cation-π contacts with the surrounding amino acids, as we previously detected in other series of TAAR1 ligands<sup>8,9</sup>

These findings were supported by further homology modelling and docking studies we performed within the murine and human TAAR1 and TAAR5 receptors with respect to T<sub>1</sub>AM ligand<sup>10</sup>, and were also in accordance with the key role played by a proper basic feature included in any TAAR1
ligand, as reported in literature\textsuperscript{11}. Notably, this information was definitively validated by the mutagenesis data involving the D102 residue, reported by Reese\textsuperscript{12}.

In order to rationalize the pharmacological results and refine the computational models, \textit{in silico} docking studies on the most active compounds (1, 2, 7 and 8) were also performed, analysing their interactions with the putative receptor binding site (see SI, Table S1).

In agreement with the experimental data, our docking calculations revealed that the presence of the ethylamine chain appears to be the most effective choice, in particular within those series of compounds bearing a mono methyl-substituted inner ring (1, 2 and 8). Based on a comparison with the prototype T1AM, the most promising newly designed analogues were overturned within the receptor binding site, maintaining in any case the key contact with D102 which is required for TAAR1 agonism (see SI, Figure S1)\textsuperscript{12}.

Notably, the most effective compounds 1 and 2 were characterized by a reversed docking mode when compared to that previously described for SG2\textsuperscript{4}. Briefly, we originally highlighted the key role played by the protonated amino group of the ethyloxyamine side chain of SG2 in H-bonding with D102 and Y291, while the inner-phenyl group was engaged in $\pi-\pi$ stacking with Y287. On the contrary, 1 (and 2) displayed one H-bond between the aniline portion in the outer ring (or the hydroxyl group) and the D102 negative-charged side-chain, while the protonated basic side chain appeared to be involved in one H-bond and in a salt-bridge with T83 and D284, respectively, as shown in Figure 3a and 3b, respectively.
Figure 3. Docking mode of compounds SG2 (C atom cyan) and (a) 1 (C atom deep magenta) (b) 2 (C atom; coral) (c) 7 (C atom; light green) and (d) 8 (C atom; yellow) at the murine TAAR1 binding site. The most important residues are labelled.

The addition of a methyl-group in the inner-ring of SG2 led to a 2-fold increase of potency for 7, with an EC$_{50}$ of 134nM vs 240 nM for SG2. The docking study showed that also 7 takes an overturned positioning with respect to SG2 in the binding site of mTAAR1 (Figure 3c).
Presumably, the presence of an additional substituent into the inner ring causes a shift of the molecule within the binding site promoting the interaction of the aniline nitrogen atom in the outer ring with D102 residue, while the side chain’s nitrogen atom interacts with D284. Finally, the introduction of small alkyl groups into the outer ring of 1 as in compound 8 didn’t prevent the interaction between the nitrogen atom of aniline with D102 residue. Indeed, the EC$_{50}$ value of 8 (EC$_{50}$ = 102nM) was comparable to that of 1 (EC$_{50}$ = 119nM).

**Modulation of hepatic glucose production.**

The hyperglycemic effect observed after administration of exogenous T1AM had previously been attributed only to a modulation of insulin and/or glucagone secretion$^{13-15}$. Recently, our study$^{16}$ showed that in human hepatocarcinoma cells (HepG2) T1AM treatment directly stimulated glucose production (EC$_{50}$ = 0.84 µM), if adequate gluconeogenetic substrates were provided. Interestingly, the effect of T1AM on gluconeogenesis displayed a bell-shaped dose-response curve, suggesting a biphasic effect of T1AM on the modulation of gluconeogenesis. In the present study, the effects induced by two of the most potent new mTAAR1 agonists (i.e. 1 and 2) on gluconeogenesis were assessed. Preliminarily, HepG2 cells were incubated with each compound at three different doses (0.1, 1 and 10µM). By comparison, similar experiments were performed using T1AM. As shown in Figure 4a, incubation with 0.1 and 1 µM of compound 2 induced a significant increase in glucose production (P<0.01), while a dose of 10µM induced a decreased stimulation of glucose production. On the other hand, compound 1 showed a dose-dependent increase of glucose production, which reached statistical significance only when compound 1 was used at the highest dose (10µM). As shown in Figure 4a, treatment of HepG2 cells with T1AM (0.1, 1 and 10 µM) confirmed the bell-shaped dose–response curve observed in our previous study$^{16}$. To better define the dose dependence of 1 and 2 effects on glucose production additional experiments were carried out using a wider...
range of doses for both compounds. As shown in Figure 4b the EC$_{50}$ calculated for the effect on gluconeogenesis was in the submicromolar range for 2, and in the supramicromolar range for 1.

**Figure 4:** (a) Glucose production in HepG2 cell cultures that were incubated for 4 h in glucose production buffer after adding vehicle (DMSO) or compounds 1, 2 and T1AM. Results are expressed as mean ± S.E.M. and are normalized to the total cell protein content determined in cell lysates. (n=3 in each case). (b) Glucose production in HepG2 cell in response to increasing concentrations of compound 1 (0.1, 1, 10 and 20 µM) and 2 (0.001, 0.01, 0.1, 1 and 10 µM). Results are mean ± S.E.M. and are normalized to the total cell protein content determined in cell lysates. (n=6 in each case). *P<0.05, **P<0.01, ***P<0.001 by ANOVA.

**Modulation of plasma glucose level.**

As previously reported by us thyronamine-like analogues SG1 and SG2 demonstrated to be able to increase plasma glycaemia with a potency comparable to that of the corresponding endogenous thyronamine (i.e. T0AM and T1AM). Thus, in the present work the effects induced by 1 and 2 derivatives on plasma glucose level were also assessed.

Figure 5 shows that a single low dose (4.0 µg/kg, i.p.) of 1 or 2 significantly increases plasma glycaemia in CD-1 mice with a potency comparable to that previously shown by lead-compound SG2.
Figure 5. Effect of 1 and 2 on plasma glycaemia (mg·L⁻¹) in CD1-mice. Plasma glucose was measured 15 min after test compound or vehicle injection (i.p.) in blood collected from the tail veins of 4 h starved mice (n = 8 in each group). Results are expressed as means mean±SEM.*P < 0.05 vs vehicle. ***P < 0.001 vs vehicle.

Conclusion

Our ongoing efforts to develop thyronamine-like analogues synthetically more accessible than endogenous thyronamines yielded a new class of potent mTAAR1 diphenylmethane ligands. A medicinal chemistry optimization combined to docking studies were used starting from the molecular scaffold of our recently developed SG-compounds to design the new ligands. In particular, we investigated: (i) the replacement of the oxyethylamino side chain of SG2 with an ethylamino one (ii) the introduction of small alkyl substituents (Me, i-Pr) on both outer- and inner-rings and (iii) the recovery of the phenolic function as in T1AM. Overall these chemical modifications resulted in more potent mTAAR1 agonists when compared to T1AM, as well as, lead compound SG2. Taking into account the high structure similarity of compounds 1 and 2 with T1AM and their high affinity for mTAAR1, we further investigated their ability to modulate glucose levels using both in vitro (human HepG2 cells) and in vivo (CD1-mice) models. Both
compounds demonstrated potent functional activity. In conclusion, this paper describes for the first time the successful outcome of a hit-to-lead optimization process leading to the identification of new tools to explore the physiological and pharmacological functions of TAAR1.

