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Novel multi target-directed ligands targeting 5-HT₄ receptors with *in cellulo* antioxidant properties as promising leads in Alzheimer's disease

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KEYWORDS

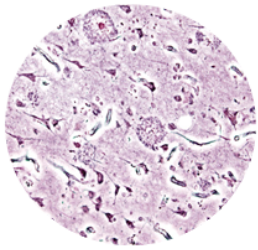
Alzheimer's Disease; 5-HT₄ receptor; antioxidant; phenol; MTDL.

Highlights

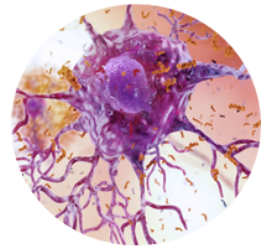
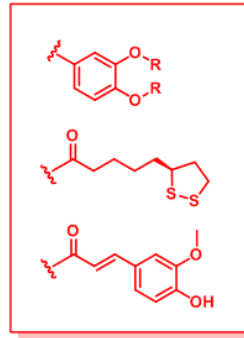
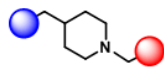
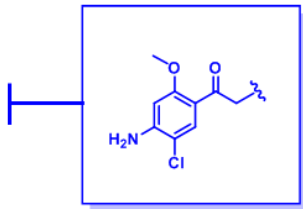
- Conception and synthesis of new MTDLs combining 5-HT4R agonist activity and antioxidant properties
- All compounds showed good %inhibition at 10⁻⁶M on 5-HT4R, with 9b and 9g showing K_i < 15 nM on 5-HT4R as well as agonist profile.
- In vitro and in cellulo antioxidant effects were evaluated
- 9b is the best compromise regarding the 5-HT4R activity and antioxidant properties

5-HT₄ R agonist

Antioxidant



Senile plaques
Formed by β -amyloid peptide



Oxidative stress

ABSTRACT

Facing the complexity of Alzheimer's disease (AD), it is now currently admitted that a therapeutic pleiotropic intervention is needed to alter its progression. Among the major hallmarks of the disease, the amyloid pathology and the oxidative stress are closely related. We propose in this study to develop original Multi-Target Directed Ligands (MTDL) able to impact at the same time A β protein accumulation and toxicity of Reactive Oxygen Species (ROS) in neuronal cells. Such MTDL were obtained by linking on a central piperidine two scaffolds of interest: a typical aminochlorobenzophenone present in numerous 5-HT₄R agonists, and diverse antioxidant chemotypes. Interestingly, the most active compound **9g** possesses a K_i of 12.7 nM towards 5-HT₄R and an antioxidant activity *in vitro* and *in cellulo*.

Introduction

Alzheimer's disease (AD) is the most common form of dementia and affects more than 50 million people worldwide.¹ More than a century after its discovery, AD is now characterized by synaptic dysfunction and synapse loss correlated with cognitive decline.² These dysfunction are supposed to be due to the presence of two peptide deposits in and around neuronal cells. The accumulation and aggregation of A β peptide is at the origin of the amyloid pathology whereas the neurofibrillary tangles are mainly composed of phosphorylated tau protein.³ These lesions are generally associated with mitochondria dysfunction, an increase inflammatory response and the presence of oxidative stress.⁴ The latter was further particularly linked to the presence of amyloid aggregates which can produce Reactive Oxygen Species (ROS) in the presence of metal ions and which ultimately leads to neuronal cell death.⁵ The control of ROS production is therefore of particular interest in the prevention or the treatment of AD.

Despite these progresses, the discovery and development of treatments with disease-modifying effect on AD remain one of the greatest challenges in drug discovery.⁶ After numerous failures in clinical trials with compounds targeting mainly amyloid⁷ or tau pathologies,⁸ it is now currently admitted that a pleiotropic approach is needed for this multifactorial neurodegenerative disease.⁹ In order to impact several biological targets at the same time, single pleiotropic compounds, known as Multi-Target Directed Ligands (MTDLs),^{10,11,12} have been developed during the last decade. Among them, many examples include compounds able to act on oxidative stress and on another target.^{13,14,15} On the other hand, many examples have been described in order to limit amyloid pathology with numerous examples of MTDL acting on β -secretase inhibition,¹⁶ or inhibiting A β aggregation.¹⁴ In addition to those classical targets, the serotonergic system appears

of particular interest, notably *via* the modulation of 5-HT₄ and 5-HT₆ receptors.^{17,18} The activation of 5-HT₄ receptors (5-HT₄R) with partial agonists, such as RS67333, is of particular interest as it can promote the non-amyloidogenic cleavage of Amyloid Protein Precursor (APP) and subsequently decrease the amyloid load in AD transgenic mice models.^{19,20} In this context, we have in the past modulated the structure of RS67333 in order to introduce another target of choice for the treatment of AD such as acetylcholinesterase (AChE) inhibitory activity^{21,22} or 5-HT₆R antagonism.²³ These two studies demonstrated that the replacement of the butyl chain of RS67333 by diverse alkyl moieties has limited impact on both the affinity and the pharmacological profile towards 5-HT₄R. A similar strategy has been recently followed in order to generate 5-HT₆R antagonists bearing antioxidant activities.²⁴

In this context, we would like to assess the possibility to introduce, on the central piperidine ring of the targeted structures (Figure 1), various chemical moieties known to exhibit antioxidant activities. We postulate that such MTDL would be of particular interest combining, for the first time in a unique compound, antioxidant properties as well as 5-HT₄R agonism. Several antioxidant scaffolds were chosen for this proof of concept: polyphenol,²⁵ hydroxycinnamic acid²⁶ and lipoic acid.^{27,28} Following this strategy, the two pharmacophores, will be connected through a permanent linker in order to optimize the central distribution of the antioxidant moiety. Indeed, the presence of the piperidine as a linker appears essential for the 5-HT₄R activity and the generated compounds should possess physicochemical properties for CNS application.

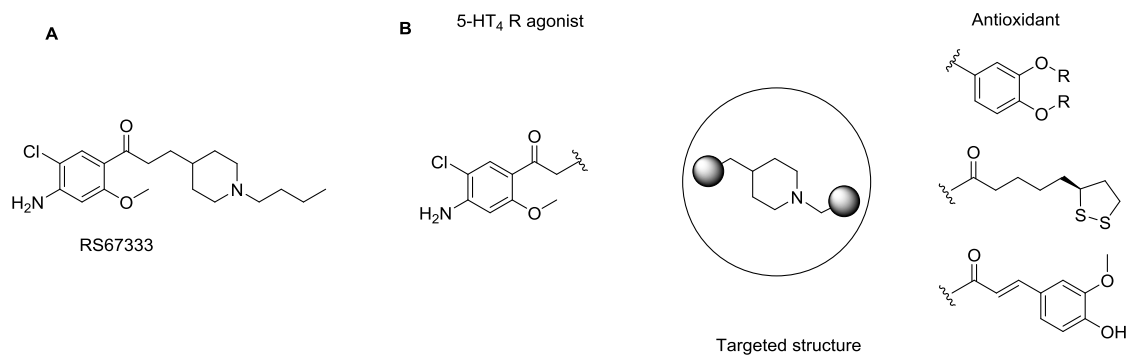


Figure 1. Chemical structure of RS67333 **A** and targeted structure of the MTDL **B**.

The influence of these substitutions on 5-HT₄R affinity and the antioxidant activity of the resulting compounds could thus be assessed *in vitro* and *in cellulo*.

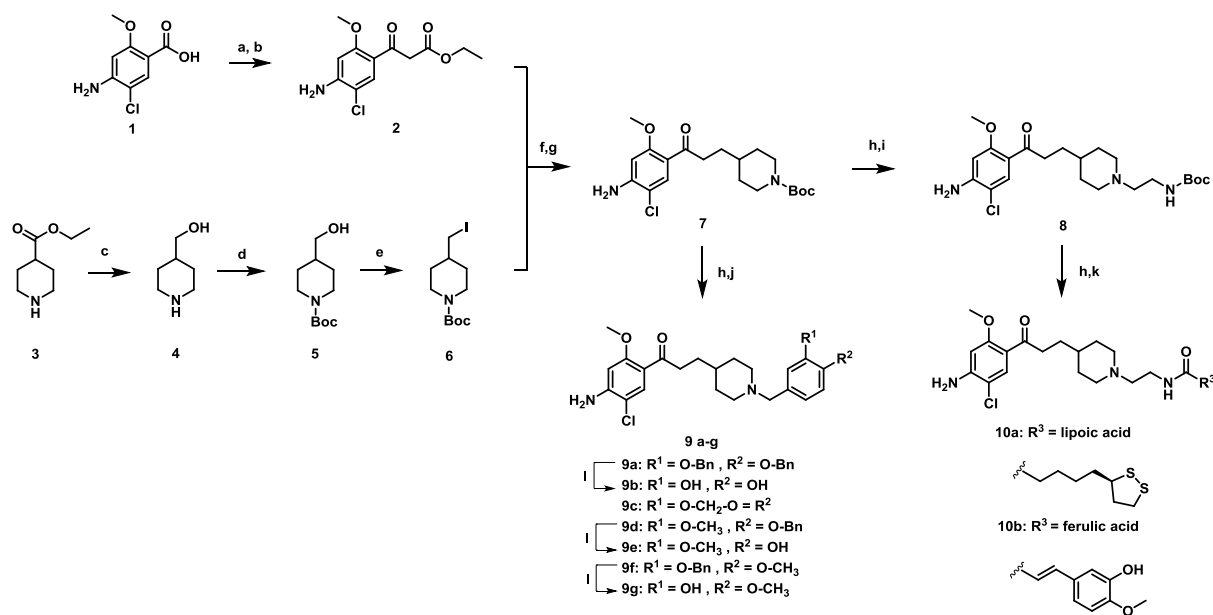
Results

Chemistry

According to our previous chemical studies, the precursor **7** appears as a central scaffold in the synthesis of RS67333.^{22,23} Compound **7** was synthesized in a three-step sequence through formation of a β -keto ester **2** by reaction between acid **1**, activated *in situ* with CDI, and potassium 3-ethoxy-3-oxopropanoate.^{29,30} Subsequent alkylation with a previously synthesized piperidine chain **6**, and followed by reaction with KOH in an EtOH/H₂O mixture provided a saponification-decarboxylation reaction to afford **7**.²² From this precursor **7**, different moieties were linked, such as catechol, vanillin or isovanillin widely known for their antioxidant properties.^{31,32} The first compounds **9a**, **9c**, **9d**, and **9f** were obtained by alkylation with the corresponding bromobenzyl derivatives at room temperature. A final debenzylation step was performed with HBr 33% in solution in acetic acid at 0°C to obtain free alcohols derivatives **9b**, **9e**, and **9g** respectively.

The second goal was to link ferulic and lipoic acid (LA) described as powerful antioxidants in the literature.^{33,34,35} In order to link LA and ferulic acid while maintaining the basicity of the nitrogen of the piperidine, an ethylamine linker was added. According to literature,²³ an ethylamine linker has no influence on the 5-HT₄R affinity and allowed the availability of a free terminal amine for peptidic coupling. Once the intermediate **8** was obtained, several coupling reactions were performed with EDCI, HOBt as activators, to synthesize coupled products **10a** and **10b**. Due to the sensitivity of LA to air, light, and heat, reactions were performed in dry, cold and dark conditions.

Scheme 1. Synthesis of the final derivatives **9-10**

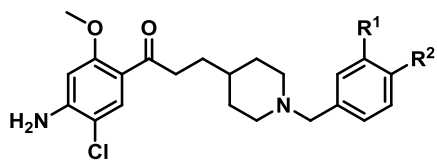


Reagents, conditions: (a) CDI, THF; (b) KO₂CCH₂CO₂Et, MgCl₂, THF, 40 ° C; (c) LiAlH₄, Et₂O, reflux; (d) (Boc)₂O, DCM; (e) PPh₃, I₂, Imidazole, THF; (f) K₂CO₃, DMF; (g) KOH, EtOH/H₂O; (h) TFA, DCM; (i) tert - butyl N - (2 - bromoethyl)carbamate, K₂CO₃, ACN ; (j) Bromobenzylalcohol, K₂CO₃, DCM ; (k) EDCl, HOBT, Et₃N, 0 ° C to rt, in the dark ; (l) HBr 33% in AcOH, DCM, 0 ° C..

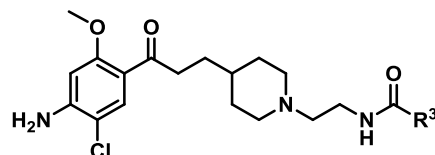
***In vitro* evaluation**

As previously mentioned, the main goal of our study was to discover a hit that will have sufficient antioxidant properties, as well as a good affinity toward 5-HT₄R. All the synthesized compounds, were tested on 5-HT₄R according to the Grossman et al method.³⁶ The affinity toward the receptor was evaluated using a displacement assay of the tritiated ligand [³H]-GR113808, a specific and highly potent 5-HT₄R antagonist used to label specific binding sites in human brain.

Table 1. Binding to h5-HT₄R and *in vitro* antioxidant activities of final derivatives



9 a-g



10a: R³ = lipoic acid

10b: R³ = ferulic acid

Cmpd	R ¹	R ²	R ³	cLogP*	<i>h5-HT₄R</i>				<i>DPPH</i>	<i>ORAC</i>
					% inhibition at 10 ⁻⁸ M	% inhibition at 10 ⁻⁶ M	K _i (nM)	IC ₅₀ (nM)	EC ₅₀ at 120 min (μM)	(μM _{compound} / μM Trolox®)
9a	O-Bn	O-Bn	-	6.78	3%	89%	-	-	>250	< 0.2
9b	OH	OH	-	3.48	76%	100%	5.9 ± 0.7	7.5 ± 0.7	19.6 ± 0.8	3.40 ± 0.02
9c	O-CH ₂ -O		-	3.84	22%	100%	-	-	>250	< 0.2
9d	O-CH ₃	O-Bn	-	5.55	0%	97%	-	-	>250	< 0.2
9e	O-CH ₃	OH	-	3.92	7%	95%	-	-	180.3 ± 25.5	3.88 ± 0.55
9f	O-Bn	O-CH ₃	-	5.62	7%	88%	-	-	>250	< 0.2
9g	OH	O-CH ₃	-	3.94	64%	100%	12.7 ± 2.1	14.5 ± 5.8	85.5 ± 2.0	3.41 ± 0.59
10a	-	-	Lipoic acid	4.98	26%	99%	-	-	>250	0.76 ± 0.11
10b	-	-	Ferulic acid	3.65	39%	100%	101 ± 8.0	101 ± 3.6	45.5 ± 2.0	1.47 ± 0.25
Serotonin	-	-	-	-	0%	65%	246 ± 31	323 ± 25	-	-
Trolox	-	-	-	-	-	-	-	-	22.9 ± 0.9	1
Quercetin	-	-	-	-	-	-	-	-	9.3 ± 1.5	6.50 ± 0.73

* calculated logP with ALOGPS 2.1.

Those compounds were also developed for their antioxidant properties and preliminary antioxidant *in vitro* test was also performed. Our attention was focused on two tests, DPPH, and ORAC.³⁷ The ORAC_{Fl} assay was conducted in an aqueous medium according to well-described procedure. It evaluates the antioxidant activity via a Hydrogen Atom Transfert (HAT) mechanism. Calculating the AUC of the fluorescence decrease for 1.5 hours, quantitative and kinetic aspects are both taken into account. Here, phenol gifted compounds showed a moderate antioxidant activity compared with a TEAC ranged between Trolox[®] (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid), the reference, and quercetin (Table 1), a natural polyphenol antioxidant. The DPPH assay was performed in an organic solvent and enabled the evaluation of antioxidant activity following a Single-Electron-Transfer (SET) mechanism. According to recent literature, our initial procedure was completed here by slight modifications to ensure a complete evaluation of our compounds. First calculation of the specific absorbance of DPPH subtracting the absorbance of others components in the solution was realized. The maximum effect was normalized using a high dose of Trolox[®] (375 μ M) to get the absolute EC₅₀.³⁸ The kinetic analysis to reach the plateau of % DPPH RSA at infinite time was determined and EC₅₀ were calculated using linear or non-linear regression ($R^2 > 0.98$) from the dose-effect curves.

In these conditions, Trolox[®] and quercetine reached their maximal antioxidant activity very quickly (immediately and few minutes respectively) compared to other active compounds ($T_{1/2}$ about 30 min). Their dose-effect curve was a linear ($R^2 > 0.99$). In our phenol series, when DPPH EC₅₀ was calculable, EC₅₀ values obtained after 120 min were very spread from 19.6 to >250 μ M, varying as a terminal chemical pattern.

In cellulo evaluation

The pharmacological profile of the selected compounds was assayed towards h5-HT₄R transiently expressed in COS-7 cells (Figure 2). Three compounds behaved as partial agonists of the receptor with EC₅₀ of 2.8 ± 1.3 nM, 1.4 ± 0.8 nM and 4.5 ± 2.1 nM for **9b**, **9g** and **10b**, respectively (Figure 4). Compound **10a** also behaved as a partial agonist of the 5-HT₄R, but its irregular profile suggested that it may engage other targets in the COS-7 and no clear pharmacological profile was established. Compound **10b** presented an E_{max} of $47 \pm 8\%$ that is similar to the one of RS67333, the 5-HT₄R agonist of reference. Compounds **9b** and **9g** showed greater efficacy than the reference to induce cAMP production with E_{max} of $72 \pm 10\%$ and $57 \pm 3\%$, respectively.