**Experimental Section**

**Chemistry.**

*General Material and Methods.* Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references; coupling constants J are reported in hertz. $^1$H NMR and $^{13}$C NMR spectra of all compounds were obtained with a Bruker TopSpin 3.2 400 MHz spectrometer. $^{13}$C NMR spectra were fully decoupled. The following abbreviations are used: singlet (s), doublet (d), triplet (t), double–doublet (dd), and multiplet (m). The elemental compositions of the compounds agreed to within ±0.4% of the calculated values. Chromatographic separation was performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. The ≥95% purity of the tested compounds was confirmed by combustion analysis. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F$_{254}$) sheets that were visualized under a UV lamp. The microwave-assisted procedures were carried out with a CEM Discover LabMate microwave. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. Commercially available chemicals were purchased from Sigma-Aldrich.

4-(4-(2-Aminoethyl)-2-methylbenzyl)aniline (1). To a suspension of LiAlH$_4$ (4.80 mL, 4.80 mmol) in THF was added dropwise a solution of AlCl$_3$ (639.6 mg, 4.80 mmol) in THF (20 mL), and the mixture was stirred for 5 min at r.t. A solution of 25 (142.0 mg, 0.53 mmol) in THF was added dropwise, and the mixture was heated at reflux for 12 h. The mixture was cooled to 0°C with an ice bath and added dropwise with water and then with 10% aqueous HCl. The mixture was extracted with diethyl ether, and the aqueous layer was made alkaline with NaOH 2N and extracted with
The organic phase was separated, washed with brine, dried, filtered, and concentrated. The crude was purified by conversion in the corresponding hydrochloride salt. White solid. mp: 153-157°C. (67% yield). \(^1\)H NMR (CD\(_3\)OD): δ 2.21 (s, 3H, CH\(_3\)); 2.94 (t, 2H, \(J = 7.8\) Hz; CH\(_2\)); 3.17 (t, 2H, \(J = 7.8\) Hz; CH\(_2\)); 4.03 (s, 2H, CH\(_2\)); 7.07-7.15 (m, 3H, Ar); 7.29 (d, 2H, \(J = 8.4\) Hz, Ar); 7.34 (d, 2H, \(J = 8.4\) Hz, Ar) ppm. \(^{13}\)C NMR (CD\(_3\)OD): δ 143.44, 138.48, 138.37, 136.40, 131.89, 131.66, 131.35, 129.71, 127.54, 124.11, 41.99, 39.31, 34.09, 19.70 ppm. Anal. (C\(_{16}\)H\(_{20}\)N\(_2\)·HCl) C, H, N. % Calcd: 79.96 (C); 8.39 (H); 11.66 (N). % Found: 79.88 (C); 8.55 (H); 11.42 (N).

4-(4-(2-Aminoethyl)-2-methylbenzyl)phenol (2). Compound 2 was synthesized from 30 (109 mg, 0.46 mmol), LiAlH\(_4\) (4.13 mL; 4.13 mmol) and AlCl\(_3\) (550.8 mg, 4.13 mmol) in THF (18 mL) following the same procedure described above for the preparation of 1. Yellow solid. mp: 138-140°C. (30% yield). \(^1\)H NMR (CD\(_3\)OD): δ 2.18 (s, 3H, CH\(_3\)); 2.70 (t, 2H, \(J = 7.2\) Hz; CH\(_2\)); 2.87 (t, 2H, \(J = 7.2\) Hz; CH\(_2\)); 3.81 (s, 2H, CH\(_2\)); 6.64 (d, 2H, \(J = 8.4\) Hz, Ar); 6.84 (d, 2H, \(J = 8.4\) Hz, Ar); 6.94-7.03 (m, 3H, Ar) ppm. \(^{13}\)C NMR (CD\(_3\)OD): δ 146.29, 139.28, 137.93, 137.82, 131.69, 131.64, 131.10, 130.31, 127.21, 116.88, 54.79, 39.22, 38.30, 19.74 ppm. Anal. (C\(_{16}\)H\(_{19}\)NO) C, H, N. % Calcd: 79.63 (C); 7.94 (H); 5.80 (N). % Found: 79.77 (C); 7.81 (H); 5.92 (N).

N-(4-(4-(2-Aminoethyl)-2-methylbenzyl)phenyl)acetamide (3). To a solution of 1 (50 mg, 0.18 mmol) in saturated NaHCO\(_3\) solution (0.06 mL) was added Ac\(_2\)O (0.02 mL, 0.18 mmol). The mixture was left stirring at r.t. for 1 h, then, the mixture was dissolved in water and extracted with CHCl\(_3\). The organic layer was dried and concentrated under reduced pressure. The crude product was purified by precipitation from MeOH/Et\(_2\)O to give 3. White solid. mp: 132-136°C. (60% yield). \(^1\)H NMR (CD\(_3\)OD): δ 2.10 (s, 3H, CH\(_3\)); 2.20 (s, 3H, COCH\(_3\)); 2.92 (t, 2H, \(J = 7.7\) Hz; CH\(_2\)); 3.15 (t, 2H, \(J = 7.7\) Hz; CH\(_2\)); 3.91 (s, 2H, CH\(_2\)); 7.04 (d, 2H, \(J = 8.4\) Hz, Ar); 7.05-7.11 (m, 3H, Ar); 7.34 (d, 2H, \(J = 8.4\) Hz, Ar) ppm. \(^{13}\)C NMR (CD\(_3\)OD): δ 171.57, 139.37, 138.31, 137.77, 137.62, 135.91, 131.71, 131.52, 129.93, 127.33, 121.33, 42.01, 39.37, 34.09, 23.77, 19.74 ppm. Anal.
(C₁₆H₂₀N₂·HCl) C, H, N. % Calcd: 79.96 (C); 8.39 (H); 11.66 (N). % Found: 79.88 (C); 8.55 (H); 11.42 (N).

**4-(4-(2-Aminoethyl)-2,6-dimethylbenzyl)aniline (4).** Compound 4 was synthesized from 26 (109 mg, 0.46 mmol), LiAlH₄ (4.13 mL; 4.13 mmol) and AlCl₃ (550.8 mg, 4.13 mmol) in THF (18 mL) following the same procedure described above for the preparation of 1. The crude was purified by conversion in the corresponding hydrochloride salt. White solid, mp: 143-145°C. (68% yield). 

**1H NMR (CD₃OD): δ 2.22 (s, 6H, CH₃); 2.91 (t, 2H, J = 7.7 Hz; CH₂); 3.18 (t, 2H, J = 7.7 Hz; CH₂); 4.11 (s, 2H, CH₂); 7.01 (s, 2H, Ar); 7.17 (d, 2H, J = 8.6 Hz, Ar); 7.30 (d, 2H, J = 8.6 Hz, Ar) ppm.**

**13C NMR (CD₃OD): δ 142.77, 138.85, 136.31, 136.14, 130.58, 129.68, 129.61, 124.13, 42.00, 34.98, 34.12, 20.23 ppm.** Anal. (C₁₇H₂₂N₂·HCl) C, H, N. % Calcd: 80.27 (C); 8.72 (H); 11.01 (N). % Found: 80.40 (C); 8.59 (H); 11.31 (N).

**4'-(2-Aminoethyl)-2'-methyl-[1,1'-biphenyl]-4-amine (5).** Compound 5 was synthesized from 27 (109 mg, 0.46 mmol), LiAlH₄ (4.13 mL; 4.13 mmol) and AlCl₃ (550.8 mg, 4.13 mmol) in THF (18 mL) following the same procedure described above for the preparation of 1. The crude was purified by conversion to the corresponding hydrochloride salt. White solid, mp: 165-167°C. (70% yield). 

**1H NMR (CD₃OD): δ 2.25 (s, 3H, CH₃); 2.99 (t, 2H, J = 7.8 Hz; CH₂); 3.22 (t, 2H, J = 7.8 Hz; CH₂); 7.19 (d, 2H, J = 1.2 Hz, Ar); 7.25 (s, 1H, Ar); 7.51-7.46 (m, 4H, Ar) ppm.**  

**13C NMR (CD₃OD): δ 144.11, 140.55, 137.72, 137.02, 131.99, 131.21, 130.72, 127.48, 124.08, 41.92, 34.19, 20.43 ppm. Anal. (C₁₅H₁₈N₂·HCl) C, H, N. % Calcd: 79.61 (C); 8.02 (H); 12.38 (N). % Found: 80.40 (C); 8.59 (H); 11.31 (N).

**4'-(2-Aminoethyl)-2',6'-dimethyl-[1,1'-biphenyl]-4-amine (6).** Compound 6 was synthesized from 28 (109 mg, 0.46 mmol), LiAlH₄ (4.13 mL; 4.13 mmol) and AlCl₃ (550.8 mg, 4.13 mmol) in THF (18 mL) following the same procedure described above for the preparation of 1. The crude was purified by conversion to the corresponding hydrochloride salt. White solid, mp: 171-173°C. (73% yield). 

**1H NMR (CD₃OD): δ 2.00 (s, 3H, CH₃); 2.95 (t, 2H, J = 7.8 Hz; CH₂); 3.20 (t, 2H, J = 7.8 Hz; CH₂);
CH$_2$); 7.07 (s, 2H, Ar); 7.30 (d, 2H, $J$ = 8.4 Hz, Ar); 7.53 (d, 2H, $J$ = 8.4 Hz, Ar) ppm. $^{13}$C NMR (CD$_3$OD): $\delta$ 143.35, 140.41, 137.50, 137.27, 132.17, 130.72, 128.97, 124.52, 41.98, 34.21, 20.84 ppm. Anal. (C$_{16}$H$_{20}$N$_2$·HCl) C, H, N. % Calcd: 79.96 (C); 8.39 (H); 11.66 (N). % Found: 79.90 (C); 8.49 (H); 11.71 (N).

4-(4-(2-Aminoethoxy)-2,6-dimethylbenzyl)aniline (7). Compound 7 was synthesized from 33 (186.2 mg, 0.66 mmol), LiAlH$_4$ (6.87 mL, 6.87 mmol) and AlCl$_3$ (915.6 mg, 6.87 mmol) in THF (25 mL) following the same procedure described above for the preparation of 1. The crude was purified by conversion to the corresponding hydrochloride salt. White solid, mp: 156-158°C. (75% yield). $^1$H NMR (CD$_3$OD): $\delta$ 2.20 (s, 6H, CH$_3$); 3.37 (t, 2H, $J$ = 5.0 Hz; CH$_3$); 4.07 (s, 2H, CH$_2$); 4.23 (t, 2H, $J$ = 5.0 Hz; CH$_2$); 6.76 (s, 2H, Ar); 7.15 (d, 2H, $J$ = 8.4 Hz, Ar); 7.30 (d, 2H, $J$ = 8.4 Hz, Ar) ppm. $^{13}$C NMR (CD$_3$OD): $\delta$ 156.50, 141.82, 138.32, 129.24, 129.09, 128.16, 122.72, 114.04, 63.74, 39.07, 33.21, 19.10 ppm. Anal. (C$_{17}$H$_{22}$N$_2$O·HCl) C, H, N. % Calcd: 75.52 (C); 8.20 (H); 10.36 (N). % Found: 75.60 (C); 8.07 (H); 10.40 (N).

4-(4-(2-Aminoethyl)-2-methylbenzyl)-N-ethyl-2-isopropylaniline (8). Compound 8 was synthesized from 37 (130.8 mg, 0.41 mmol), LiAlH$_4$ (1.84 mL, 1.84 mmol) and AlCl$_3$ (244.8 mg, 1.84 mmol) in THF (17 mL) following the same procedure described above for the preparation of 1. The crude was purified by conversion in the corresponding hydrochloride salt. Brown solid, mp: 148-150°C. (67% yield). $^1$H NMR (CD$_3$OD): $\delta$ 1.28 (d, 6H, $J$ = 6.8 Hz, CH$_3$); 1.37 (t, 3H, $J$ = 7.2 Hz, CH$_3$); 2.22 (s, 3H, CH$_3$); 2.93 (t, 2H, $J$=7.8 Hz, CH$_2$); 3.05-3.12 (m, 1H, CH); 3.16 (t, 2H, $J$ = 7.8 Hz, CH$_2$); 3.43 (q, 2H, $J$ = 7.2 Hz, CH$_2$); 4.04 (s, 2H, CH$_2$); 7.06-7.14 (m, 4H, Ar); 7.32 (d, 1H, $J$=8.4 Hz, Ar); 7.36 (d, 1H, $J$ = 1.6 Hz, Ar) ppm. $^{13}$C NMR (CD$_3$OD): $\delta$ 144.42, 143.51, 138.47, 138.36, 136.41, 131.87, 131.56, 131.00, 129.52, 128.88, 127.54, 124.53, 49.90, 41.97, 39.45, 34.11, 28.70, 24.43, 19.72, 11.38 ppm. Anal. (C$_{21}$H$_{30}$N$_2$·HCl) C, H, N. % Calcd: 81.24 (C); 9.74 (H); 9.02 (N). % Found: 81.36 (C); 9.65 (H); 9.11 (N).
N-(4-(2-Aminoethyl)-2-methylbenzyl)-2-isopropylphenyl)-N-ethylacetamide (9). Compound 9 was synthesized from 8 (45.6 mg, 0.15 mmol), saturated NaHCO₃ solution (0.05 mL) and Ac₂O (0.01 mL, 0.15 mmol) following the same procedure described above for the preparation of 3. The crude solid was collected by filtration and purified by conversion to the corresponding hydrochloride salt. Yellow solid. (30% yield). ¹H NMR (CD₃OD): δ 1.08-1.20 (m, 9H, CH₃, CH₂CH₂); 1.73 (s, 3H, COCH₃); 2.24 (s, 3H, CH₃); 2.90-2.96 (m, 3H, CH₂, CH); 3.02-3.07 (m, 1H, CH₂); 3.17 (t, 2H, J = 7.4 Hz, CH₂); 4.02 (s, 2H, CH₂); 4.11-4.18 (m, 1H, CH₂); 6.97-7.05 (m, 2H, Ar); 7.07-7.17 (m, 3H, Ar); 7.27 (s, 1H, Ar) ppm. ¹³C NMR (CD₃OD): δ 173.03, 146.93, 143.12, 138.91, 138.71, 138.36, 136.21, 131.81, 131.53, 130.08, 128.87, 128.30, 127.47, 45.29, 42.01, 39.67, 34.10, 28.83, 24.48, 24.13, 22.59, 19.77, 12.99 ppm. Anal. (C₂₃H₃₂N₂O·HCl) C, H, N. % Calcd: 78.36 (C); 9.15 (H); 7.95 (N). % Found: 78.45 (C); 9.02 (H); 7.84 (N).