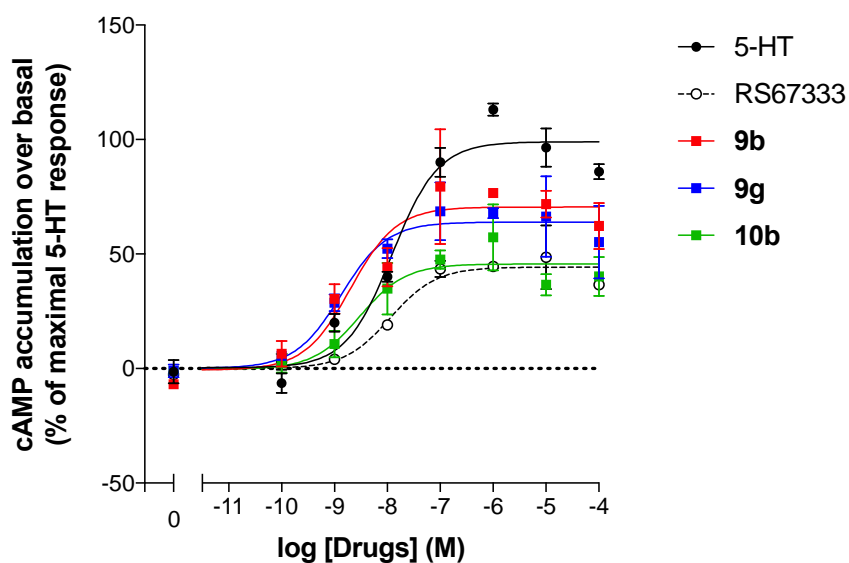


Figure 2. Determination of the 5-HT₄R pharmacological profiles for compounds **9b**, **9g** and **10b**, by quantification of cAMP accumulation. The agonism activity of the compounds was determined with serotonin (5-HT) and the partial agonist RS67333 as references. Data presented are the mean of three independent experiments performed in duplicate.

The first *in vitro* antioxidant assays allowed us to select **9b**, **9g**, **10a-b**, to complete their antioxidant evaluation in a cellular context. To define the suitable concentration, the cytotoxicity of **9b**, **9g**, **10a-b** was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay³⁹ in a neuroblastoma cell line, SH-SY5Y. As seen on Figure 3, treatment of the cells with compounds **9b**, **9g**, **10a** (0.5 to 10 μ M) did not show cytotoxicity. A little decrease of 12% of viability compared to control cells was seen with compound **10b**. While at a dose of 50 μ M all compounds seem to show a small cytotoxicity with a decreasing of 37%, 40%, 48%, 68% for **9b**, **9g**, **10a**, **10b** respectively. Therefore, a single dose of 10 μ M was chosen to perform the *in cellulo* antioxidant assay.

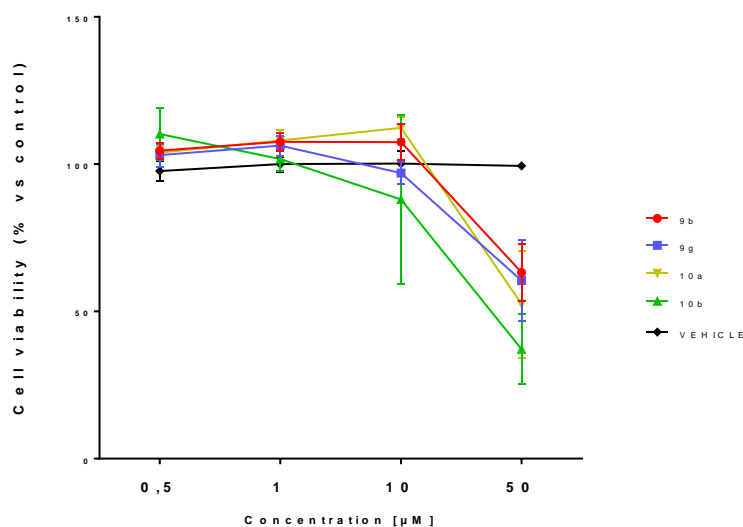


Figure 3 Figure 3 Cell viability determined by MTT assay. Cells were grown into 96-well plates and treated with **9b**, **9g**, **10a** and **10b** for 24 hours at the concentration of 0.5, 1, 10 and 50 μ M or vehicle (DMSO). Data are presented as percentage of viable cells in comparison with vehicle treated controls. Error bars indicate \pm SEM.

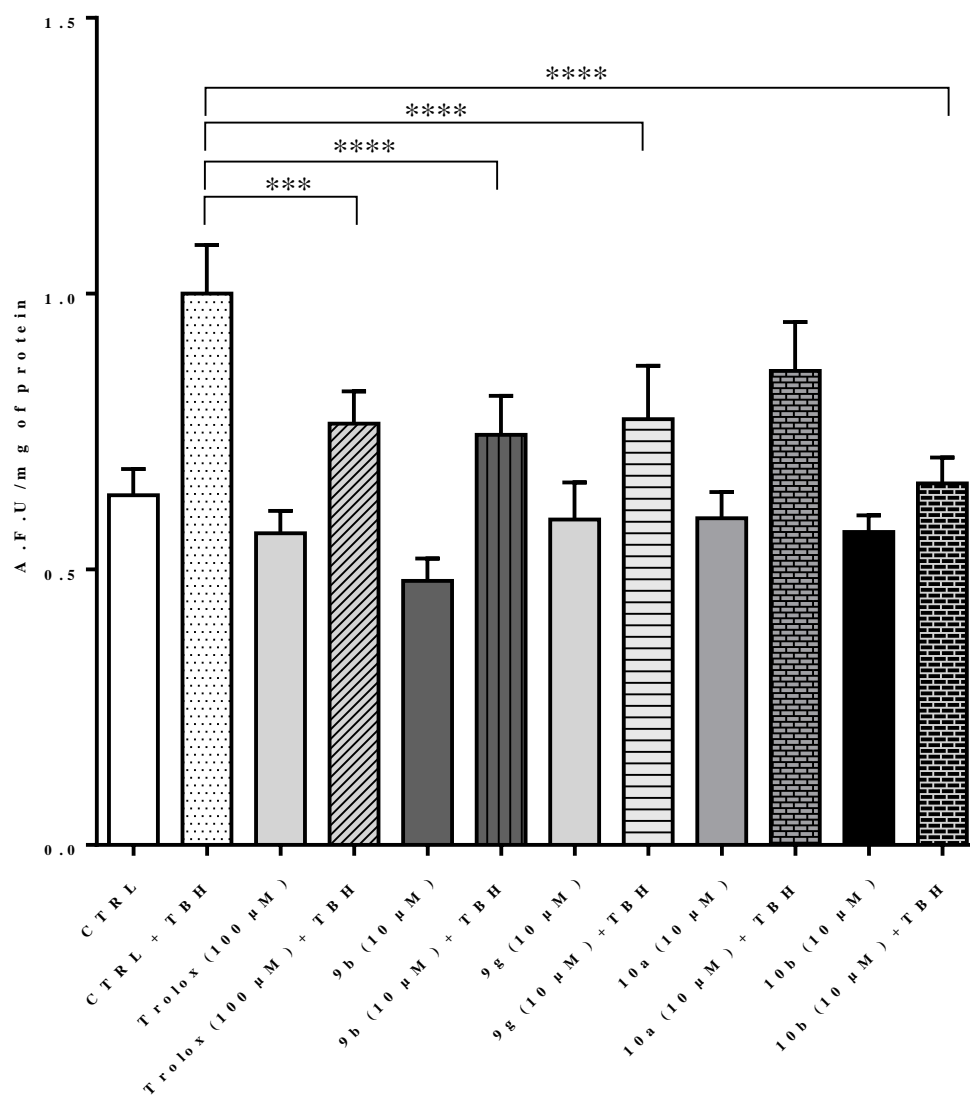


Figure 4 Reactive oxygen species determination in live SH-SY5Y cells. Cells were grown in 96 wells plates and incubated for 24 hours with compounds **9b**, **9g**, **10a** and **10b**, Trolox or vehicle (CTRL). Oxidative stress was induced by treating the cells with 100μM TBH for 30 minutes and detected by staining the cells with the fluorogenic probe H₂DCF-DA. Data are presented as Arbitrary Fluorescence Units normalized on protein content measured by Lowry method. Error bars indicate ± SEM. *** P < 0.001, **** P < 0.0001

The antioxidant activity of selected compounds was evaluated on live SH-SY5Y cells using the fluorogenic probe H₂DFCDA. The cell-permeant H₂DFCDA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterases. Upon oxidation by

ROS, the nonfluorescent H₂DCF is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) as described by Wang in 1999.⁴⁰ The level of oxidative stress is directly correlated with the intensity of DCF fluorescence.

To evaluate the antioxidant activity, cells were incubated for 24 hours with the selected compounds, Trolox or vehicle. Then cells were incubated with 10 μ M H₂DCFDA probe for 30 minutes. After this time the cells were carefully washed with PBS and the oxidative stress was induced by 30 minutes treatment with tert-butyl hydroperoxide (t-BuOOH or TBH). The fluorescence intensity was normalized on protein content (Figure 4).

Discussion

We have demonstrated in our previous study²² that the introduction of a non-substituted benzyl group instead of the butyl group present in RS67333 has little impact on affinity towards the 5-HT₄R and on Acetylcholinesterase inhibition. Based on these results a first series of hydroxyl or methoxy substituted benzylated derivatives was generated. In a previous study, we have demonstrated that the replacement of the butyl substituent of the piperidine of RS67333 by benzyl or methylenecyclohexyl moiety leads to potent AChE inhibitors with good physicochemical properties and in vivo promnesiant activities.²² In order to verify the impact of these modulations on drugability parameters, cLogP were determined. If the protected benzylated compounds **9a**, **9d** and **9f** appear particularly lipophilic, all final compounds are compatible with optimal CNS parameters.⁴¹ If benzyl piperidine scaffolds are well known pharmacophores for cholinesterase interaction,⁴² we have verified that the RS67333 analogs were selective towards butyrylcholinesterase.²² Docking studies in AChE binding site revealed that the orientation of those ligands was similar to Donepezil and reproduced its interactions. In particular, the benzyl

moiety is located at the bottom of the aromatic gorge of AChE and engaged in a hydrophobic interaction with Trp86. We postulate that the introduction of polar or bulky substituents on the benzyl group of our final compounds will be detrimental for their activities. In order to verify this hypothesis, all final compounds **9a-g** and **10a-b** were evaluated as inhibitors of human AChE. As expected, these modulations were particularly detrimental for the affinity of the ligands towards AChE (See Table S1 in Supporting Informations). Concerning the impact of these modulations on 5-HT₄R activity, as shown in Table 1, all compounds exhibited good inhibition rates at 10⁻⁶ M. However, the benzyl protecting group present on the scaffold of compounds **9a**, **9d** and **9f** has detrimental impact on affinity towards 5-HT₄R receptors probably due to its steric hindrance. On the contrary, smaller substituents lead to enhanced activity. In this assay only **9b**, **9g**, and **10b** exhibited more than 35% of inhibition rate at 10⁻⁸ M allowing us to calculate further data such as IC₅₀ and K_i. Surprisingly the vanillin derivative **9e** showed a decreased potency compared to the isovanillin analog **9g**. On the contrary, **9b** and **9g** are really promising with K_i values under 15 nM. According to our hypothesis, compounds **9b**, **9g** and **10a** behave as partial agonist for the 5-HT₄R, the first two being able to induce cAMP production with a greater efficiency than the reference RS67333 (Figure 2).

All compounds were then engaged in two complementary *in vitro* antioxidant biological assays. In the DPPH assay, a SET mechanism test, hydroxylated compounds **9b**, **9e**, **9g** and **10b** were the only one to exert antioxidant activity, the catechol **9b** being the most potent. In this assay, the lipoic acid derivative **10a** did show limited antioxidant activity. In the ORAC evaluation, the antioxidant activities of all compounds were compared to Trolox[®], a widely used antioxidant reference compound. The same compounds **9b**, **9e** and **9g** appeared to be 3-fold more potent than Trolox[®], whereas ferulic derivative **10g** demonstrated similar activity than the reference. Both

test confirm that the *in vitro* activity is clearly linked to the introduction of a phenol group as all benzylated compounds are inactive in this assay.

Based on the results we have chosen to test the *in cellulo* antioxidant activity of these compounds **9b**, **9g**, **10a** and **10b**. Compound **9e** was not selected because of its lower affinity towards 5-HT₄R. As already demonstrated for prucalopride, another 5-HT₄R agonist, we have chosen to evaluate this antioxidant and neuroprotective activities in SH-SY5Y neuroblastoma cell line.⁴³ Lipoic acid antioxidant activity is dependent of mitochondrial activity⁴⁴ and therefore cannot be highlighted by an *in vitro* assay. For that reason, **10a** was also selected despite no sign of *in vitro* antioxidant effect.

A non-toxic dose of 10µM was chosen for the *in cellulo* antioxidant activity assay, after a general MTT viability assay. Oxidative stress was generated using TBH and the ability of the compounds to limit ROS induction was compared with the well-known antioxidant compound Trolox®.⁴⁵

Trolox® was used at 100 µM and, as expected, exerts a significant reduction of ROS production. Used at a concentration of 10µM in this assay, compounds **9b**, **9g** and **10b** used at a concentration of 10µM significantly decreased ROS production by at least 50% compared to the control group. However, no activity was obtained in this assay with the lipoic acid substituted compound **10a**.

These results are in accordance with the pharmacological profile of the compounds on 5-HT₄R, towards which no clear profile was established for compound **10a**. The three other compounds demonstrated clear agonism toward the receptor with the catechol substituted compound **9b** and the isovanillin substituted compound **9g** being interesting candidates to further *in vivo* evaluation.

Conclusion

This study demonstrates for the time the possibility to generate a series of potent 5-HT₄R ligands with promising antioxidant activity. Among them compound **9g** appears as the best compromise with a K_i of 12.7 nM toward 5-HT₄R and an antioxidant activity *in cellulo*. If the balance of activity between the *in cellulo* or *in vitro* antioxidant assay (micro molar range) and the *in vitro* assay for 5-HT₄R affinity (nanomolar range) might be improved, these series of compound show great promises in more challenging models. In the experiment, the amount of oxidative stress needs to be high enough to be visible with DCF-DA. In the brain, the level of oxidative stress caused by AD is not properly quantified and is lower than the one induced by t-BuOOH. On the base of the obtained data, it seems plausible that, with a suitable pharmacokinetic and a proper Blood Brain Barrier crossing according to its adequate LogP, **9g** could have an antioxidant effect at a nanomolar concentration and will be tested in a near future in preclinical animal models of AD.

Experimental section

Chemistry. All commercially available compounds were used without further purification. Melting points were determined on a Stuart melting point apparatus (SMP50). Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ on aluminium plates (Merck) and visualized with UV light (254 nm). Flash chromatography was conducted on a VWR SPOT II Essential instrument with silica gel 60 (40-63µm). Column size and flow rate followed the manufacturer's recommendations. NMR spectra were recorded at 295K, at 400 or 500 MHz (Bruker Avance III 400/500 MHz) for ¹H NMR and at 100 or 126 MHz for ¹³C NMR in chloroform-*d*, methanol-*d*₄ or DMSO-*d*₆ with chemical shifts (δ) given in parts per million (ppm) relative to TMS as internal standard and recorded. The following abbreviations are used to describe peak splitting patterns when appropriate: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet. Coupling constants *J* are reported in hertz units (Hz). Infrared spectra (IR) were obtained on a PERKIN-ELMER FT-IR spectrometer and are reported in terms of frequency of absorption (cm⁻¹) using KBr discs. High-resolution mass spectra (HRMS) were obtained by electrospray (ESI, sampling cone 50V, capillary 0.3kV) on a Xevo G2-XS QToF WATERS mass spectrometer. LC-MS (ESI) analyses were realized with Waters Alliance 2695 as separating module using the following gradients: A (95%)/B (5%) to A (5%)/B (95%) in 4.00min. This ratio was hold during 1.50 min before return to initial conditions in 0.50 min. Initial conditions were then maintained for 2.00 min (A = H₂O, B = CH₃CN; each containing HCOOH : 0.1 %; column XBridge BEH C18 2.5 µm/3.0 x 50 mm; flow rate 0.8 mL/min). MS were obtained on a SQ detector by positive ESI. Mass spectrum data are reported as m/z.

General procedure for deprotection/alkylation of the piperidine moiety A

To a stirred solution of tert-butyl piperidine-1-carboxylate derivative (1.0 eq.) in DCM (20 mL/mmol) was added TFA (2 mL/mmol). The resulting mixture was stirred at room temperature for 1h. Removal of the solvent under vacuum afforded the crude product, which was directly engaged in the next step. The residue obtained (1.0 eq.) was dissolved in DCM or ACN (10 mL/mmol) and bromo-benzyl-alcohol derivatives (1.1-1.5 eq.) and K_2CO_3 (10.0 eq.) were added. The resulting mixture was stirred at RT or 70°C depending on the product for 2-72h, then concentrated *in vacuo*. The crude was directly purified by chromatography on silica gel column and concentrated under reduced pressure to afford alkylated compound (isolated yields).

The general procedure to obtain unprotected catechol B

Appropriate benzylated product (1 eq) was stirred in bromhydric acid 33 % diluted in acetic acid (10 mL.mmol⁻¹, 0.10M) and DCM (50 mL.mmol⁻¹, 0.02M) at 0°C for 1 to 4h. The mixture was evaporated in vacuo at rt. Then the crude was directly purified on silica gel column (DCM/MeOH; from 100:0 to 80:20).

Ethyl 3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropanoate (2)

To a solution of 4-amino-5-chloro-2-methoxybenzoic acid (5.00 g, 24.8 mmol, 1.0 eq.) in THF (200 mL, 0.12 M) was added CDI (4.42 g, 27.3 mmol, 1.1 eq.) portionwise. The mixture was stirred at rt for 4h. Then, ethyl potassium malonate (5.06 g, 29.7 mmol, 1.2 eq.) and $MgCl_2$ (2.83 g, 29.7 mmol, 1.2 eq.) were added portionwisely. The resulting mixture was stirred 16h at 40°C.