N-(4-(2-Acetamidoethyl)-2-methylbenzyl)-2-isopropylphenyl)-N-ethylacetamide (10). Compound 10 was synthesized from 8 (45.6 mg, 0.15 mmol), saturated NaHCO₃ solution (0.05 mL) and Ac₂O (0.02 mL, 0.30 mmol) following the same procedure described above for the preparation of 3. The reaction mixture was evaporated and the crude dissolved in AcOEt and washed twice with water. The evaporation of the organic collected phases gave final product as a white oil. (20% yield). ¹H NMR (CD₃OD): δ 1.10-1.20 (m, 9H, CH₃, CH₂CH₂); 1.73 (s, 3H, COCH₃); 1.90 (s, 3H, COCH₃); 2.21 (s, 3H, CH₃); 2.74 (t, 2H, J = 7.4 Hz, CH₂); 2.90-2.96 (m, 1H, CH); 3.01-3.09 (m, 1H, CH₂); 3.37 (t, 2H, J = 7.4 Hz, CH₂); 4.00 (s, 2H, CH₂); 4.11-4.20 (m, 1H, CH₂); 6.99-7.09 (m, 5H, Ar); 7.25 (d, 1H, J = 1.2 Hz, Ar) ppm. ¹³C NMR (CD₃OD): δ 173.03, 146.93, 143.12, 138.91, 138.71, 138.36, 136.21, 131.81, 131.53, 130.08, 128.87, 128.30, 127.47, 45.29, 42.01, 39.71, 36.09, 28.86, 24.50, 24.16, 22.60, 22.52, 19.78, 13.00 ppm. Anal. (C₂₅H₃₄N₂O₂) C, H, N. % Calcd: 76.10 (C); 8.71 (H); 7.10 (N). % Found: 76.25 (C); 8.53 (H); 7.21 (N).

N-(4-(2-Aminoethoxy)benzyl)phenylacetamide (11). Compound 11 was synthesized from SG1 (49.7 mg, 0.20 mmol), saturated NaHCO₃ solution (0.07 mL) and Ac₂O (0.03 mL, 0.20 mmol).
following the same procedure described above for the preparation of 3. The crude product was purified by precipitation from MeOH/Et₂O to give 11. White solid, mp: 132-136°C. (60% yield). ¹H NMR (CD₃OD): δ 2.10 (s, 3H, COCH₃); 3.34 (t, 2H, J = 5.2 Hz; CH₂); 3.87 (s, 2H, CH₂); 4.19 (t, 2H, J = 5.2 Hz; CH₂); 6.92 (d, 2H, J = 8.8 Hz, Ar); 7.10-7.14 (m, 4H, Ar); 7.42 (d, 2H, J = 8.4 Hz, Ar) ppm. ¹³C NMR (CD₃OD): δ 171.59, 157.83, 138.90, 137.82, 136.02, 130.93, 130.03, 121.40, 115.70, 65.29, 41.30, 40.39, 23.76 ppm. Anal. (C₁₇H₂₀N₂·HCl) C, H, N. % Calcd: 71.81 (C); 7.09 (H); 9.85 (N). % Found: 71.88 (C); 7.15 (H); 9.92 (N).

N-(2-(4-(4-Acetamidobenzyl)phenoxy)ethyl)acetamide (12). Compound 12 was synthesized from SG1 (47.0 mg, 0.19 mmol), saturated NaHCO₃ solution (0.14 mL) and Ac₂O (0.04 mL, 0.38 mmol) following the same procedure described above for the preparation of 3. The crude product was purified by precipitation from MeOH/Acetone to give 12. White solid, mp: 152-153°C. (60% yield). ¹H NMR (DMSO): δ 2.00 (s, 3H, COCH₃); 2.08 (s, 3H, COCH₃); 3.34-3.38 (m, 2H, CH₂); 3.79 (s, 2H, CH₂); 3.91 (t, 2H, J = 5.6 Hz; CH₂); 6.84 (d, 2H, J = 8.8 Hz, Ar); 7.08-7.10 (m, 4H, Ar); 7.45 (d, 2H, J = 8.8 Hz, Ar); 8.06 (br t, NH₂); 9.83 (br s, NH) ppm. ¹³C NMR (CD₃OD): δ 173.59, 171.54, 158.58, 139.08, 137.78, 135.20, 130.82, 130.06, 121.39, 115.59, 67.53, 41.32, 40.26, 23.70, 22.46 ppm. Anal. (C₁₉H₂₂N₂O₃·HCl) C, H, N. % Calcd: 69.92 (C); 6.79 (H); 8.58 (N). % Found: 69.88 (C); 6.67 (H); 8.61 (N).

N-(4-(4-(2-Aminoethoxy)-2-methylbenzyl)phenyl)acetamide (13). Compound 13 was synthesized from SG2 (50.0 mg, 0.19 mmol), saturated NaHCO₃ solution (0.07 mL) and Ac₂O (0.02 mL, 0.19 mmol) following the same procedure described above for the preparation of 3. The crude product was purified by precipitation from MeOH/Et₂O to give 13. White solid, mp: 132-136°C. (60% yield). ¹H NMR (CD₃OD): δ 2.10 (s, 3H, CH₃); 2.19 (s, 3H, COCH₃); 3.34 (t, 2H, J = 5.0 Hz; CH₂); 3.89 (s, 2H, CH₂); 4.20 (t, 2H, J = 5.0 Hz; CH₂); 6.77 (dd, 1H, J = 2.4, 8.4 Hz, Ar); 6.83 (d, 1H, J = 2.4 Hz, Ar); 7.03 (d, 2H, J = 8.4 Hz, Ar); 7.06 (d, 1H, J = 8.4 Hz, Ar); 7.41 (d, 2H, J = 8.4 Hz, Ar) ppm. ¹³C NMR (CD₃OD): δ 171.58, 158.00, 139.19, 138.03, 137.67, 133.61, 132.00, 129.79,
121.32, 117.73, 112.83, 65.18, 40.41, 38.93, 23.79, 19.96 ppm. Anal. (C_{18}H_{22}N_{2}O_{2}:HCl) C, H, N. % Calcd: 72.46 (C); 7.43 (H); 9.39 (N). % Found: 72.58 (C); 7.55 (H); 9.42 (N).

**N-(2-(4-(4-Acetamidobenzyl)-3-methylphenoxy)ethyl)acetamide (14).** Compound 14 was synthesized from **SG2** (50.0 mg, 0.19 mmol), saturated NaHCO$_3$ solution (0.14 mL) and Ac$_2$O (0.04 mL, 0.38 mmol) following the same procedure described above for the preparation of 3. The crude product was purified by precipitation from MeOH/Et$_2$O to give 14. White solid, mp: 139-141°C. (56% yield). $^1$H NMR (CD$_3$OD): δ 1.95 (s, 3H, CH$_3$); 2.09 (s, 3H, COCH$_3$); 2.16 (s, 3H, COCH$_3$); 3.53 (t, 2H, $J = 5.6$ Hz; CH$_2$); 3.87 (s, 2H, CH$_2$); 4.00 (t, 2H, $J = 5.6$ Hz; CH$_2$); 6.70 (dd, 1H, $J = 2.4$, 8.4 Hz, Ar); 6.74 (d, 1H, $J = 2.4$ Hz, Ar); 6.99-7.03 (m, 3H, Ar); 7.41 (d, 2H, $J = 8.4$ Hz, Ar) ppm. $^{13}$C NMR (CD$_3$OD): δ 173.60, 171.53, 158.78, 139.01, 138.27, 137.66, 132.85, 131.94, 129.83, 121.32, 117.66, 112.72, 67.42, 40.30, 38.98, 23.70, 22.47, 19.96 ppm. Anal. (C$_{20}$H_{24}N$_2$O$_2$·HCl) C, H, N. % Calcd: 70.56 (C); 7.11 (H); 8.23 (N). % Found: 70.43 (C); 7.05 (H); 8.22 (N).

**N-(4-(4-(2-(3-Carbamimidoylguanidino)ethoxy)-2-methylbenzyl)phenyl)acetamide (15).** A mixture of 13 (32.3 mg, 0.16 mmol) and dicyandiamide (13.5 mg, 0.16 mmol) was heated to 160 °C for 100 min. The reaction mixture initially melts, then re-solidifies. The reaction was cooled to room temperature. The crude product was purified by crystallization from EtOH/Et$_2$O. Brown oil. (52% yield). $^1$H NMR (CD$_3$OD): δ 2.09 (s, 3H, CH$_3$); 2.15 (s, 3H, COCH$_3$); 3.60 (t, 2H, $J = 5.2$ Hz; CH$_2$); 3.86 (s, 2H, CH$_2$); 4.05 (t, 2H, $J = 5.2$ Hz; CH$_2$); 6.65-6.77 (m, 3H, Ar); 7.01 (d, 2H, $J = 8.0$ Hz, Ar); 7.41 (d, 2H, $J = 8.0$ Hz, Ar) ppm. $^{13}$C NMR (CD$_3$OD): δ 171.60, 162.12, 161.01, 158.56, 139.04, 138.20, 137.63, 133.00, 131.94, 129.80, 121.34, 117.63, 112.73, 67.55, 42.15, 38.94, 23.74, 19.97 ppm. Anal. (C$_{20}$H$_{26}$N$_6$O$_2$) C, H, N. % Calcd: 62.81 (C); 6.85 (H); 21.97 (N). % Found: 62.95 (C); 6.73 (H); 21.76 (N).

**4-(4-(2-(3-Carbamimidoylguanidino)ethoxy)-2-methylbenzyl)aniline (16).** To a solution of the amide 15 (0.57 mmol) in MeOH was added dropwise an aqueous solution of HCl 1N (0.2 mL); the
resulting solution was refluxed for 2h, then, after cooling, the solvent was evaporated. The residue was purified by precipitation from EtOH/Et₂O. Brown oil. (60% yield). ¹H NMR (CD₃OD): δ 2.17 (s, 3H, CH₃); 3.79 (t, 2H, J = 4.8 Hz; CH₂); 3.99 (s, 2H, CH₂); 4.21 (t, 2H, J = 4.8 Hz; CH₂); 6.79 (dd, 1H, J = 2.6, 8.2, Ar); 6.83 (d, 1H, J = 2.6 Hz, Ar); 7.08 (d, 1H, J = 8.2 Hz, Ar), 7.26 (d, 2H, J = 8.6 Hz, Ar), 7.32 (d, 2H, J = 8.6 Hz, Ar) ppm. Anal. (C₁₈H₂₄N₆O) C, H, N. % Calcd: 63.51 (C); 7.11 (H); 24.69 (N). % Found: 63.72 (C); 7.05 (H); 24.53 (N).

4-(Chloromethyl)-2-methyl-1-(4-nitrobenzyl)benzene (21). To a solution of alcohol 17 (96.0mg, 0.35 mmoli) in CHCl₃ at 0°C was added SOCl₂ (3.50 mmol; 0.25 mL). The reaction mixture was stirred for 2 h at room temperature, then, the solvent was evaporated. The residue was dissolved in H₂O and alkalized with NaOH 1N; and the aqueous layer was extracted with DCM. The organic phase was dried and the solvent was evaporated to give the corresponding crude 21, which was used without further purification. Yellow oil. (80% yield). ¹H NMR (CDCl₃): δ 2.22 (s, 3H, CH₃); 4.08 (s, 2H, CH₂); 4.57 (s, 2H, CH₂); 7.09 (d, 1H, J = 7.6 Hz, Ar); 7.19-7.24 (m, 2H, Ar); 7.26 (d, 2H, J=7.4 Hz, Ar); 8.13 (d, 2H, J = 7.4 Hz, Ar) ppm. Anal. (C₁₅H₁₄ClNO₂) C, H, N. % Calcd: 65.34 (C); 5.12 (H); 5.08 (N). % Found: 65.52 (C); 5.03 (H); 5.24 (N).

5-(Chloromethyl)-1,3-dimethyl-2-(4-nitrobenzyl)benzene (22). Compound 22 was synthesized from 18 (224 mg, 0.87 mmol) and SOCl₂ (0.63 mL, 8.70 mmol) in CHCl₃ (25 mL) following the same procedure described above for the preparation of 21. The crude was used without further purification. White oil. (76% yield). ¹H NMR (CDCl₃): δ 2.22 (s, 6H, CH₃); 4.14 (s, 2H, CH₂); 4.56 (s, 2H, CH₂); 7.13 (s, 2H, Ar); 7.15 (d, 2H, J = 8.5 Hz Ar); 8.11 (d, 2H, J = 8.5 Hz Ar) ppm. Anal. (C₁₆H₁₆ClNO₂) C, H, N. % Calcd: 66.32 (C); 5.57 (H); 4.83 (N). % Found: 66.49 (C); 5.59 (H); 5.11 (N).

4-(Chloromethyl)-2-methyl-4′-nitro-1,1′-biphenyl (23). Compound 23 was synthesized from 19 (125 mg, 0.51 mmol) and SOCl₂ (0.37 mL, 5.10 mmol) in CHCl₃ (25 mL) following the same procedure described above for the preparation of 21. The crude was used without further purification. Yellow
oil. (83% yield). \( ^1 \)H NMR (CDCl\(_3\)): \( \delta \) 2.27 (s, 3H, CH\(_3\)); 4.61 (s, 2H, CH\(_2\)); 7.21 (d, 2H, \( J = 7.8 \) Hz, Ar); 7.31 (d, 1H, \( J = 7.8 \) Hz, Ar); 7.34 (s, 1H, Ar); 7.48 (d, 2H, \( J = 8.8 \) Hz, Ar); 8.29 (d, 2H, \( J = 8.8 \) Hz Ar) ppm. Anal. (C\(_{14}\)H\(_{12}\)ClNO\(_2\)) C, H, N. % Calcd: 64.25 (C); 4.62 (H); 5.35 (N). % Found: 63.91 (C); 4.68 (H); 5.40 (N).