Solvent were evaporated under vacuum. The residue was diluted with water and then extracted with DCM three times. Organics layers were combined and washed with $NaHCO_3$ sat. solution, then brine, and dried over $MgSO_4$. Solvent was evaporated under vaccum to afford a crude

product which was purified on silica gel column (Cyclohexane/EtOAc from 100:0 to 60:40). The compound was obtained as a light yellow powder (3.76 g, 60% yield). ¹H NMR (CDCl₃, 400 MHz) δ 7.92 (s, 1H), 6.23 (s, 1H), 4.55 (s, 2H), 4.17 (q, *J* = 7.1 Hz, 2H), 3.87 (s, 2H), 3.82 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (CDCl₃, 100 MHz) δ 189.6, 168.6, 159.9, 148.7, 132.5, 117.3, 111.7, 97.0, 60.8, 55.4, 50.5, 14.2 MS *m/z* [M+H]⁺ 272.51 IR (neat, cm⁻¹) ν 3463, 3363, 3223, 2983, 1725, 1648, 1622, 1573, 1479, 1457 1326, 1220, 837, 654 Mp 117°C. Spectral and analytical data matched with literature.²¹

(piperidin-4-yl)methanol (4)

To a solution of ethyl isonipecotate (2 mL, 13.0 mmol, 1.0 eq.) in Et₂O (150 mL) was added portion-wise LiAlH₄ (1.48 g, 39.0 mmol, 3.0 eq.) at 0°C and the reaction mixture was refluxed for 7h. The mixture was then cooled to 0°C and the reaction was then slowly quenched with 1.5 mL of water, 1.5 mL of 15% NaOH solution followed by 4.5 mL of water to afford a granular inorganic precipitate. The white solid was filtered and washed several times with EtOAc. Evaporation of the filtrate under reduced pressure provided 1.38 g (92% yield) of compound (1) as a colorless oil. ¹H NMR (CDCl₃, 400 MHz) δ 3.45 (d, *J* = 5.9 Hz, 2H), 3.08 (m, 2H), 2.59 (dt, *J* = 12.0 Hz, *J* = 3.0 Hz, 2H), 1.96 (br s, 1H), 1.71 (m, 2H), 1.60-1.55 (m, 1H), 1.17-1.07 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 66.9, 45.9 (2C), 38.8, 29.7 (2C); IR (neat, cm⁻¹) ν 3369 , 2923, 2855, 1636, 1531, 1425, 1266, 1036. Spectral and analytical data matched with literature.⁴⁶

***Tert*-butyl 4-(hydroxymethyl)piperidine-1-carboxylate (5)**

To a solution of (1) (1.14 g, 9.96 mmol, 1.0 eq.) in DCM (40 mL) was added di-*tert*-butyldicarbonate (2.39 g, 10.9 mmol, 1.1 eq.). The solution was stirred for 15h at room

temperature. The mixture was then diluted with water and the aqueous layer was extracted several times with DCM. The organic layer was dried over MgSO₄ and concentrated in vacuo. The crude product was purified by chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 70:30) to give 1.34 g (62% yield) of compound (**2**) as white solid.

¹H NMR (CDCl₃, 400 MHz) δ 4.10 (m, 2H), 3.48 (d, *J* = 6.1 Hz, 2H), 2.68 (m, 2H), 1.80 (br s, 1H, OH), 1.69 (m, 2H), 1.63 (m, 1H), 1.44 (s, 9H), 1.12 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 155.0, 79.5, 67.6, 43.9 (2C), 38.9, 28.7 (2C), 28.6 (3C); MS *m/z* [M+H]⁺ 216.47; [M-Boc+H]⁺ 116.47; [M-*t*-butyl+H]⁺ 160.47; IR (neat, cm⁻¹) ν 3470, 2926, 2859, 1672, 1422, 1168, 1045, 572; Mp 85°C.

***Tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate (**6**)**

To a solution of (**2**) (2.25 g, 10.5 mmol, 1.0 eq.), imidazole (858 mg, 12.6 mmol, 1.2 eq.) and triphenylphosphine (3.30 g, 12.6 mmol, 1.2 eq.) in dry THF (30 mL) was added drop-wise a solution of iodine (3.20 g, 12.6 mmol, 1.2 eq.) in dry THF (30 mL) at 0°C. The reaction mixture was stirred at room temperature for 14h. The mixture was quenched with a saturated Na₂S₂O₃ solution. The aqueous layer was extracted with EtOAc. The organic layer was washed with water, dried over MgSO₄, and concentrated in vacuo. The crude was several times diluted with cold Et₂O, and white crystals were filtered. After concentration of filtrate in vacuo, 3.13 g of the compound (**3**) was obtained as a colorless oil (92% yield).

¹H NMR (CDCl₃, 400 MHz) δ 4.11 (m, 2H), 3.10 (d, *J* = 5.8 Hz, 2H), 2.68 (m, 2H), 1.83 (m, 2H), 1.64-1.61 (m, 1H), 1.46 (s, 9H), 1.19-1.09 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 154.6, 79.5, 43.4 (2C), 38.6, 32.5 (2C), 28.4 (3C), 13.5; IR (neat, cm⁻¹) ν 2975, 2932, 2850, 1692, 1422, 1243, 1157, 965. Spectral and analytical data matched with literature.⁴⁷

Tert-butyl 4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidine-1-carboxylate

(7)

To a solution of ethyl 3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropanoate (**4**) (3.14 g, 11.6 mmol, 1.0 eq.) in DMF (30 mL) and *tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate (**3**) (4.14 g, 12.7 mmol, 1.1 eq.) was added K₂CO₃ (3.19 g, 23.10 mmol, 2 eq.). The mixture was stirred 72h at rt. The reaction mixture was diluted with water, and product was extracted with EtOAc. Combined organic layers were washed with water and brine and then dried over MgSO₄. Evaporation of the solvent gave a crude mixture which was directly dissolved in EtOH (500 mL), H₂O (100 mL) and KOH (3.23 g, 57.5 mmol, 5.5 eq.) were added. The reaction mixture was refluxed for 3h, EtOH was evaporated. The mixture was diluted with H₂O and extracted with EtOAc. The organic layer was washed with H₂O and brine. Evaporation of the solvent provided a crude product, which was purified by column on silica gel, eluting on DCM/EtOAc from 100:0 to 60:40 to give the product **5** as a colorless solid (3.11 g, 67% isolated yield).

¹H NMR (CDCl₃, 400 MHz) δ 7.79 (s, 1H), 6.26 (s, 1H), 4.46 (s, 2H), 4.18 – 3.98 (m, 2H), 3.85 (s, 3H), 2.91 (dd, *J* = 8.3, 6.9 Hz, 2H), 2.67 (t, *J* = 12.4 Hz, 2H), 1.72 – 1.64 (m, 2H), 1.63 – 1.58 (m, 2H), 1.58 – 1.54 (m, 1H), 1.45 (s, 9H), 1.10 (qd, *J* = 12.4, 4.3 Hz, 2H). ¹³C NMR (CDCl₃, 100.4 MHz) δ 198.7, 159.5, 154.9, 147.7, 132.2, 118.9, 111.3, 97.5, 79.2, 55.7, 44.0, 40.6, 35.8, 32.1, 31.1, 28.5. MS *m/z* [M+H]⁺ 397.60 ; [M-Boc+H]⁺ 297.51; [M-*t*-butyl+H]⁺ 341.55; IR (neat, cm⁻¹) ν 3470, 3353, 2975, 2930, 2851, 1673, 1624, 1588, 1421, 1366, 1311, 1250, 1216, 1173, 967, 738; Mp 136°C. Spectral and analytical data matched with literature.²¹

***Tert*-butyl *N*-(2-{4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidin-1-yl}ethyl)carbamate (8)**

Following procedure A with *tert*-butyl 4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidine-1-carboxylate (7) (2.37 g, 5.98 mmol, 1 eq.) and *tert*-butyl *N*-(2-bromoethyl)carbamate (1.61 g, 7.18 mmol, 1.2 eq.) synthesized following the literature, the reaction was stirred at rt for 16h. After a purification by chromatography on silica gel column (Cyclohexane/EtOAc, gradient 100:0 to 0:100), the compound (8) was obtained as a light yellow solid (780 mg, 30% isolated yield).

¹H NMR (CDCl₃, 400 MHz) δ 7.71 (s, 1H), 6.20 (s, 1H), 4.98 (br s, 1H, NH), 4.49 (s, 2H, NH₂), 3.77 (s, 3H), 3.14 (t, *J* = 6.0 Hz, 2H), 2.86 – 2.72 (m, 4H), 2.35 (t, *J* = 6.0 Hz, 2H), 1.92 – 1.76 (m, 2H), 1.67 – 1.54 (m, 2H), 1.56 – 1.44 (m, 2H), 1.38 (s, 9H), 1.24 – 1.08 (m, 3H). ¹³C NMR (CDCl₃, 100.0 MHz) δ 199.0, 159.4, 156.0, 147.7, 132.2, 118.9, 111.2, 97.5, 79.1, 57.5, 55.6, 53.8 (2C), 40.9, 37.4, 35.6, 32.2 (2C), 31.3, 28.5 (3C); MS *m/z* [M+H]⁺ 441.8; IR (neat, cm⁻¹) ν 3486, 3376, 3325, 2980, 2925, 2855, 1690, 1625, 1573, 1467, 1367, 1311, 1175, 1216, 1114, 969, 610; Mp 119°C; HRMS/ESI: *m/z* calcd. for C₂₂H₃₄ClN₃O₄ [M+H]⁺ 440.2316, found 440.2324;

1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-[(3,4-dibenzyloxyphenyl)methyl]-4-piperidyl]propan-1-one (9a)

Following procedure A with *tert*-butyl 4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidine-1-carboxylate (7) (200 mg, 0.50 mmol, 1 eq.) and 1,2-bis(benzyloxy)-4-(bromomethyl)benzene (230 mg, 0.60 mmol, 1.2 eq.) synthesized following the literature, the reaction was stirred at RT for 10h. After a purification by chromatography on silica gel column

(DCM/MeOH, gradient 100:0 to 90:10), the compound (**9a**) was obtained as a light yellow solid (141 mg, 47% isolated yield).

¹H NMR (CDCl₃, 400 MHz) 7.78 (s, 1H), 7.49 – 7.40 (m, 4H), 7.40 – 7.24 (m, 6H), 7.01 (d, *J* = 2.0 Hz, 1H), 6.87 (d, *J* = 8.2 Hz, 1H), 6.81 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.25 (s, 1H), 5.18 (s, 2H), 5.14 (s, 2H), 4.51 (s, 2H), 3.82 (s, 3H), 3.49 (s, 2H), 2.95 – 2.79 (m, 4H), 1.97 (t, *J* = 10.5 Hz, 2H), 1.66 (d, *J* = 10.5 Hz, 2H), 1.64 – 1.51 (m, 2H), 1.47 – 1.20 (m, 4H). ¹³C NMR (CDCl₃, 100 MHz) δ 198.9, 159.54, 148.6, 148.4, 147.8, 137.3, 137.3, 132.2, 129.5, 128.5 (2C), 128.4 (2C), 127.8 (2C), 127.7 (2C), 127.5, 127.3, 122.7, 118.7, 116.6, 114.6, 111.2, 97.5, 71.3, 71.2, 62.5, 55.6, 53.3 (2C), 40.7, 35.0, 31.5 (2C), 31.0; MS *m/z* [M+H]⁺ 599.7; IR (neat, cm⁻¹) ν 3482, 3368, 3220, 2921, 2847, 2798, 1618, 1585, 1508, 1453, 1419, 1257, 1214, 1184, 1015, 735, 695; Mp 104.5°C. HRMS/ESI: *m/z* calcd. for C₃₆H₃₉ClN₂O₄ [M+H]⁺ 599.2677, found 599.2673;

1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-[(3,4-dihydroxyphenyl)methyl]-4-piperidyl]propan-1-one (9b**)**

From general procedure B with 1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-[(3,4-dibenzoyloxyphenyl)methyl]-4-piperidyl]propan-1-one **9a** (125 mg, 0.21 mmol) to afford 71 mg of 1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-[(3,4-dihydroxyphenyl)methyl]-4-piperidyl]propan-1-one **9b** as a pale yellow powder with a yield of 81%. ¹H NMR (MeOD, 400 MHz) δ 7.66 (s, 1H), 6.92 (s, 1H), 6.85 – 6.80 (m, 2H), 6.45 (s, 1H), 4.06 (s, 2H), 3.86 (s, 3H), 3.39 (d, *J* = 11.8 Hz, 2H), 2.97 – 2.81 (m, 4H), 1.95 (m, 2H), 1.66 – 1.52 (m, 3H), 1.51 – 1.36 (m, 2H). ¹³C NMR (MeOD, 100 MHz) δ 198.78, 160.32, 150.43, 146.74, 145.57, 131.46, 122.57, 120.61, 117.59, 116.27, 115.16, 110.26, 96.64, 60.35, 54.75, 51.86 (2C), 39.86, 33.28, 30.16,

29.05 (2C); MS m/z $[M+H]^+$ 419.8; IR (neat, cm^{-1}) ν 3432, 3366, 3206, 2938, 2725, 1619, 1584, 1453, 1419, 1295, 1261, 1216, 1184. Mp 115.9°C. HRMS/ESI: m/z calcd. for $\text{C}_{22}\text{H}_{28}\text{ClN}_2\text{O}_4$ $[M+H]^+$ 419.1738, found 419.1737;

1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-(1,3-benzodioxol-5-ylmethyl)-4-piperidyl]propan-1-one (9c)

tert-butyl 4-(3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl)piperidine-1-carboxylate (200 mg, 0.50 mmol, 1 eq.) was dissolved in 4 mL of DCM, then 1 mL of TFA was added. The solution was stirred 15 min at RT, then the solvent was reduced under pressure. The resulting yellow oil was dissolved in DMF (10 mL), then K_2CO_3 (691.0 mg, 5.00 mmol, 10 eq.) and 5-(bromomethyl)benzo[*d*][1,3]dioxole (129.4 mg, 0.60 mmol, 1.2 eq.) were added dropwisely. The reaction mixture was warmed at 110°C for 3h.

Then water was added and the solution was extracted three times with EtOAc, then dried over MgSO_4 , concentrated under vacuum to afford a crude product which was purified on neutral alumine column with a 100% DCM isocratic gradient. The product was obtained as a yellowish powder (65 mg, 30% isolated yield) ^1H NMR (CDCl_3 , 400 MHz) δ 7.77 (s, 1H), 6.84 (s, 1H), 6.74 (d, $J = 7.5$ Hz, 1H), 6.72 (d, $J = 7.5$ Hz, 1H), 6.24 (s, 1H), 5.93 (s, 2H), 4.46 (br s, 2H), 3.82 (s, 3H), 3.38 (s, 2H), 2.93 – 2.80 (m, 4H), 1.90 (m, 2H), 1.87 (m, 1H), 1.66 (d, $J = 9.2$ Hz, 2H), 1.58 (dd, $J = 7.2, 6.4$ Hz, 2H), 1.31 – 1.15 (m, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 199.1, 159.5, 147.7, 147.5, 146.4, 132.5, 132.2, 122.3, 118.9, 111.2, 109.6, 107.8, 100.8, 97.5, 63.3, 55.6, 53.8 (2C), 40.9, 35.6, 32.3 (2C), 31.3; MS m/z $[M+H]^+$ 431.6; IR (neat, cm^{-1}) ν 3470, 3358, 3216, 3013, 2922, 2873, 2854, 2810, 2775, 1715, 1642, 1619, 1573, 1440, 1421, 1332, 1312, 1261,

1214, 1178, 986, 932, 738, 567; Mp 124°C. HRMS/ESI: m/z calcd. for C₂₃H₂₈ClN₂O₄ [M+H]⁺ 431.1738, found 431.1747;

1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-{[4-(benzyloxy)-3-methoxyphenyl]methyl}piperidin-4-yl)propan-1-one (9d)

Following procedure A with **7** (200 mg, 0.50 mmol, 1 eq.) and 1-(benzyloxy)-4-(bromomethyl)-2-methoxybenzene (185 mg, 0.60 mmol, 1.2 eq.), the reaction was stirred at RT for 16h. After a purification by chromatography on silica gel column (DCM/MeOH, gradient 100:0 to 80:20), the compound (**9d**) was obtained as a light yellow oil (74 mg, 28% isolated yield).