4-(Chloromethyl)-2,6-dimethyl-4'-nitro-1,1'-biphenyl (24). Compound 24 was synthesized from 20 (134 mg, 0.52 mmol) and SOCl\(_2\) (0.38 mL, 5.20 mmol) in CHCl\(_3\) (25 mL) following the same procedure described above for the preparation of 21. The crude was used without further purification. White oil. (81% yield). \( ^1 \)H NMR (CDCl\(_3\)): \( \delta \) 2.02 (s, 6H, CH\(_3\)); 4.58 (s, 2H, CH\(_2\)); 7.17 (s, 2H, Ar)); 7.33 (d, 2H, \( J = 8.8 \) Hz Ar); 8.31 (d, 2H, \( J = 8.8 \) Hz Ar) ppm. Anal. (C\(_{15}\)H\(_{14}\)ClNO\(_2\)) C, H, N. % Calcd: 65.34 (C); 5.12 (H); 5.08 (N). % Found: 65.40 (C); 5.59 (H); 5.31 (N).

2-(4-(4-Aminobenzyl)-3-methylphenyl)acetonitrile (25). To a solution of chloro compound 21 (78.0 mg; 0.46 mmol) in CH\(_3\)CN (0.62 mL) was added NaCN (45.1 mg; 0.92 mmol) in H\(_2\)O (0.21 mL). The mixture was submitted to microwave irradiation (150W, 100°C, 20 min). After cooling the solution was extracted with DCM. Organic phase was dried and evaporated to dryness. Yellow oil. (86% yield). \( ^1 \)H NMR (CDCl\(_3\)): \( \delta \) 2.22 (s, 3H, CH\(_3\)); 3.71 (s, 2H, CH\(_2\)); 4.07 (s, 2H, CH\(_2\)); 7.09-7.18 (m, 3H, Ar); 7.25 (d, 2H, \( J = 8.6 \) Hz, Ar); 8.13 (d, 2H, \( J = 8.6 \) Hz, Ar) ppm. Anal. (C\(_{16}\)H\(_{14}\)N\(_2\)O\(_2\)) C, H, N. % Calcd: 72.16 (C); 5.12 (H); 10.52 (N). % Found: 72.34 (C); 5.02 (H); 10.65 (N).

2-(3,5-Dimethyl-4-(4-nitrobenzyl)phenyl)acetonitrile (26). Compound 26 was synthesized from 22 (193 mg, 0.70 mmol) and NaCN (68.5 mg, 1.40 mmol) in CH\(_3\)CN/H\(_2\)O (0.94 mL/0.32 mL) following the same procedure described above for the preparation of 25. The crude was used without further purification. White oil. (70% yield). \( ^1 \)H NMR (CDCl\(_3\)): \( \delta \) 2.22 (s, 6H, CH\(_3\)); 3.70 (s, 2H, CH\(_2\)); 4.13 (s, 2H, CH\(_2\)); 7.06 (s, 2H, Ar); 7.14 (d, 2H, \( J = 8.6 \) Hz Ar); 8.11 (d, 2H, \( J = 8.6 \) Hz Ar) ppm. Anal. (C\(_{17}\)H\(_{16}\)N\(_2\)O\(_2\)) C, H, N. % Calcd: 72.84 (C); 5.75 (H); 9.99 (N). % Found: 72.60 (C); 5.60 (H); 10.02 (N).
2-(2-Methyl-4’-nitro-[1,1’-biphenyl]-4-yl)acetonitrile (27). Compound 27 was synthesized from 23 (95.0 mg, 0.61 mmol) and NaCN (59.8 mg, 1.22 mmol) in CH$_3$CN/H$_2$O (0.82 mL/0.30 mL) following the same procedure described above for the preparation of 25. The crude was used without further purification. Yellow oil. (86% yield). $^1$H NMR (CDCl$_3$): δ 2.28 (s, 3H, CH$_3$); 3.78 (s, 2H, CH$_2$); 7.21-7.31 (m, 3H, Ar); 7.47 (d, 2H, $J = 7.0$ Hz, Ar); 8.29 (d, 2H, $J = 7.0$ Hz Ar) ppm. Anal. (C$_{15}$H$_{12}$N$_2$O$_2$) C, H, N. % Calcd: 71.42 (C); 4.79 (H); 11.10 (N). % Found: 71.41 (C); 4.80 (H); 11.15 (N).

2-(2,6-Dimethyl-4’-nitro-[1,1’-biphenyl]-4-yl)acetonitrile (28). Compound 28 was synthesized from 24 (101 mg, 0.36 mmol) and NaCN (35.3 mg, 0.72 mmol) in CH$_3$CN/H$_2$O (0.49 mL/0.16 mL) following the same procedure described above for the preparation of 25. The crude was used without further purification. Pale yellow oil. (81% yield). $^1$H NMR (CDCl$_3$): δ 2.02 (s, 6H, CH$_3$); 3.73 (s, 2H, CH$_2$); 7.10 (s, 2H, Ar); 7.32 (d, 2H, $J = 8.6$ Hz Ar); 8.31 (d, 2H, $J = 8.6$ Hz Ar) ppm. Anal. (C$_{16}$H$_{14}$N$_2$O$_2$) C, H, N. % Calcd: 72.16 (C); 5.30 (H); 10.52 (N). % Found: 72.40 (C); 5.59 (H); 10.31 (N).

2-(4-(4-Aminobenzyl)-3-methylphenyl)acetonitrile (29). A solution of 25 (136 mg, 0.5 mmol) in AcOH (0.5mL) was hydrogenated in the presence of 10% Pd-C (28.3 mg), for 12 h. Then the catalyst was filtered off, and the solvent was removed to dryness to give a crude product that was used in the subsequent step without any further purification. Yellow oil. (80% yield). $^1$H NMR (CDCl$_3$): δ: 2.24 (s, 3H, CH$_3$); 3.68 (s, 2H, CH$_2$); 6.61 (d, 2H, $J = 8.4$ Hz, Ar); 6.88 (d, 2H, $J =8.4$ Hz, Ar); 7.06-7.12 (m, 3H, Ar) ppm. Anal. (C$_{16}$H$_{16}$N$_2$) C, H, N. % Calcd: 81.32 (C); 6.82 (H); 11.85 (N). % Found: 81.45 (C); 6.73 (H); 11.98 (N).

2-(4-(4-Hydroxybenzyl)-3-methylphenyl)acetonitrile (30). To a mixture of aniline derivative 29 (63.0 mg, 0.26 mmol) in H$_2$O was added dropwise with H$_2$SO$_4$$_{con}$ (0.06 mL) and the mixture was stirred at room temperature for 20min. Then a solution NaNO$_2$ (17.9 mg, 0.26 mmol) in H$_2$O (0.19 ml) was added dropwise to the reaction mixture. The resulting solution was stirred for 1 h at 100°C.
The mixture was cooled to room temperature, and the residue diluted with AcOEt and washed with brine. The collected organic layers were dried and evaporated to give compound 30 that was directly used in the next step. Yellow oil. (60% yield). $^1$H NMR (CDCl$_3$) δ: 2.22 (s, 3H, CH$_3$); 3.84 (s, 2H, CH$_2$); 4.06 (s, 2H, CH$_2$); 7.14-7.19 (m, 3H, Ar); 7.27-7.33 (m, 4H, Ar) ppm. Anal. (C$_{16}$H$_{15}$NO) C, H, N. % Calcd: 80.98 (C); 6.37 (H); 5.90 (N). % Found: 80.72 (C); 6.51 (H); 5.84 (N).