¹H NMR (CDCl₃, 400 MHz) δ 7.78 (s, 1H), 7.49 – 7.28 (m, 5H), 6.96 – 6.88 (m, 1H), 6.81 (d, *J* = 8.2 Hz, 1H), 6.75 (dd, *J* = 8.2, 1.9 Hz, 1H), 6.24 (s, 1H), 5.13 (s, 2H), 4.46 (s, 2H), 3.89 (s, 3H), 3.82 (s, 3H), 3.43 (s, 2H), 2.98 – 2.78 (m, 4H), 1.92 (s, 2H), 1.67 (m, 2H), 1.59 (m, 2H), 1.28 (s, 3H). ¹³C NMR (CDCl₃, 100 MHz) δ 199.1, 159.5, 149.4, 147.7, 147.2, 137.3, 132.2, 129.5, 128.5(2C), 127.78, 127.23 (2C), 121.4, 118.9, 113.5, 112.9, 111.2, 97.5, 71.1, 63.2, 56.1, 55.6, 53.8 (2C), 40.9, 35.6, 32.2 (2C), 31.3. MS m/z [M+H]⁺ 523.6; IR (neat, cm⁻¹) ν 3.449, 2.919, 2.853, 2.788, 1.616, 1.586, 1.463, 1.450, 1.414, 1.261, 1.216, 1.177, 735; HRMS/ESI: m/z calcd. for C₃₀H₃₆ClN₂O₄ [M+H]⁺ 523.2364, found 523.2363;

1-(4-amino-5-chloro-2-methoxyphenyl)-3-{1-[(4-hydroxy-3-methoxyphenyl)methyl]piperidin-4-yl}propan-1-one (9e)

From general procedure B with **9d** (80 mg, 0.16 mmol, 1.0 eq.) to afford **9e** as yellow powder with a yield of 85%. ¹H NMR (MeOD, 400 MHz) δ 7.66 (s, 1H), 7.10 (d, *J* = 2.0 Hz, 1H), 6.91 (dd, *J* = 8.1, 2.0 Hz, 1H), 6.85 (d, *J* = 8.1 Hz, 1H), 6.45 (s, 1H), 4.13 (s, 2H), 3.90 (s, 3H), 3.86 (s, 3H), 3.40 (d, *J* = 12.6 Hz, 2H), 3.00 – 2.79 (m, 4H), 1.96 (d, *J* = 13.4 Hz, 2H), 1.62 (m, 3H), 1.46 (m, 2H). ¹³C NMR (MeOD, 100 MHz) δ 198.78, 160.3, 150.4, 148.0, 147.9, 131.5, 124.0, 120.5, 116.3, 115.1, 114.0, 110.3, 96.7, 60.5, 55.1, 54.8, 51.9 (2C), 39.9, 33.3, 30.2, 29.1 (2C); MS *m/z* [M+H]⁺ 432.8; MP: 120.5°C; IR (neat, cm⁻¹) ν 3360, 3220, 2932, 2855, 1620, 1586, 1420, 1254, 1216, 1177, 1136, 1084, 735; HRMS/ESI: *m/z* calcd. for C₂₃H₃₀ClN₂O₄⁺ [M+H]⁺ 433.1894, found 433.1894;

1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-{3-(benzyloxy)-4-hydroxyphenyl}methyl)piperidin-4-yl)propan-1-one (9f)

Following procedure A with **7** (200 mg, 0.05 mmol, 1 eq.) and 2-(benzyloxy)-4-(bromomethyl)-1-methoxybenzene (185 mg, 0.60 mmol, 1.2 eq.), the reaction was stirred at rt for 16h. After a purification by chromatography on silica gel column (DCM/MeOH, gradient 100:0 to 80:20), the compound (**9f**) was obtained as a light yellow oil (100 mg, 38% isolated yield). ¹H NMR (CDCl₃, 400 MHz) δ 7.78 (s, 1H), 7.44 (d, *J* = 7.2 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 7.28 (d, *J* = 7.2 Hz, 1H), 6.94 (s, 1H), 6.83 (s, 2H), 6.25 (s, 1H), 5.17 (s, 2H), 4.46 (s, 2H), 3.87 (s, 3H), 3.84 (s, 3H), 3.45 (s, 2H), 2.87 (m, 4H), 1.96 – 1.84 (m, 2H), 1.65 (d, *J* = 10.2 Hz, 2H), 1.62 – 1.56 (m, 2H), 1.28 (s, 3H). ¹³C NMR (CDCl₃, 100 MHz) δ 199.0, 159.5, 149.0, 147.9, 147.7, 137.1, 132.1, 129.3, 128.5 (2C), 127.8, 127.5 (2C), 122.3, 118.7, 115.5, 111.4, 111.2, 97.5, 70.9, 62.7, 56.0, 55.7, 53.4 (2C), 40.8, 35.2, 31.7 (2C), 31.1; MS *m/z* [M+H]⁺ 523.6; IR (neat, cm⁻¹) ν 3360, 2923,

2834, 2788, 1618, 1587, 1463, 1450, 1414, 1261, 1216, 1177, 735; HRMS/ESI: m/z calcd. for $C_{30}H_{36}ClN_2O_4$ $[M+H]^+$ 523.2364, found 523.2365;

1-(4-amino-5-chloro-2-methoxyphenyl)-3-{1-[(3-hydroxy-4-methoxyphenyl)methyl]piperidin-4-yl}propan-1-one (9g)

From general procedure B with **9f** (80 mg, 0.16 mmol, 1 eq.) to afford acetate salt of **9g** as a pale yellow powder with a yield of 70%. 1H NMR ($CDCl_3$, 400 MHz) δ 7.76 (s, 1H), 6.95 (d, $J = 2.0$ Hz, 1H), 6.86 – 6.76 (m, 2H), 6.26 (s, 1H), 4.54 (br s, 2H), 3.86 (s, 3H), 3.82 (s, 3H), 3.74 (s, 2H), 3.16 (d, $J = 11.5$ Hz, 2H), 2.87 (t, $J = 7.4$ Hz, 2H), 2.27 (t, $J = 11.5$ Hz, 2H), 2.04 (s, 3H), 1.74 – 1.72 (m, 2H), 1.62 – 1.60 (m, 2H), 1.54 – 1.43 (m, 2H), 1.37 (m, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 198.5, 176.9, 159.6, 147.9, 147.1, 145.9, 132.2, 125.78, 122.2, 118.6, 116.8, 111.3, 110.7, 97.5, 61.0, 55.9, 55.7, 52.1 (2C), 40.4, 34.2, 30.5, 30.1 (2C), 22.9; MS m/z $[M+H]^+$ 432.8; MP: 80.8°C; IR (neat, cm^{-1}) ν 3360, 3220, 2932, 2855, 1620, 1586, 1420, 1254, 1216, 1177, 1136, 1084, 735; HRMS/ESI: m/z calcd. for $C_{23}H_{31}ClN_2O_4$ $[M+H]^+$ 433.1894, found 433.1894;

***N*-(2--{4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidin-1-yl}ethyl)-5-[(3R)-1,2-dithiolan-3-yl]pentanamide (10a)**

To a stirred solution of **8** (125 mg, 0.28 mmol, 1.1 eq.) in DCM (20 mL/mmol) was added TFA (2 mL/mmol). The resulting mixture was stirred at room temperature for 1h. Removal of the solvent under vacuum afforded the crude product, which was directly engaged in the next step. To a solution of LA (53 mg, 0.26 mmol, 1.0 eq.) in DCM (5 mL) were successively added

hydroxybenzotriazole (HOBt) (42 mg, 0.31 mmol, 1.2 eq.) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI) (49 mg, 0.31 mmol, 1.2 eq.), the mixture was stirred at 0°C in the dark for 30min, then the free amine was added, and the reaction was stirred at rt for 16h. Solvent was evaporated under vacuum then the product was purified on silica gel column with DCM/MeOH/NH₃ (97.5:2.5:0.25), **10a** was obtained as white powder with a yield of 44%. ¹H NMR (CDCl₃, 400 MHz) δ 7.81 (s, 1H), 6.28 (s, 1H), 6.11 (br s, 1H), 4.49 (s, 2H), 3.87 (s, 3H), 3.59 (dq, *J* = 8.5, 6.5 Hz, 1H), 3.35 (q, *J* = 5.7 Hz, 2H), 3.24 – 3.07 (m, 2H), 2.97 – 2.82 (m, 4H), 2.47 (m, 3H), 2.24 – 2.17 (m, 2H), 2.01 – 1.90 (m, 3H), 1.76 – 1.65 (m, 6H), 1.62 (dt, *J* = 8.5, 6.5 Hz, 2H), 1.57 – 1.42 (m, 2H), 1.33 – 1.17 (m, 3H). ¹³C NMR (CDCl₃, 100 MHz) δ 198.9, 172.7, 159.5, 147.7, 132.2, 118.9, 111.3, 97.6, 56.87, 56.5, 55.7, 53.7 (2C), 40.9, 40.3, 38.5, 36.5, 36.1, 35.6, 34.7, 32.3, 31.2 (2C), 28.9, 25.5; MS *m/z* [M+H]⁺ 527.8; MP: 122.5°C; IR (neat, cm⁻¹) ν 3217, 2926, 2844, 1642, 1586, 1462, 1419, 1316, 1254, 1250, 1170; HRMS/ESI: *m/z* calcd. for C₂₃H₃₉ClN₃O₃S₂ [M+H]⁺ 528.2121, found 528.2118;

(2E)-N-(2-{4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidin-1-yl}ethyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enamide (10b)

Following the same procedure as **10a**, from *N*-(2-{4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidin-1-yl}ethyl)carbamate (**8**) (125 mg, 0.28 mmol, 1.1 eq.) and ferulic acid (39 mg, 0.20 mmol, 1. eq.) with EDCI, HOBt, and Et₃N. The crude was purified on silica gel column with DCM/MeOH/NH₃ (92.5:7.5:0.75) to afford a bright yellow powder with a yield of 42%. ¹H NMR (CDCl₃, 400 MHz) δ 7.72 (s, 1H), 7.43 (d, *J* = 15.5 Hz, 1H), 6.96 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.90 (d, *J* = 2.0 Hz, 1H), 6.80 (d, *J* = 8.2 Hz, 1H), 6.37 (br s, 1H), 6.23 – 6.07 (m, 2H), 4.40 (br s,

2H), 3.84 (s, 3H), 3.77 (s, 3H), 3.46 – 3.41(m, 2H), 2.91 (d, $J = 11.0$ Hz, 2H), 2.86 – 2.82 (m, 2H), 2.51 (t, $J = 5.8$ Hz, 2H), 2.45 – 2.43 (m, 1H), 1.98 – 1.96 (m, 3H), 1.67 (d, $J = 9.2$ Hz, 2H), 1.57 – 1.51 (m, 2H), 1.27 (s, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 198.8, 166.2, 159.5, 147.7, 147.4, 146.8, 140.7, 132.2, 127.4, 122.3, 118.9, 118.3, 114.8, 111.3, 109.5, 97.5, 57.1, 55.9, 55.7, 53.8 (2C), 40.7, 36.0, 35.3, 31.8 (2C), 31.0; MS m/z $[\text{M}+\text{H}]^+$ 515.77; MP: 85°C; IR (neat, cm^{-1}) ν 3435, 2938, 2837, 1647, 1616, 1585, 1511, 1459, 1419, 1264, 1214, 1156; HRMS/ESI: m/z calcd. for $\text{C}_{27}\text{H}_{35}\text{ClN}_3\text{O}_5$ $[\text{M}+\text{H}]^+$ 516.2265, found 516.2264.

***In vitro* evaluation**

DPPH radical scavenging activity

The antiradical activity of the tested compounds was assessed using a miniaturized DPPH (2,2-diphenyl-1-picrylhydrazyl radical) assay.^{48,49} The tested compounds were diluted from stock solution in absolute ethanol and incubated in the presence of 100 μM DPPH (Sigma-Aldrich, Saint-Quentin Fallavier, France) solution at room temperature in a 96-wells microplate (Sterilin®, Fisher Scientific SAS, Illkirsh, France) in the dark. The absorbance at 517 nm was then measured every 10 minutes min using Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) and used to calculate the specific absorbance of DPPH in each solution subtracting absorbance of all other components in the solution as following:

$$\begin{aligned}
 - \quad A_{max}^{DPPH} &= Abs_{DPPH\ alone} - Abs_{blank} \\
 - \quad A_{sample}^{DPPH} &= Abs_{mix\ DPPH/compound} - Abs_{compound} \\
 - \quad A_{min}^{DPPH} &= Abs_{mix\ DPPH/Trolox\ at\ 375\ \mu M} - Abs_{Trolox\ at\ 375\ \mu M}
 \end{aligned}$$

where A_{max}^{DPPH} is the maximal absorbance of DPPH alone, A_{sample}^{DPPH} is the specific absorbance of DPPH in a mix with a tested compound at given concentration, A_{min}^{DPPH} is the specific absorbance of DPPH in a mix with a Trolox® from Sigma-Aldrich

(Saint-Quentin Fallavier, France) at 375 μ M considered as the minimal absorbance. Then, the DPPH radical scavenging activity were calculated according to the following formula:

$$\% \text{ DPPH RSA} = \frac{A_{\text{max}}^{\text{DPPH}} - A_{\text{sample}}^{\text{DPPH}}}{A_{\text{max}}^{\text{DPPH}} - A_{\text{min}}^{\text{DPPH}}} \times 100$$

Assays were conduct in triplicate at 10 concentration levels (0, 10, 12.5, 15, 25, 50, 75, 100, 125, 150, 250 μ M) of the tested compound. Absorbance calculations were carried out using Microsoft Excel and regressions were performed using GraphPad Prismm 6.01 (La Jolla, CA, USA). The kinetic absorbance data were fitted with a one-phase exponential association model allowing us to calculate the time of half-reaction and the plateau of the maximum effect at each concentration. The EC₅₀ values (concentrations required to obtain 50% scavenging effect) were calculated plotting concentration versus % DPPH RSA at 60, 120, plateau and fitting the data with linear model for Trolox®, one or two-phase exponential association model for other compounds. EC₅₀ and SD values were the mean of two independent experiments. Trolox® was tested as positive control.

ORAC assay

The antioxidant activity of the synthezized compounds was assessed using the Fluorescein Oxygen Radical Absorbance Capacity (ORACFI) assay.^{48,49,50} Trolox® (Sigma-Aldrich, Saint-Quentin Fallavier, France) and other compounds stock solutions were prepared at 10 mM in DMSO and diluted in 10 mM phosphate buffer to obtain working solution at 6 concentration levels. 25 μ L of buffer or Trolox® or compounds solution were mixed in a black microplate with 150 μ L of fluorescein (Sigma-Aldrich, Saint-Quentin Fallavier, France) at 8 nM and incubated 30

min at 37° C in a multimodal microplate reader (Synergy 2, Biotek, Winooski, VT, USA). Then, 25 µL of AAPH (2,2' -Azobis(2-methylpropionamide) dihydrochloride, Sigma-Aldrich) solution at 75 mM were added and mixed in each well before the kinetic. Measurements of fluorescence ($\lambda_{ex}=485/20$ nm, $\lambda_{em}=520/20$ nm) were performed every minute during 2 hours. Each point was measured in triplicate. The AUC and the Net AUC of the standards and samples were determined using Gen5 Data Analysis Software using equations (1) and (2) respectively.

$$- \quad AUC = \frac{F_1}{F_1} + \frac{F_2}{F_1} + \frac{F_3}{F_1} + \dots + \frac{F_n}{F_1} \quad (1)$$

$$- \quad NetAUC = AUC_{sample} - AUC_{blank} \quad (2)$$

Where F1 is the fluorescence reading at the initiation of the reaction and Fn is the last measurement. The standard curve was obtained by plotting the Net AUC of different compound concentrations against their concentration using GraphPad® Prism statistical software (version 6.01, GraphPad Software, La Jolla, CA, USA). ORAC values of samples were then calculated dividing the slope of standard curve of each compounds by value obtained for Trolox®. ORAC value are given as mean \pm standard deviation of three independent experiments.

Affinity as Regards 5-HT₄ Receptors

For competition studies, 2.5 µg of proteins (5-HT₄B membrane preparations, HTS110M, Eurofins. Eurofins's 5-HT₄B membrane preparations are crude membrane preparations made by use of their proprietary stable recombinant cell lines to ensure high-level of GPCR surface expression.) were incubated in duplicate at 25 °C for 60 min in the absence or the presence of 10⁻⁶ or 10⁻⁸M of each compound together with 0.2 nM [³H]-GR 113808 (NET 1152, Perkin Elmer)

in 25 mM Tris buffer (pH 7.4, 25°C). At the end of the incubation, homogenates were filtered through Whatman GF/C filters (Alpha Biotech) presoaked with 0.5% polyethylenimine using a Brandel cell harvester. Filters were subsequently washed three times with 1 mL of ice-cold 25 mM Tris buffer (pH 7.4, 4 °C). Non-specific binding was evaluated in parallel, in the presence of 30 µM serotonin.

For some of these compounds, affinity constants were calculated from five-point inhibition curves using the GraphPad Prism 6 software and expressed as $K_i \pm SD$.

In cellulo experiment

Cell lines

SH-SY5Y cells (ATCC, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM, Euroclone, Milan, Italy) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (supplements were purchased from Sigma-Aldrich, St. Louis, MO USA). Cells were grown in a humidified incubator with 95% air and 5% CO₂ at 37°C.

Determination of cAMP production

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% dialyzed fetal calf serum (dFCS) and antibiotics. Cells were transiently transfected by electroporation with plasmid encoding HA-tagged 5-HT₄R (100 ng/10⁶ cells), then seeded in 96-well plates (35,000 cells/well). 24 hours after transfection, cells were exposed to the indicated concentrations of 5-HT₄R ligands in the presence of 0.1 mM of the phosphodiesterase inhibitor RO201724, at 37°C in 100 µL of HBS (20 mM HEPES; 150 mM NaCl; 4.2 mM KCl; 0.9 mM

CaCl₂; 0.5 mM MgCl₂; 0.1% glucose; 0.1% BSA). After 10 min, cells were then lysed by addition of the same volume of Triton-X100 (0.1%). Quantification of cAMP production was performed by HTRF[®] by using the cAMP Dynamic kit (Cisbio Bioassays) according to the manufacturer's instructions.

MTT ASSAY

The effect of **9b**, **9g**, **10a**, **10b** on the growth of SH-SY5Y cell line was assessed by MTT-based cell viability test, previously described by Mosmann.³⁹ Briefly, cells were seeded in 96-well plates at a density of 5x10³ cells/well and incubated overnight at 37°C, 5% CO₂ in humidified atmosphere to allow adhesion. After this time, cells were treated for 24 hours with the compounds or vehicle (DMSO) at different concentrations, ranging from 0.5 μM to 50μM, dissolved in complete DMEM.

Then, cells were carefully washed with PBS and treated with 300 μM MTT dissolved in DMEM and incubated for 2 hours at 37°C and 5% CO₂. Then, the aqueous solution was removed and the intracellular formazan salts were dissolved in DMSO for 15 minutes at room temperature. Finally, the formazan absorbance from each well was measured at λ= 570 nm, using a microplate reader (Victor2 1420 multilabel counter, Perkin Elmer).

ROS assay

SH-SY5Y cells were seeded in 96-well plates (OptiPlate black, Perkin Elmer) at 5x10³ cells/well and incubated overnight at 37°C and 5 % CO₂ to allow adhesion in complete culture medium. After this time, cells were treated for 24 hours with 10 μM **9f**, **9g**, **10a**, **10b**, 100μM Trolox or vehicle (DMSO). After this time, cells were washed with PBS and stained for 30 minutes with 10 μM H2DCFDA probe (2',7'-dichlorodihydrofluorescein diacetate, Thermo Fisher) for ROS

detection. Then, cells were carefully washed and treated for 30 minutes with 100 μM TBH (tert-Butyl hydroperoxide solution, Luperox, Sigma-Aldrich) to induce oxidative stress. After this time, cells were washed with PBS and stained for 30 minutes with 10 μM H₂DCFDA probe (2',7'-dichlorodihydrofluorescein diacetate, Thermo Fisher) for ROS detection.

Finally, cells were washed three times with PBS supplemented with Glucose 5 mM and CaCl₂ 1 mM. The DCF fluorescence was measured ($\lambda_{\text{exc}} = 485 \text{ nm}$; $\lambda_{\text{em}} = 535 \text{ nm}$) with a plate reader (Enspire, PerkinElmer). Data were normalized on protein content determined by Lowry method.

ASSOCIATED CONTENT

Synthesis and spectroscopic characterization of intermediates and analytical analysis of final compounds.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

AD, Alzheimer's disease; DCF, dichlorofluorescein; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HAT, Hydrogen Atom Transfer; LA, Lipoic Acid; MTDL, multi-target directed ligands; ROS, Reactive Oxygen Species; SET, Single-Electron-Transfer; TBH, tert-butyl hydroxyperoxide; TEAC, Trolox Equivalent Antioxidant Capacity.

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Novel multi target-directed ligands targeting 5-HT₄ receptors with *in cellulo* antioxidant properties as promising leads in Alzheimer's disease

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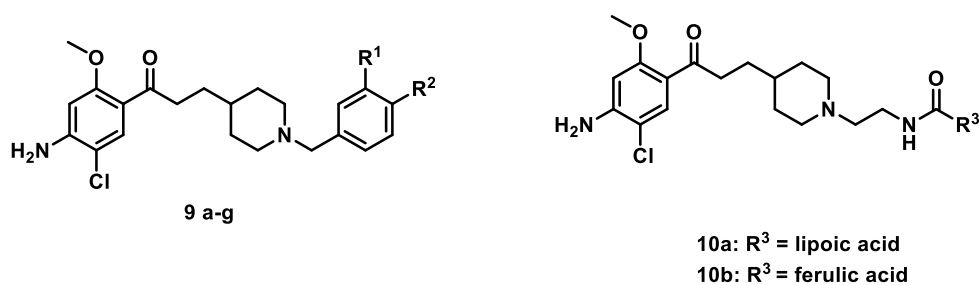
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Contents of Supplementary Information

Inhibition properties of AChE for compound 9a-g and 10a-b p2

Analytical spectrum for compound 9a-g and 10a-b p4

Table S1. Inhibition of *hAChE* of final derivatives

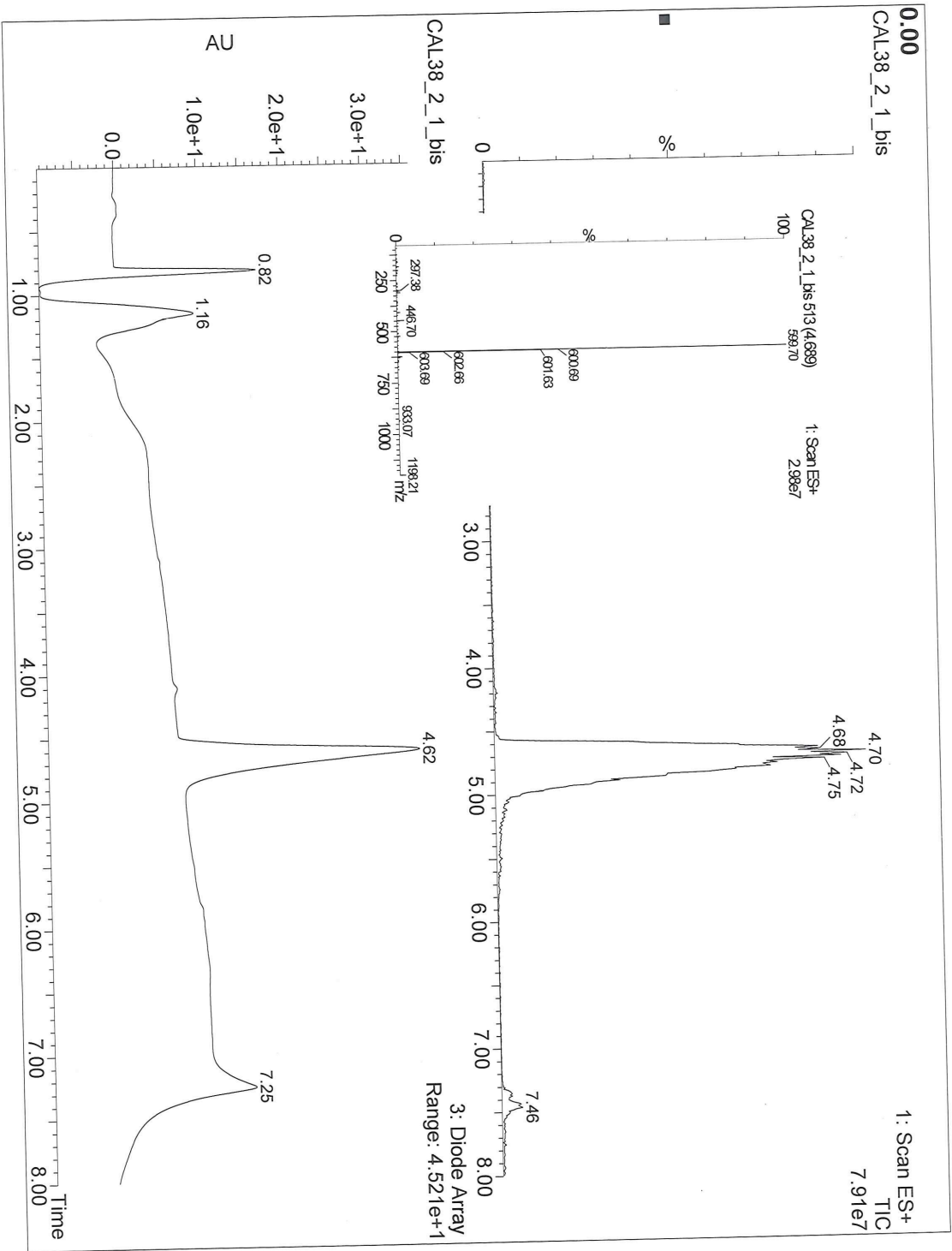
Cmpd	R ¹	R ²	R ³	cLogP*	<i>hAChE</i>	
					% inhibition at 10 ⁻⁶ M	IC ₅₀ (nM)
9a	O-Bn	O-Bn	-	6.78	32%	NT
9b	OH	OH	-	3.48	70%	137.9± 5.2
9c	O-CH ₂ -O		-	3.84	41%	NT
9d	O-CH ₃	O-Bn	-	5.55	48%	NT
9e	O-CH ₃	OH	-	3.92	67%	535 ± 49
9f	O-Bn	O-CH ₃	-	5.62	8%	NT
9g	OH	O-CH ₃	-	3.94	11%	NT
10a	-	-	Lipoic acid	4.98	15%	NT
10b	-	-	Ferulic acid	3.65	23%	NT
Donepezil	-	-	-	-	97%	12.1± 2.1

***In vitro* tests of AChE biological activity.** Inhibitory capacity of compounds on AChE biological activity was evaluated through the use of the spectrometric method of Ellman.^[1] Acetyl-iodide and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. AChE from human erythrocytes (buffered aqueous solution, ≥500 units/mg protein (BCA), Sigma Aldrich) was diluted in 20 mM HEPES buffer pH 8, 0.1% Triton X-100 such as to have enzyme solution with 0.25 unit/mL enzyme activity. In the procedure, 100 μL of 0.3 mM DTNB dissolved in phosphate buffer pH 7.4 were added into the 96 wells plate followed by 50 μL of test compound solution and 50 μL of enzyme (0.05

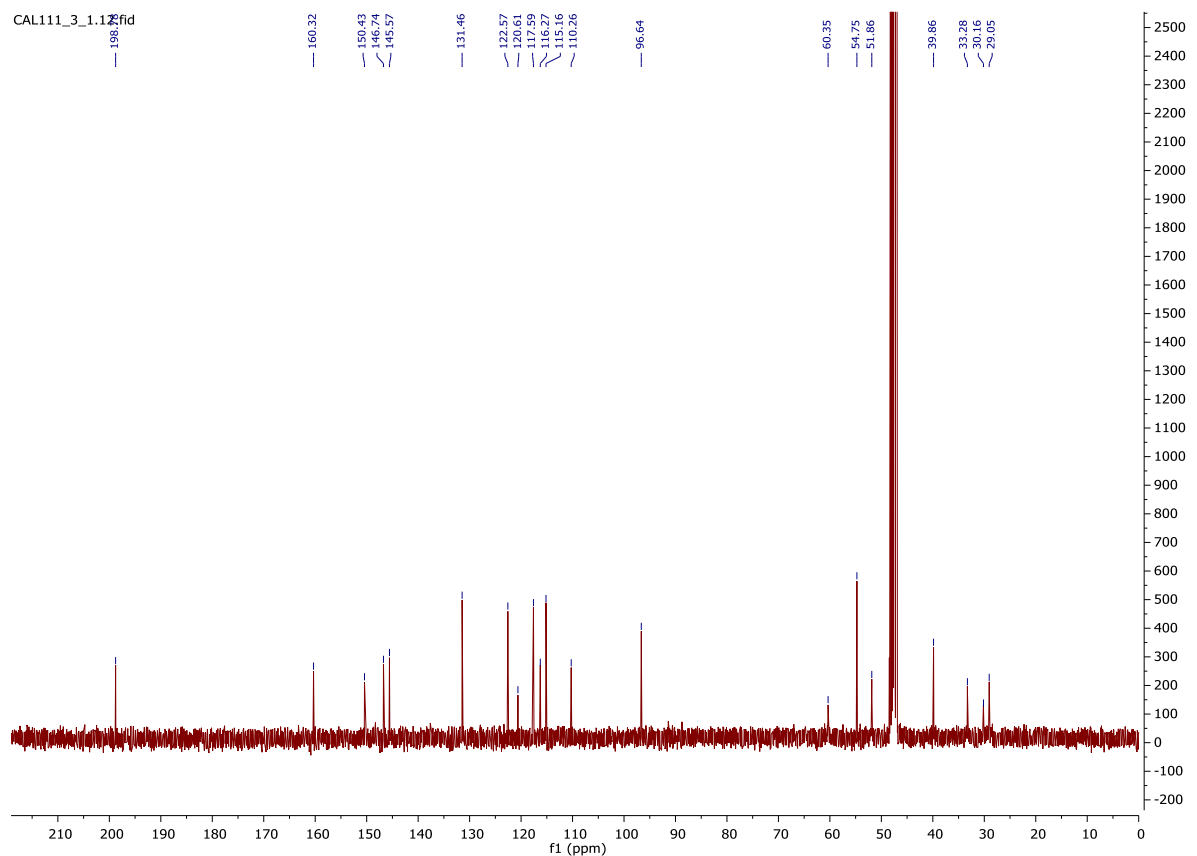
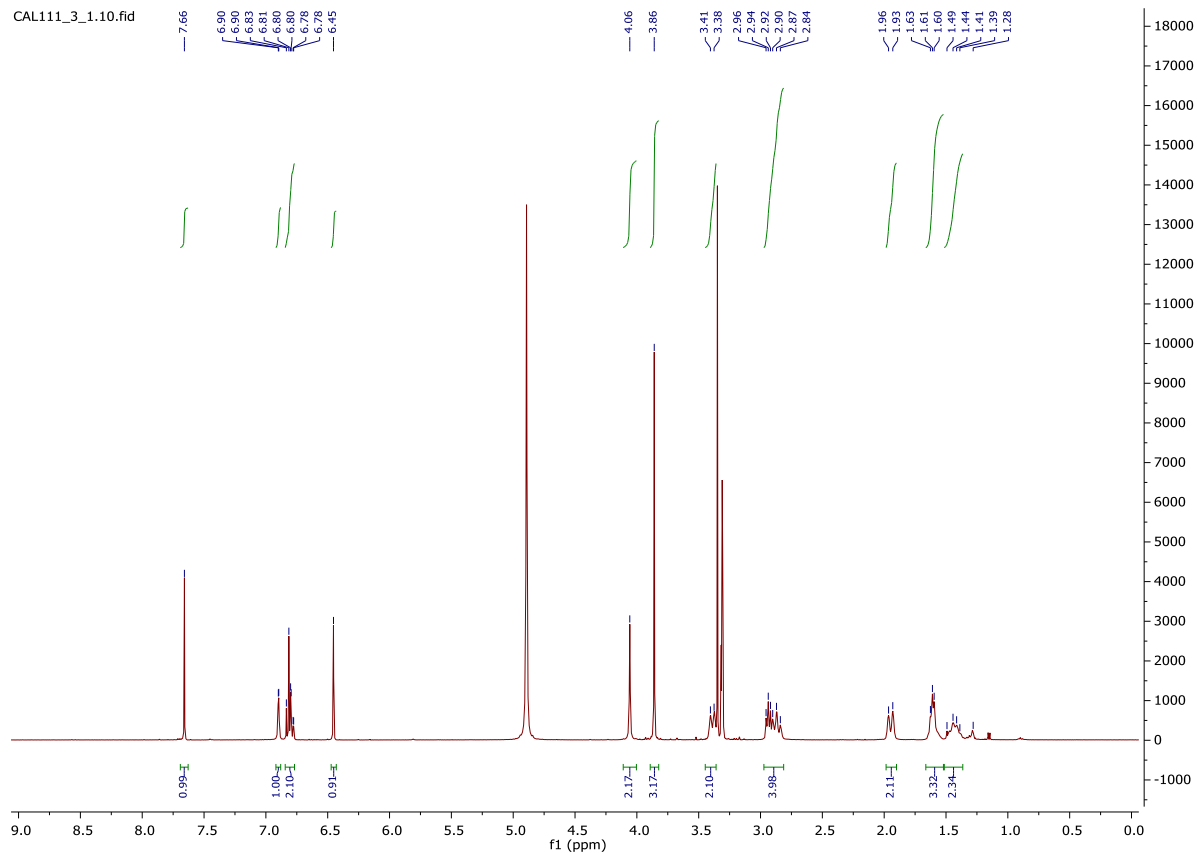
^[1] Ellman, G. L.; Courtney, K. D.; Andres Jr., V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, 7, 88–95.

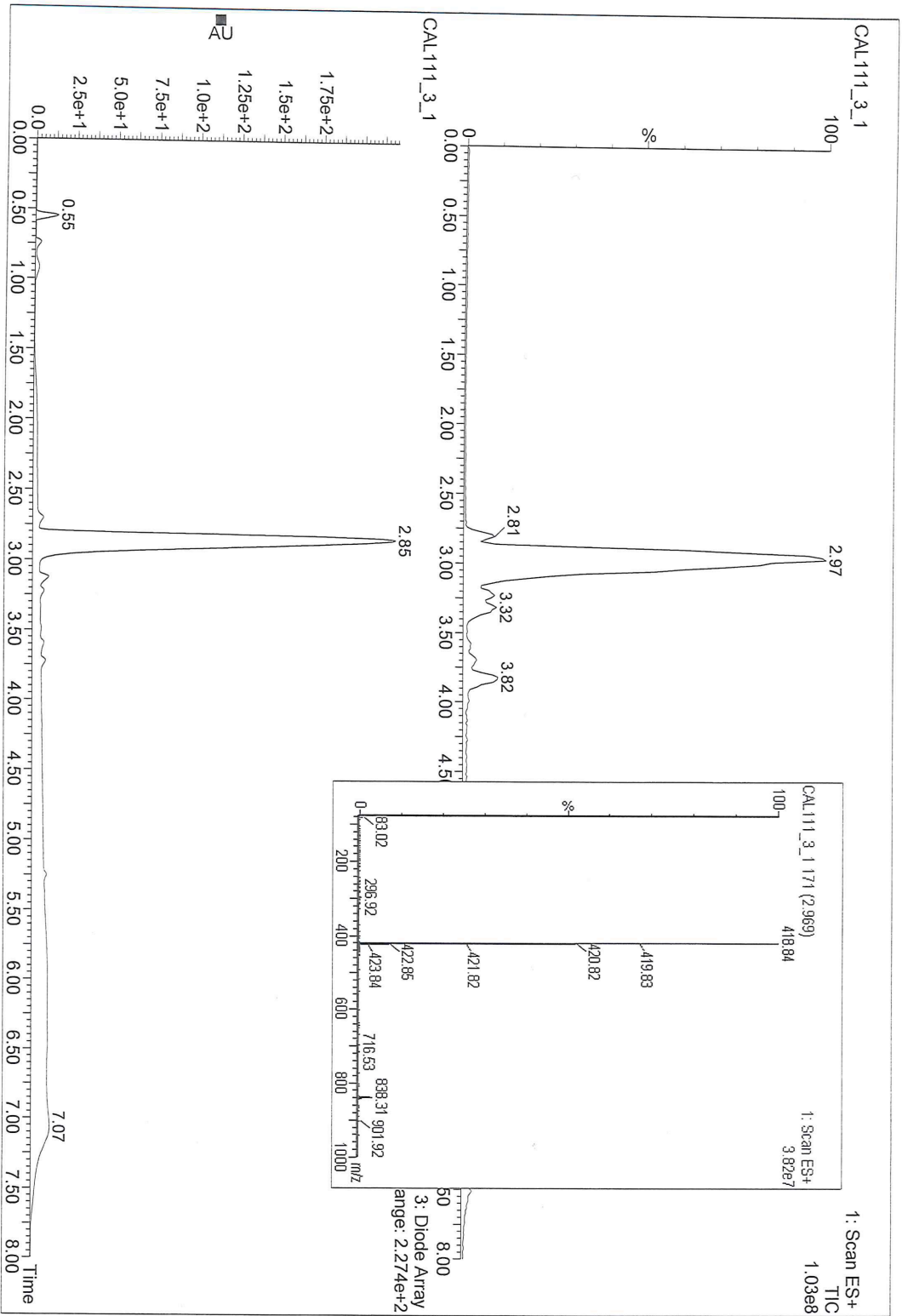
U final). After 5 min of preincubation at 25 °C, the reaction was then initiated by the injection of 50 µL of 10 mM acetylthiocholine iodide solution. The hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine, at a wavelength of 412 nm using a 96-well microplate plate reader (TECAN Infinite M200, Lyon, France). Test compounds were dissolved in analytical grade DMSO. Donepezil was used as a reference standard. The rate of absorbance increase at 412 nm was followed every minute for 10 min. Assays were performed with a blank containing all components except acetylthiocholine, in order to account for non-enzymatic reaction. The reaction slopes were compared and the percent inhibition due to the presence of test compounds was calculated by the following expression: $100 - (v_i/v_0 \times 100)$ where v_i is the rate calculated in the presence of inhibitor and v_0 is the enzyme activity.