5-Methoxy-1,3-dimethyl-2-(4-nitrobenzyl)benzene (31). To a solution of 4-methoxy-2,6-dimethylphenylboronic acid (473 mg, 2.63 mmol) in acitone/H$_2$O 1:1 (4 mL) was added, under nitrogen flux, p-nitrobenzyl bromide (569 mg, 2.63 mmol), K$_2$CO$_3$ (1.24 g, 6.58 mmol) and a catalytic amount of PdCl$_2$. The resulting mixture was stirred at r.t. for 62 h in a sealed vial. The crude mixture was evaporated and then diluted with water and extracted with Et$_2$O. The organic phase was dried over sodium sulfate and concentrated under vacuum. The crude residue was purified by column chromatography Petroleum Ether /AcOEt (99:1), affording 31. Yellow oil. (49% yield). $^1$H NMR (CDCl$_3$): δ 2.19 (s, 6H, CH$_3$); 3.80 (s, 3H, OCH$_3$); 4.08 (s, 2H, CH$_2$); 6.65 (s, 2H, Ar); 7.16 (d, 2H, J = 8.8 Hz, Ar); 8.09 (d, 2H, J = 8.8 Hz, Ar) ppm. Anal. (C$_{16}$H$_{17}$NO$_3$) C, H, N. % Calcd: 70.83 (C); 6.32 (H); 5.16 (N). % Found: 71.01 (C); 6.12 (H); 5.00 (N).

3,5-Dimethyl-4-(4-nitrobenzyl)phenol (32). A solution of 31 (0.39 mmol) in anhydrous DCM (1.5 mL) was cooled to −78 °C and treated dropwise with a solution of BBr$_3$ in DCM (3.94 mL, 1.24 mmol); the resulting solution was stirred at the same temperature for 5 min and at 0 °C for 1 h. The mixture was then diluted with water and extracted with DCM. Organic phase was dried and evaporated to give the product 32. Pale yellow oil. (64% yield). $^1$H NMR (CDCl$_3$): δ 2.16 (s, 6H, CH$_3$); 4.06 (s, 2H, CH$_2$); 6.59 (s, 2H, Ar) 7.16 (d, 2H, J = 8.8 Hz, Ar); 8.10 (d, 2H, J = 8.8 Hz, Ar) ppm. Anal. (C$_{15}$H$_{15}$NO$_3$) C, H, N. % Calcd: 70.02 (C); 5.88 (H); 5.44 (N). % Found: 70.00 (C); 5.91 (H); 5.63 (N).
2-(3,5-Dimethyl-4-(4-nitrobenzyl)phenoxy)acetonitrile (33). To a mixture of cesium carbonate (725 mg, 2.22 mmol) and phenol derivative 32 (0.44 mmol) in 50 mL of DMF was added BrCH₂CN (0.03 mL, 0.44 mmol). The reaction mixture was stirred for 30 min at r.t., poured into 100 mL of cold HCl 1N and extracted with AcOEt. The organic phase was dried and evaporated to give the product 33. White oil. (98% yield). ¹H NMR (CDCl₃): δ 2.22 (s, 6H, CH₃); 4.10 (s, 2H, CH₂); 4.77 (s, 2H, CH₂CN); 6.73 (s, 2H, Ar); 7.15 (d, 2H, J = 8.8, Ar); 8.11 (d, 2H, J = 8.8 Hz, Ar) ppm. Anal. (C₁₇H₁₆N₂O₃) C, H, N. % Calcd: 68.91 (C); 5.44 (H); 9.45 (N). % Found: 68.65 (C); 5.52 (H); 9.65 (N)

N-(4-(4-(Hydroxymethyl)-2-methylbenzyl)-2-isopropylphenyl)acetamide (35). To a solution of trifluoro(4-(hydroxymethyl)2-methylphenyl)borane salt (509 mg; 2.38 mmol) in dioxane/H₂O 9:1 (4 mL) was added, under nitrogen atmosphere, N-(4-(bromomethyl)-2-isopropylphenyl)acetamide 34 (513 mg; 2.38 mmol), cesium carbonate (2.30 g; 7.1 mmol) and PdCl₂dppf (34.8 mg; 0.05 mmol). The resulting mixture was stirred at 95°C for 24 h in a sealed vial. The crude mixture was evaporated and then diluted with water and extracted with DCM. The organic phase was dried over sodium sulfate and the solvent removed. The crude product was chromatographed on a silica gel column, eluting with Petroleum Ether/AcOEt (9:1). Brown oil. (50% yield). ¹H NMR (CDCl₃): δ 2.19 (s, 6H, CH₃); 2.26 (s, 3H, CH₃); 2.26 (s, 3H, CH₂CO); 2.96-3.03 (m, 1H, CH); 3.94 (s, 2H, CH₂); 4.65 (s, 2H, CH₂); 6.90 (dd, 1H, J = 1.6, 8.0 Hz, Ar), 7.05-7.09 (m, 2H, Ar); 7.13 (d, 1H, J = 8.0 Hz, Ar); 7.17 (s, 1H, Ar); 7.47 (d, 1H, J = 8.0 Hz) ppm. Anal. (C₂₀H₂₅NO₂) C, H, N. % Calcd: 77.14 (C); 8.09 (H); 4.50 (N). % Found: 77.39 (C); 8.21 (H); 4.64 (N).

N-(4-(4-Chloromethyl)-2-methylbenzyl)-2-isopropylphenyl)acetamide (36). Compound 36 was synthesized from 35 (208 mg, 0.67 mmol) and SOCl₂ (0.49 mL, 6.70 mmol) in CHCl₃ (25 mL) following the same procedure described above for the preparation of 21. The crude was used without any further purification. Brown oil. (70% yield). ¹H NMR (CDCl₃): δ 1.20 (d, 6H, J = 6.8 Hz, CH₃); 2.19 (s, 3H, CH₃); 2.26 (s, 3H, CH₂CO); 2.96-3.03 (m, 1H, CH); 3.93 (s, 2H, CH₂); 4.57
(s, 2H, CH₂); 6.90 (d, 1H, J = 6.0 Hz, Ar); 7.02-7.09 (m, 2H, Ar); 7.15 (d, 1H, J = 8.4 Hz, Ar); 7.19 (s, 1H, Ar); 7.48 (d, 1H, J = 8.4 Hz) ppm. Anal. (C₂₀H₂₄ClNO) C, H, N. % Calcd: 72.82 (C); 7.33 (H); 4.25 (N). % Found: 72.61 (C); 7.42 (H); 4.13 (N).