First screening of AChE activity was carried out at a 10^{-6} or 10^{-5} M concentration of compounds under study. For the compounds with significant inhibition ($\geq 50\%$), IC_{50} values were determined graphically by plotting the % inhibition versus the logarithm of six inhibitor concentrations in the assay solution using the Origin software.

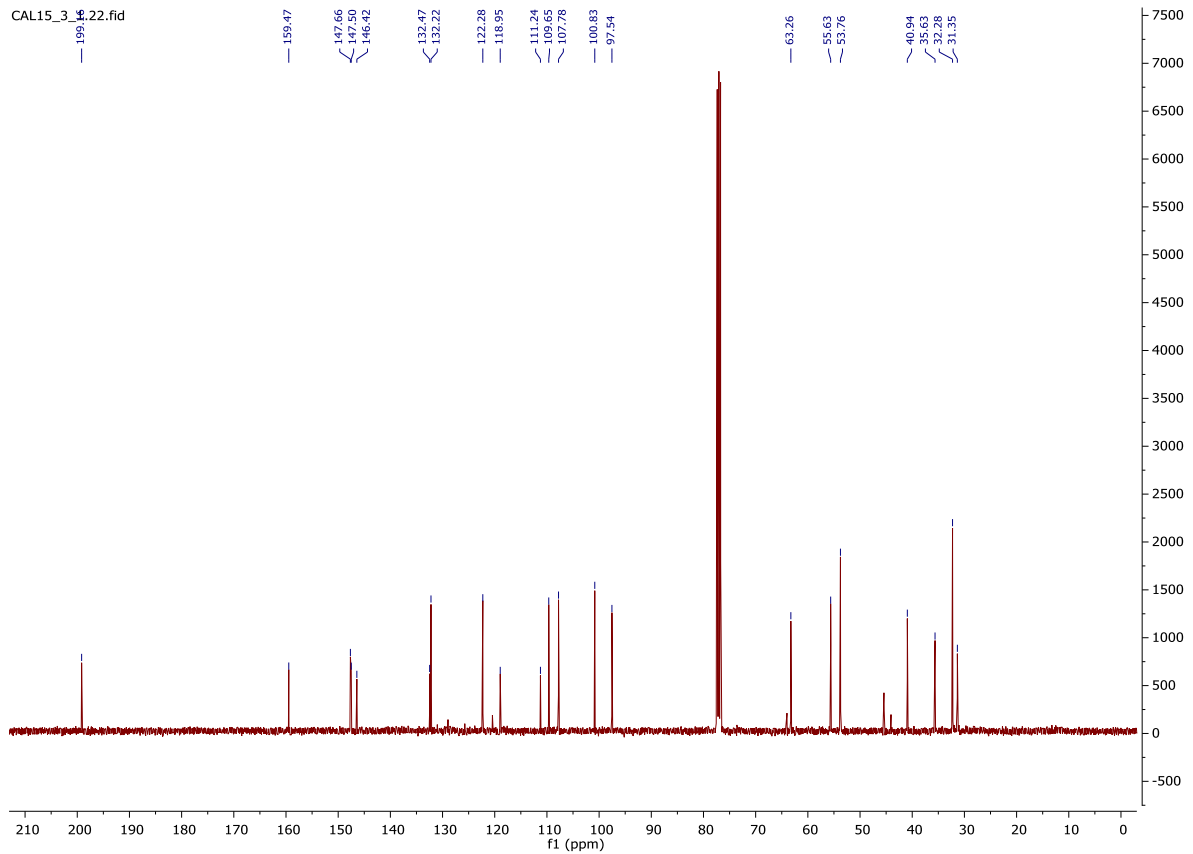
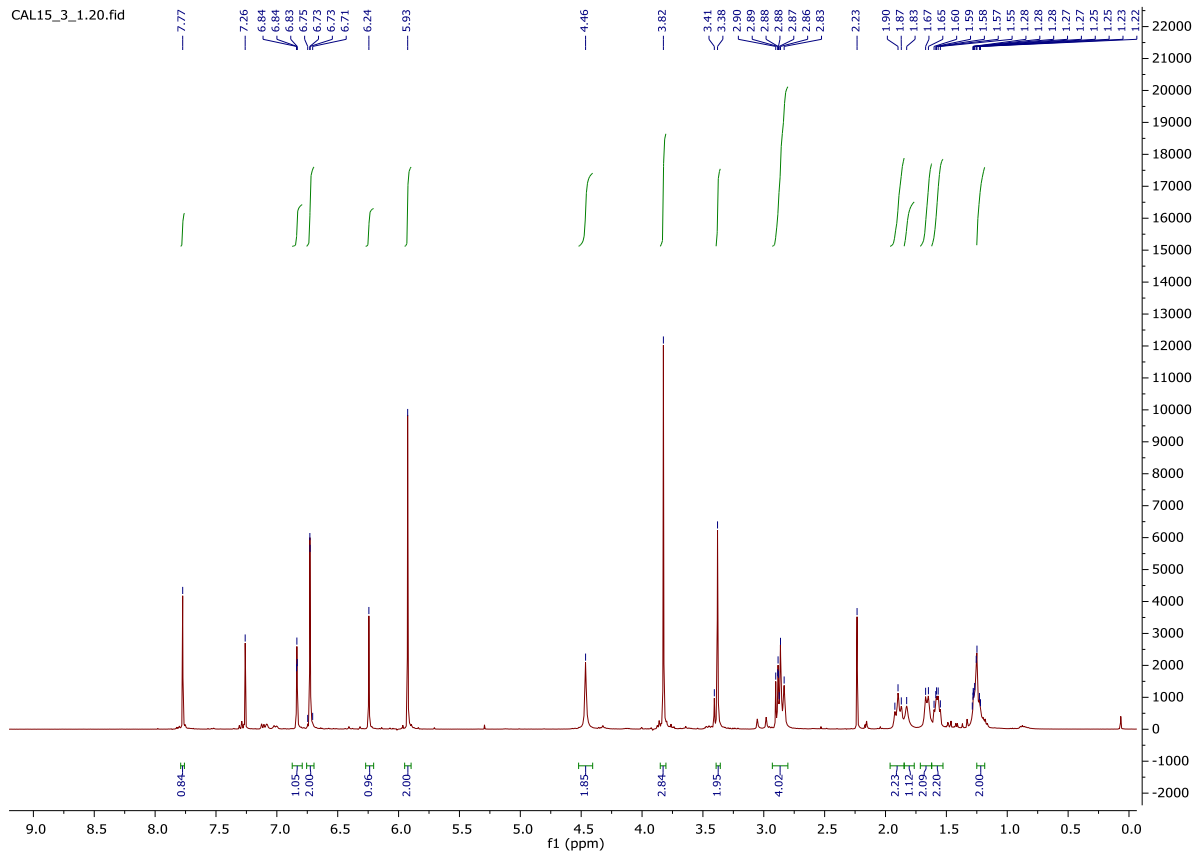


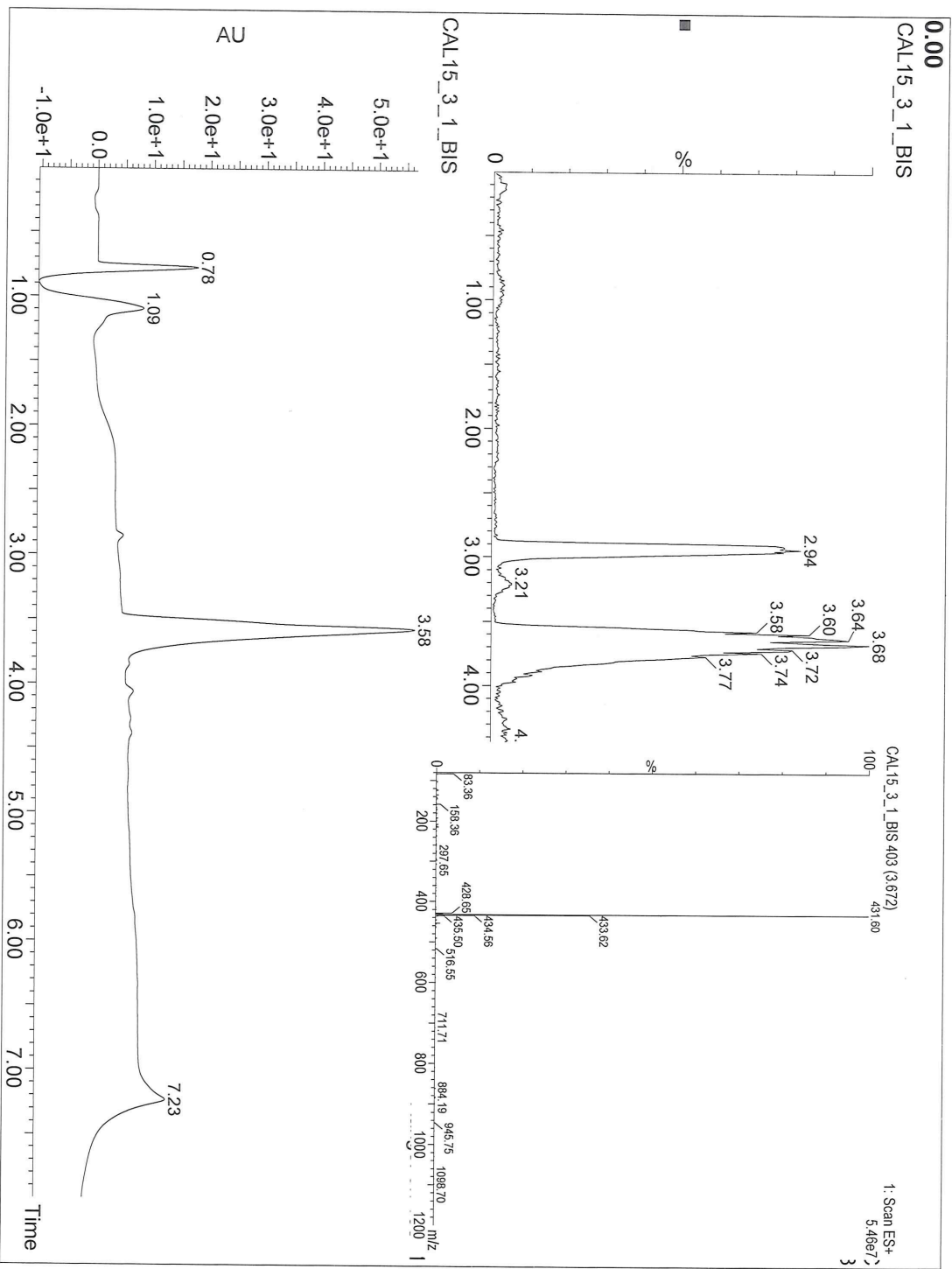
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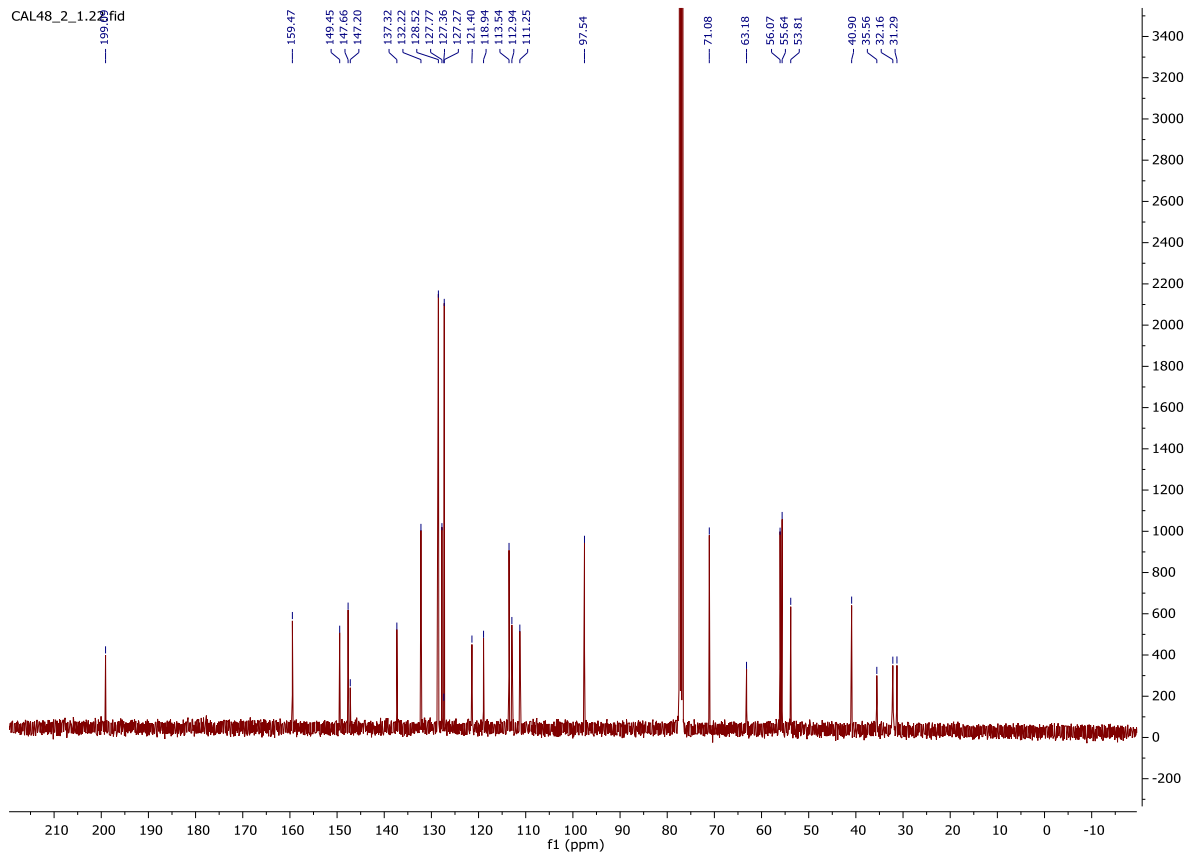
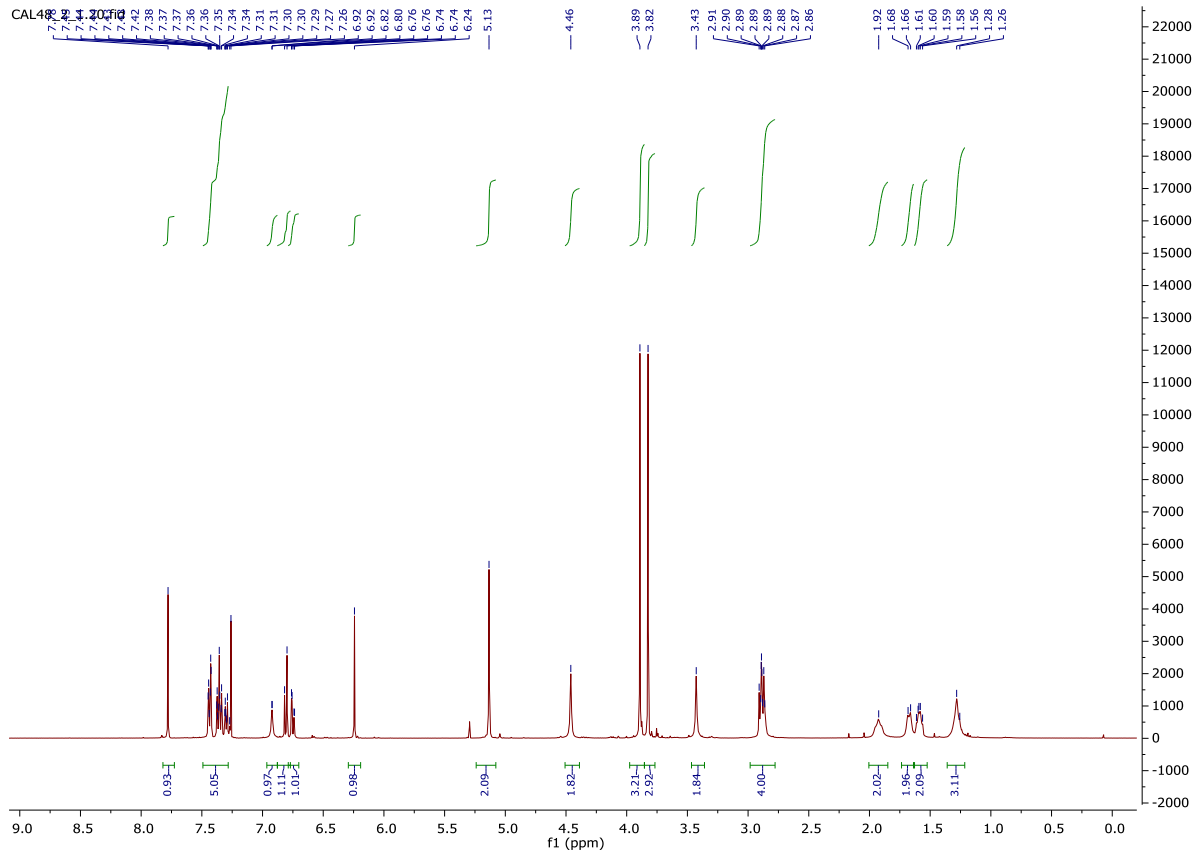