N-(4-(4-(Cyanomethyl)-2-methylbenzyl)-2-isopropylphenyl)acetamide (37). Compound 37 was synthesized from 36 (152 mg, 0.46 mmol) and NaCN (45.3 mg, 0.92 mmol) in CH₃CN/H₂O (1.86 mL/0.63 mL) following the same procedure described above for the preparation of 25. The crude was used without any further purification. Yellow oil. (88% yield). ¹H NMR (CDCl₃): δ 1.20 (d, 6H, J = 6.8 Hz, CH₃); 2.19 (s, 3H, CH₃); 2.25 (s, 3H,CH₂CO); 2.96-3.03 (m, 1H, CH); 3.70 (s, 2H, CH₂); 3.93 (s, 2H, CH₂); 6.89 (d, 1H, J = 8.0 Hz, Ar), 7.03-7.09 (m, 3H, Ar); 7.12 (s, 1H, Ar); 7.48 (d, 1H, J=8.0 Hz, Ar) ppm. Anal. (C₂₁H₂₄N₂O) C, H, N. % Calcd: 78.71 (C); 7.55 (H); 8.74 (N). % Found: 78.85 (C); 7.41 (H); 8.90 (N).

Molecular modeling

All compounds were built, parameterised (Gasteiger-Huckel method) and energy minimised within MOE using MMFF94 forcefield [MOE: Chemical Computing Group Inc. Montreal. H3A2R7 Canada. http://www.chemcomp.comp]. For all compounds, the protonated form was considered for the in silico analyses.

Docking studies. Docking studies were performed starting from the in-house homology model of the murine TAAR1 receptor⁴, built on the X-ray structure of the human β2- adrenoreceptor (PDB ID: 3PDS)¹⁷, following a protocol we previously discussed exploring the binding mode of other TAAR1 ligands⁸,⁹. Briefly, the most promising compounds were docked into the putative ligand binding site by means of the Surflex docking module implemented in Sybyl-X1.0¹⁸.

Surflex-Dock uses an empirically derived scoring function based on the binding affinities of X-ray protein-ligand complexes. The final total score listed by Surflex-Dock is a weighted sum of non-linear functions involving van der Waals surface distances between the appropriate pairs of exposed
protein and ligand atoms, including hydrophobic, polar, repulsive, entropic and solvation and crash
terms represented in terms of a total score conferred to any calculated conformer. Then, for all the
compounds, the best docking geometries (selected on the basis of the SurFlex scoring functions)
were refined by ligand/receptor complex energy minimization (CHARMM27) by means of the
MOE software.

In vitro Biological studies

Reagents. All cell culture reagents and buffers were from Invitrogen (Carlsbad, CA) and Sigma (St.
Louis, MO). Coelenterazine h was purchased from Promega (Madison, WI). Plasmid containing the
cDNA for the mTAAR1 were generously donated from Hoffman-La Roche. EPAC cAMP BRET
sensor was produced as described19.

Cell Culture and BRET experiment

Human embryonic kidney 293 cells (HEK293T) were maintained in Dulbecco’s Modified Eagle’s
medium supplemented with 10% (vol/vol) of FBS, 2 mM l-glutamine and 0.05 mg/ml of
gentamicin at 37°C in a humidified atmosphere at 95% air and 5% CO₂. Transient transfections
were performed 24 h after cells seeding using lipofectamine 2000 protocol (Invitrogen). 5µg of
mTAAR1 and 4µg of EPAC for each milliliter of transfection solution were used for the
experiments. For the BRET experiments the cells were plated, 6 hours after transfection, in poly-D-
lysine coated 96-well microplates at a density of 70,000 cells per well in phenol red free Minimum
Essential Medium containing 2% of FBS, 10 mM Hepes, 2 mM L-glutamine. The cells were then
cultured for an additional 24 h. BRET experiment was conducted as already described7. Briefly, for
time course experiments, the plate was read immediately after the addition of the agonist and for
approximately 20 minutes. All the compounds were tested for screening at the initial concentration
of 10 µM. Then, for active compounds, a dose response was performed, in order to calculate the
EC₅₀ values. All the experiments were conducted in presence of the phosphodiesterase inhibitor
IBMX (Sigma) at the final concentration of 200 µM. Readings were collected using a Tecan Infinite
instrument that allows the sequential integration of the signals detected in the 465 to 505nm and 515 to 555 nm windows using filters with the appropriate band pass and by using iControl software. The acceptor/donor ratio was calculated as previously described \(^{20}\). Curve was fitted using a non-linear regression and one site specific binding with GraphPad Prism 5. Data are representative of 4-5 independent experiments and are expressed as means±SEM.

**Cell culture and glucose production evaluation.** Human hepatocellular carcinoma cells (HepG2), obtained from American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 1 mM pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO\(_2\) and sub-cultured before confluence. To assess glucose release, HepG2 were seeded into six-well plates (5x10\(^5\) cells/well) and grown to 80% of confluence with standard medium. As previously described\(^9\), before treatment cells were washed twice with PBS and then exposed for 4 h to test compounds (1 and 2) (0.1, 1 and 10 \(\mu\)M) in 1 ml DMEM base, glucose- and phenol red-free, containing 100 U/ml penicillin,100 mg/ml streptomycin, and 4 mM L-glutamine, supplemented with 2 mM sodium pyruvate and 20 mM sodium lactate (glucose production buffer) at 37 °C in 5% CO\(_2\). Exogenous T1AM (0.1, 1 and 10 \(\mu\)M) was used as control. Control cells were incubated with supplemented DMEM containing DMSO (1–2 \(\mu\)l/well). Cell culture medium was then collected and glucose concentration was measured with a colorimetric glucose assay kit (GAHK-20, Sigma-Aldrich), following manufacturer’s instruction. Glucose concentrations were referred to the total protein content (Bradford 1976) of whole HepG2 lysates. Results are expressed as the mean ± S.E.M. Differences between groups were analyzed by ANOVA. The threshold of statistical significance was set at \(P<0.05\). GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for data processing and statistical analysis.

**Measurement of plasma glycaemia**
This investigation and animal use procedure complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23, revised 1996) and were approved by the Animal Care Committee of the Department of Pharmacology, University of Florence, in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Glycaemia was monitored in blood collected from the tail vein of 4 h fasted male mice (CD1 strain, 20-30g, from Envigo, Italy), who had received 1 and 2 (1.32, 4 and 11 µg.kg-1 i.p.) or saline (i.p.) (n = 8 in each group). Glycaemia was evaluated by a glucorefractometer 15 min after the i.p. injections, as described4. Data are expressed as mean ± SEM of independent experiments. Statistical analysis was performed by oneway ANOVA, followed by Student–Newman–Keuls multiple comparison post hoc test; the threshold of statistical significance was set at P<0.05. Data analysis was performed by GraphPad Prism 6.0 statistical program (GraphPad software, San Diego, CA, USA). The acceptor/donor ratio was calculated as previously described20. Curve was fitted using a non-linear regression and one site specific binding with GraphPad Prism 5. Data are representative of 4-5 independent experiments and are expressed as means±SEM.

ASSOCIATED CONTENT

Supporting Information. Synthetic pathways and procedures for the preparation of compound 34 and derivatives 11-16. 1H NMR and 13C NMR spectra of final compounds. The Supporting Information is available free of charge on the ACS Publications website

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Notes

The authors declare no competing financial interest.
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ABBREVIATION USED

T1AM, 3-iodothyronamine; T0AM, thyronamine; β-PEA, β-phenylethylamine; mTAAR1, murine Trace-Amine Associated-Receptor 1;

References


TOC Graphic.