1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-(1,3-benzodioxol-5-ylmethyl)-4-piperidyl]propan-1-one (9c)



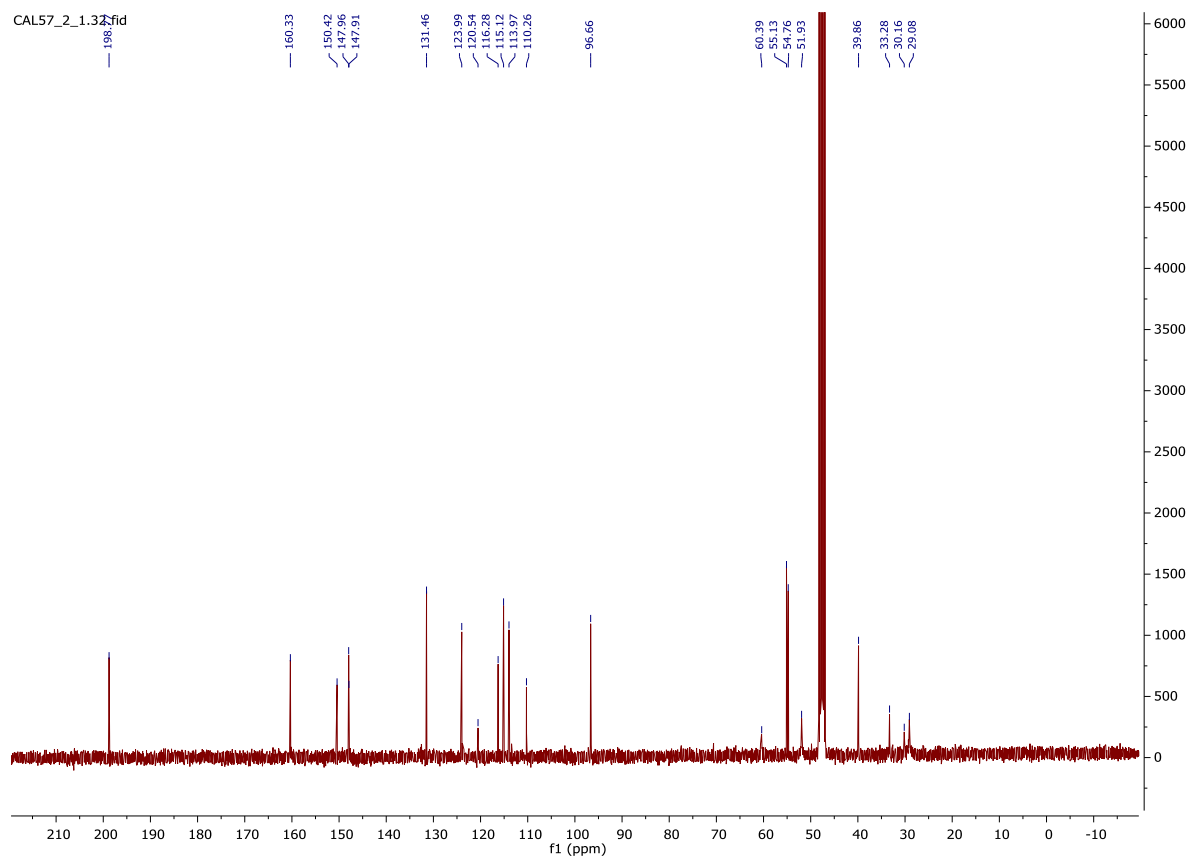
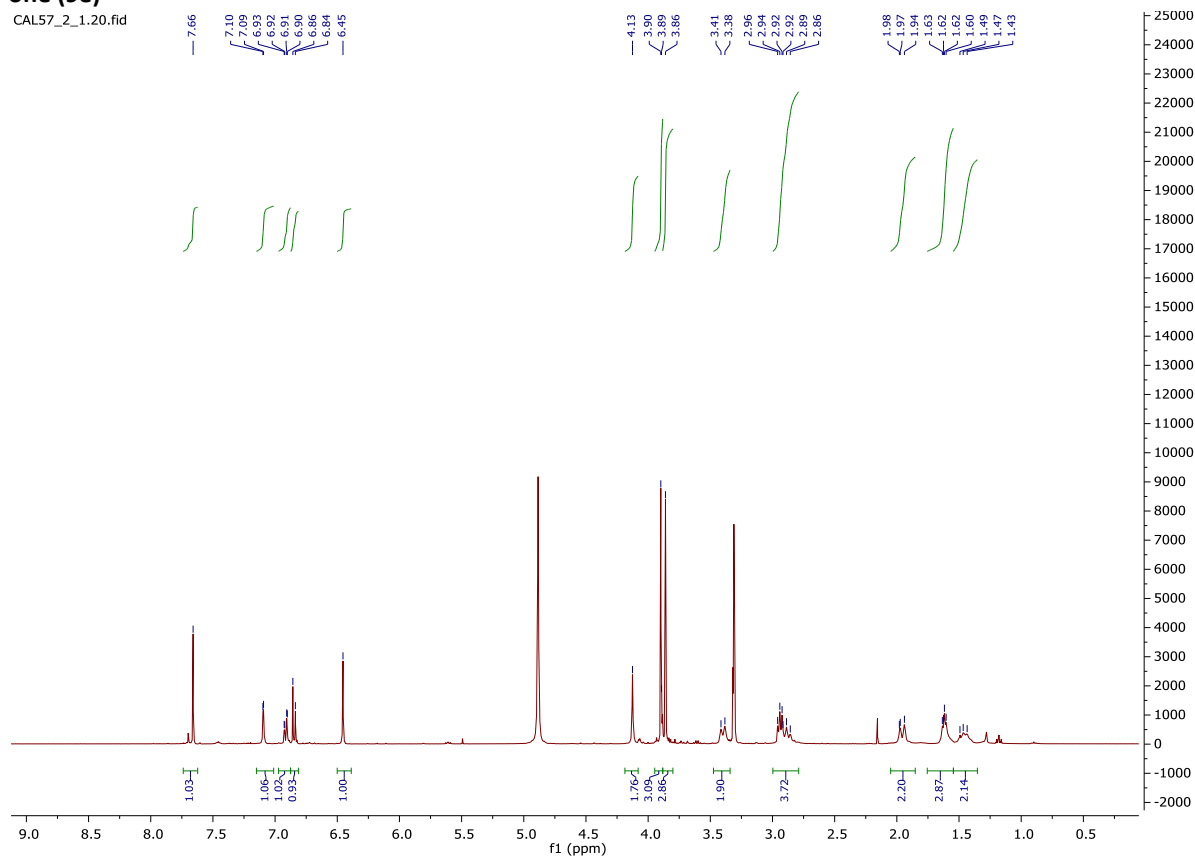


1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-[[4-(benzyloxy)-3-methoxyphenyl]methyl]piperidin-4-yl)propan-1-one (9d)



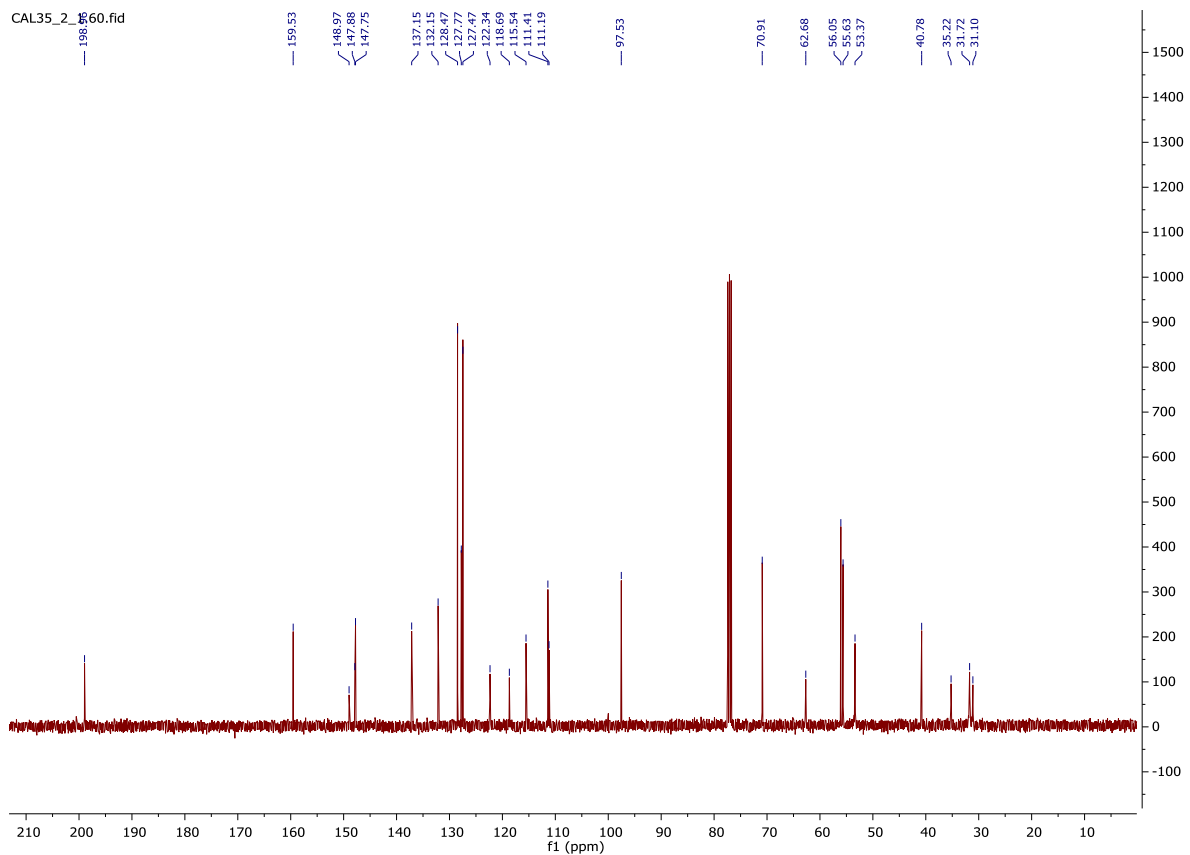
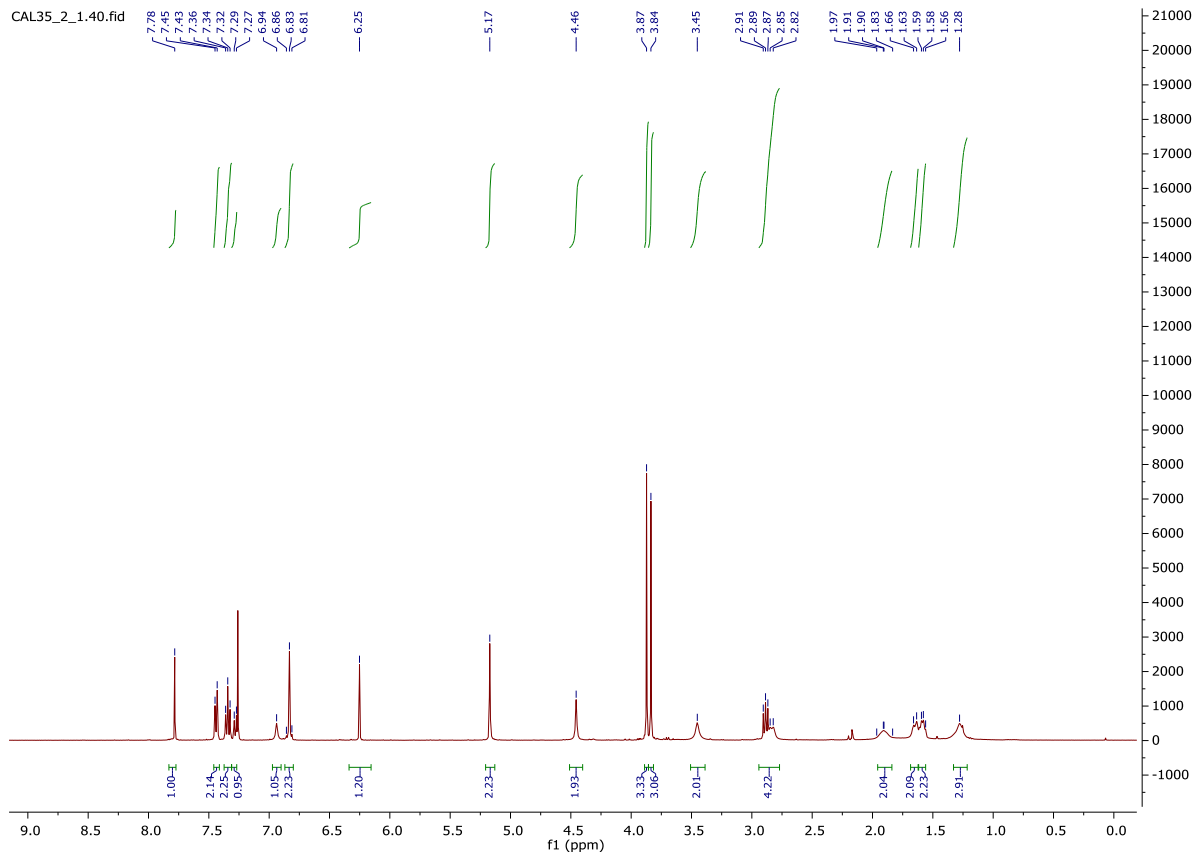
La partie de l'image avec l'ID de relation rd28 n'a pas été trouvée dans le fichier.

1-(4-amino-5-chloro-2-methoxyphenyl)-3-{1-[(4-hydroxy-3-methoxyphenyl)methyl]piperidin-4-yl}propan-1-one (9e)



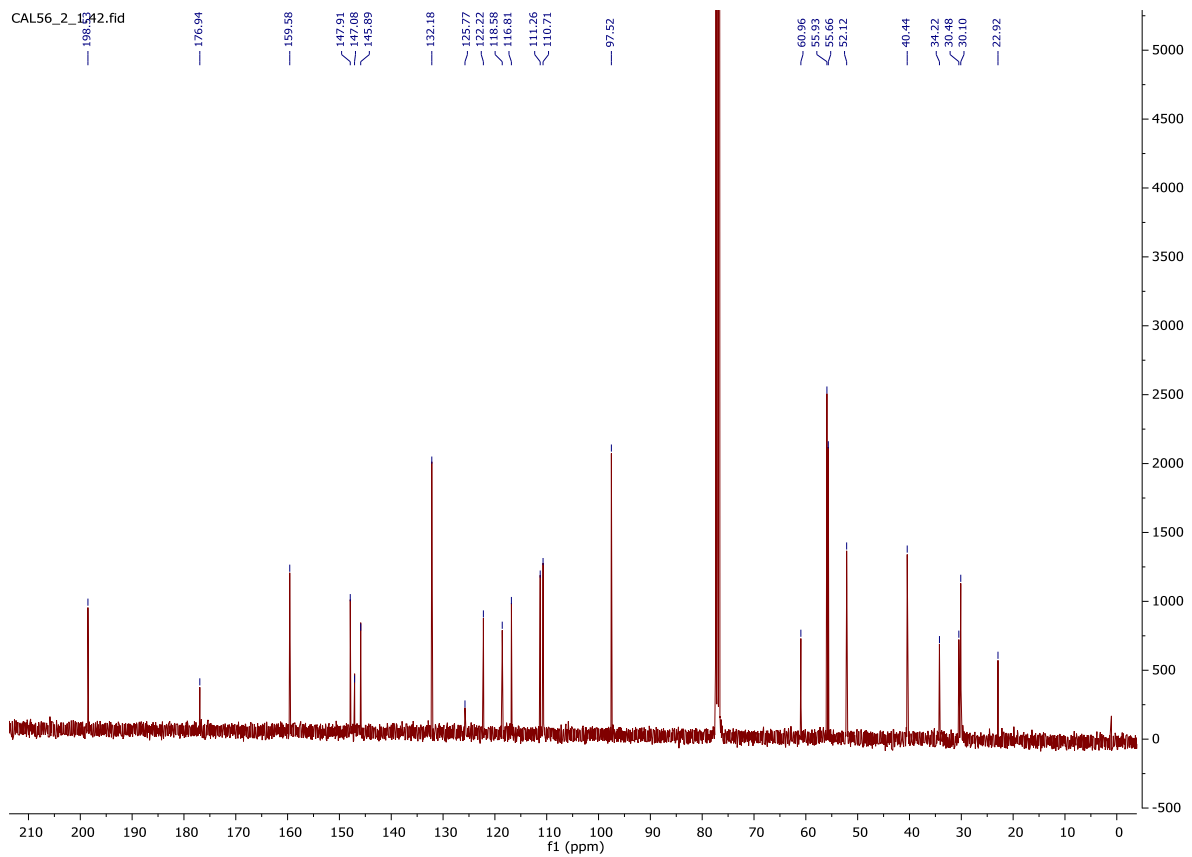
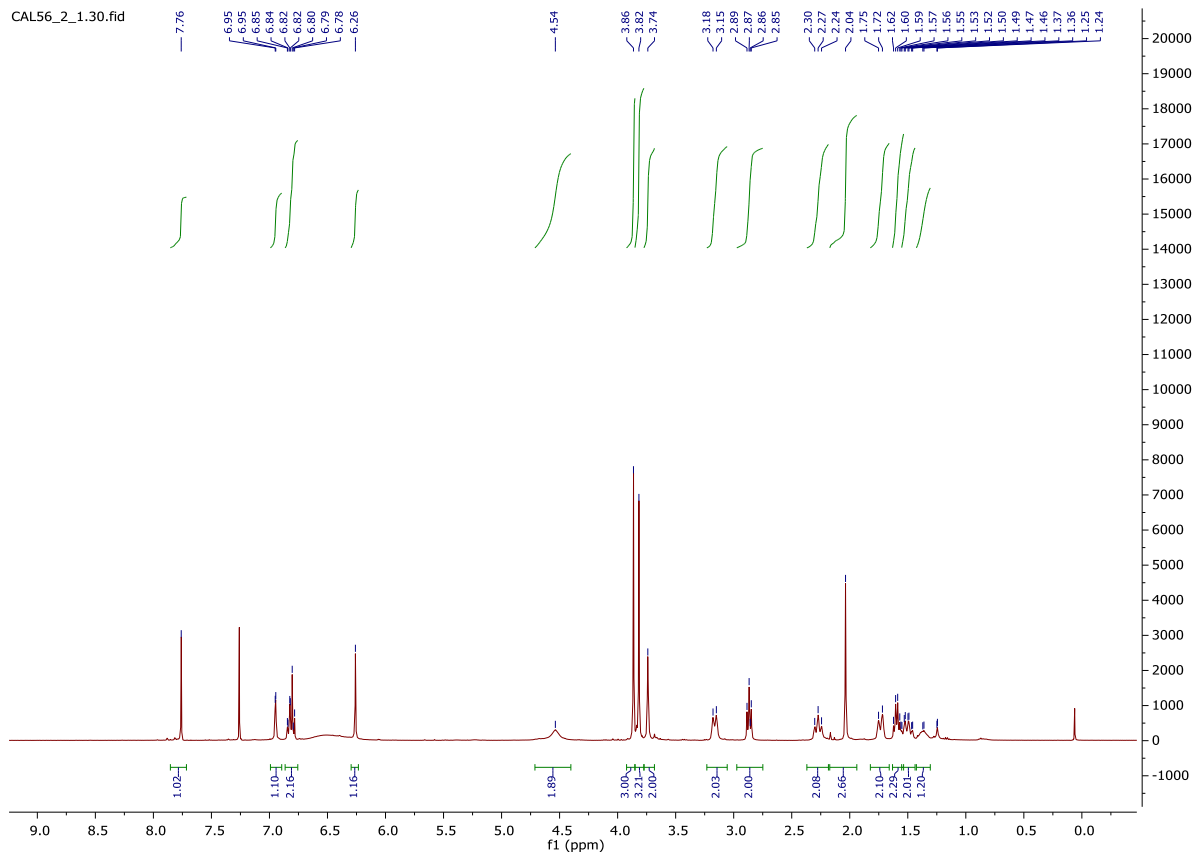
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1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-([3-(benzyloxy)-4-hydroxyphenyl]methyl)piperidin-4-yl)propan-1-one (9f)



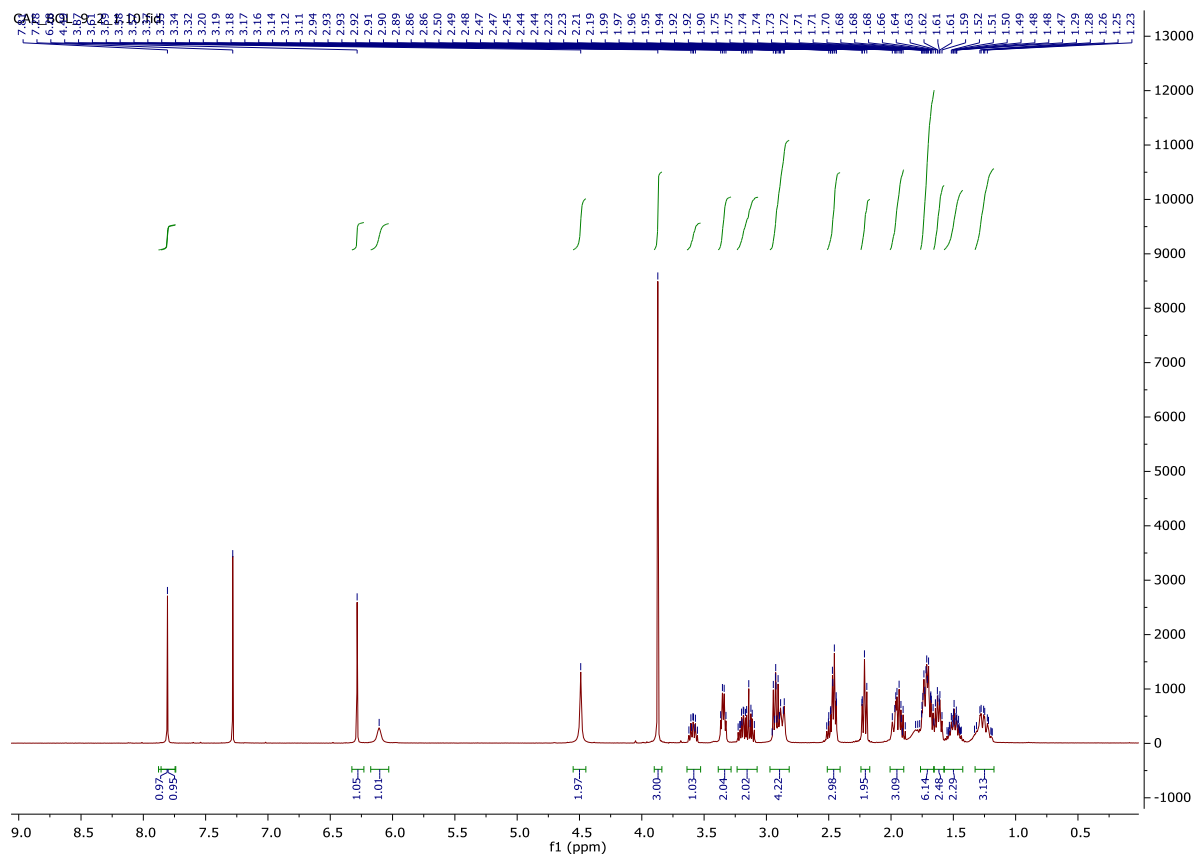
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1-(4-amino-5-chloro-2-methoxyphenyl)-3-{1-[(3-hydroxy-4-methoxyphenyl)methyl]piperidin-4-yl}propan-1-one (9g)

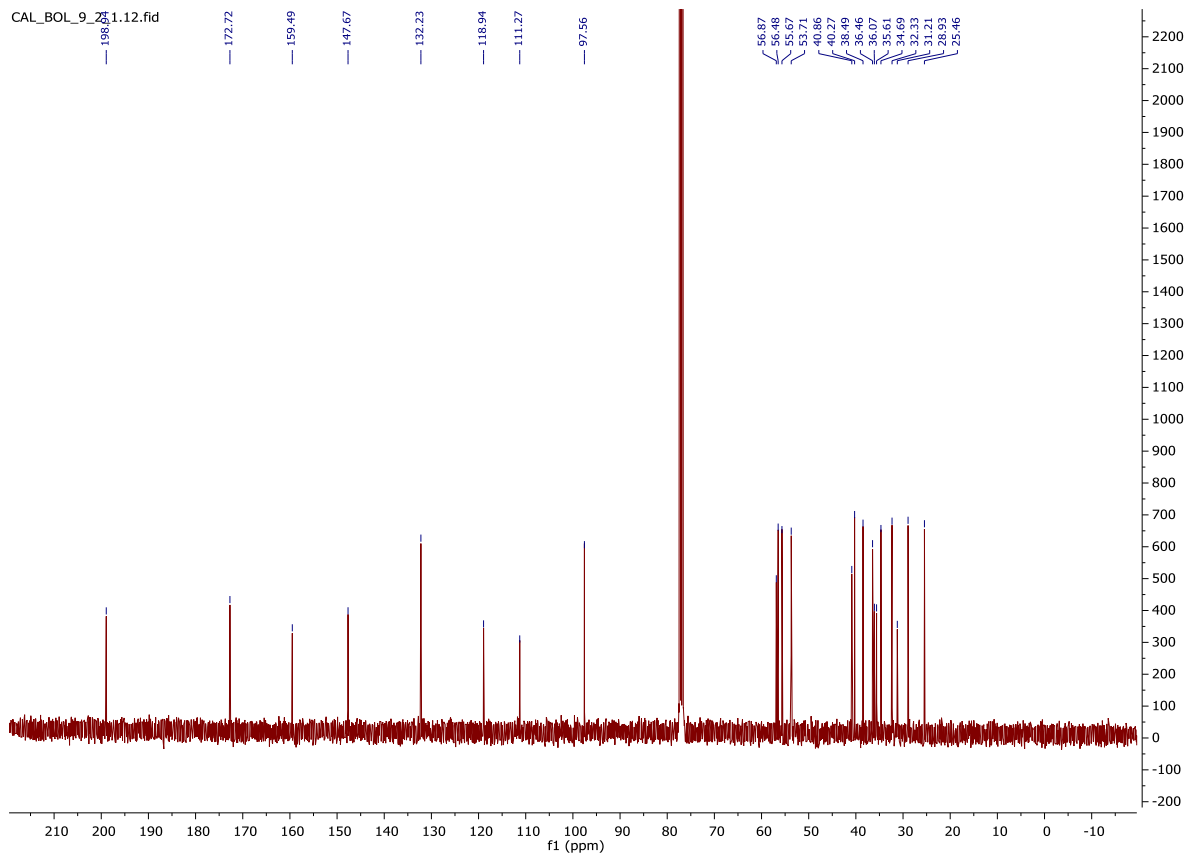


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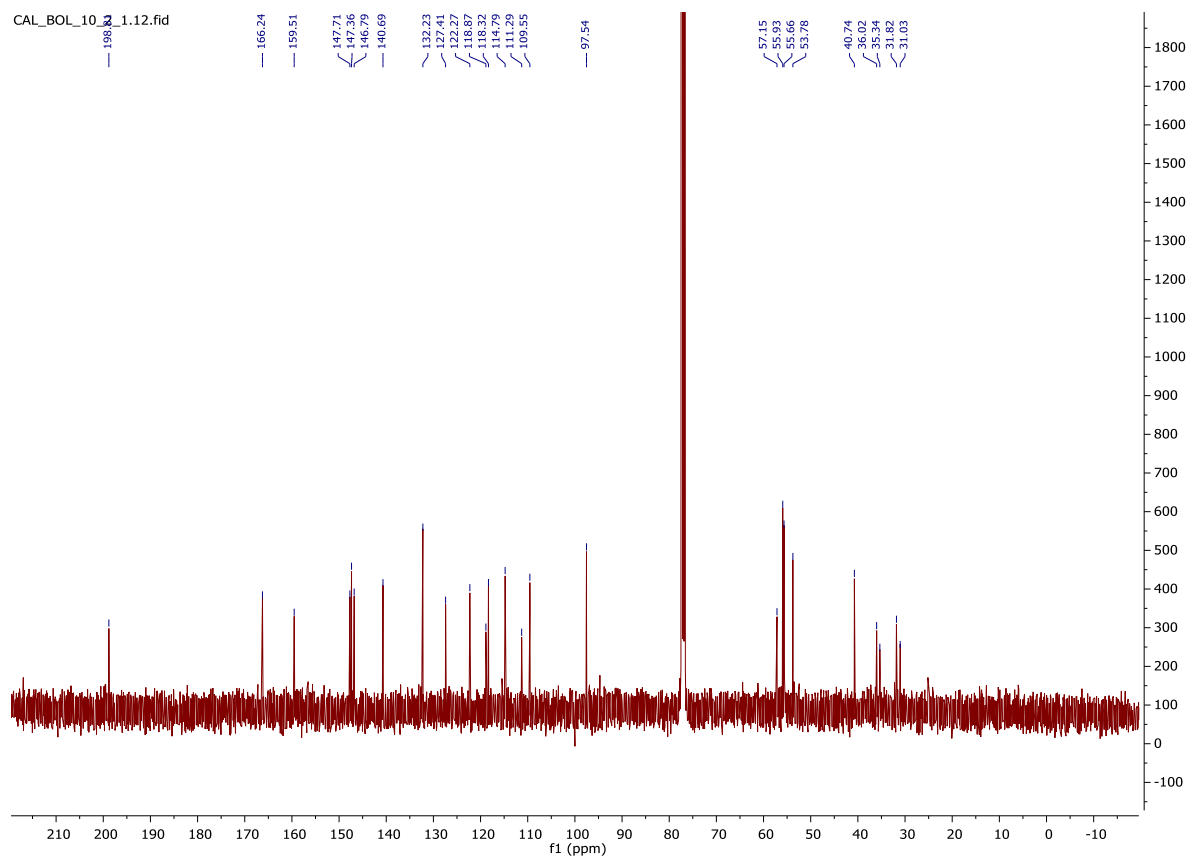
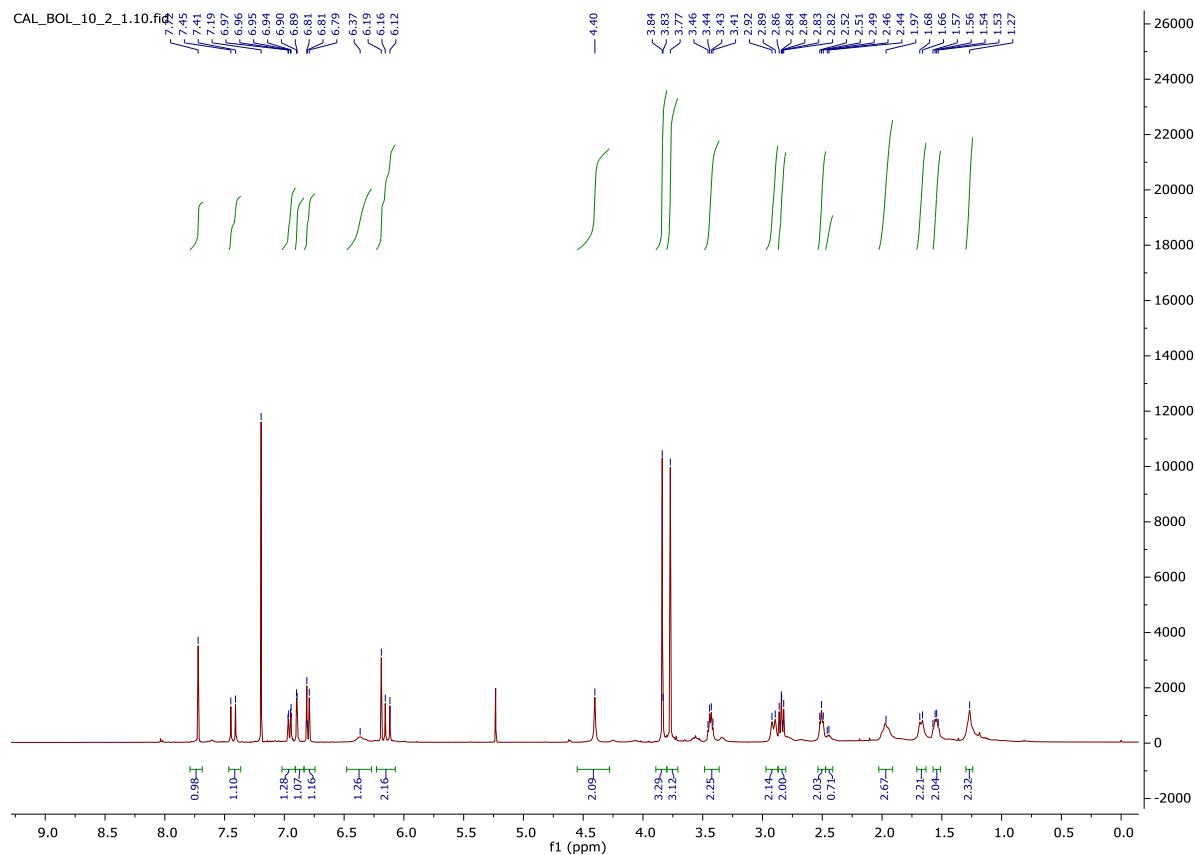
***N*-(2--{4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidin-1-yl}ethyl)-5-[(3*R*)-1,2-dithiolan-3-yl]pentanamide (10a)**



La partie de l'image avec l'ID de relation rd28 n'a pas été trouvée dans le fichier.



(2E)-N-(2-{4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl]piperidin-1-yl}ethyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enamide (10b)



La partie de l'image avec l'ID de relation rd28 n'a pas été trouvée dans le fichier.