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# *New Bifunctional Antioxidant/σ Agonist Ligands: Preliminary Chemico-physical and Biological Evaluation*

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KEYWORDS. $\sigma_1$ receptors, oxidative stress, neuroprotection, chemical and enzymatic stabilities, antioxidants, lipoic acid.

**ABSTRACT:** We previously reported bifunctional sigma-1 ($\sigma_1$) ligands endowed with antioxidant activity (**1** and **2**). In the present paper, pure enantiomers (*R*)-**1** and (*R*)-**2** along with the corresponding *p*-methoxy (**6**, **11**), *p*-fluoro derivatives (**7**, **12**) were synthesized. $\sigma_1$ and $\sigma_2$ affinities,

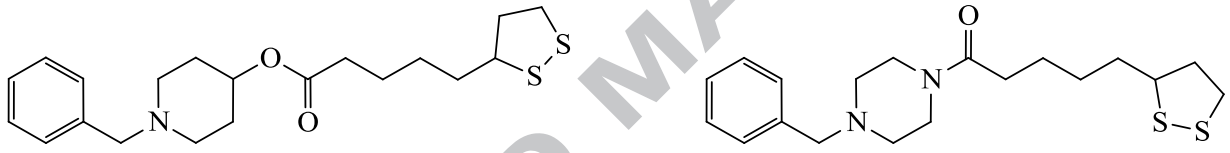
antioxidant properties, and chemico-physical profiles were evaluated. Para derivatives, while maintaining strong $\sigma_1$ affinity, displayed improved $\sigma_1$ selectivity compared to the parent compounds **1** and **2**. *In vivo* evaluation of compounds **1**, **2**, (*R*)-**1**, **7**, and **12** showed $\sigma_1$ agonist pharmacological profile. Chemico-physical studies revealed that amides **2**, **11** and **12** were more stable than corresponding esters **1**, **6** and **7** under our experimental conditions. Antioxidant properties were exhibited by fluoro derivatives **7** and **12** being able to increase total antioxidant capacity (TAC). Our results underline that *p*-substituents have an important role on $\sigma_1$ selectivity, TAC, chemical and enzymatic stabilities. In particular, our data suggest that new very selective compounds **7** and **12** could be promising tools to investigate the disorders in which $\sigma_1$ receptor dysfunction and oxidative stress are contemporarily involved.

## 1. Introduction

Originally, the sigma (σ) receptors were thought to be a subtype of opioid receptors [1]. However, further studies reclassified these receptors as a new unique binding site. In the early 90's two different receptor subtypes were recognized, termed sigma-1 ($\sigma_1$) and sigma-2 ($\sigma_2$) [2]. Both of them are highly expressed in peripheral organs and in the central nervous system, even if with slightly different distribution. They possess different molecular weight, drug selectivity patterns, and signaling pathways [2; 3]. Unlike $\sigma_2$ receptors, the $\sigma_1$ subtype has been cloned, is well characterized, and very recently was reported the crystal structure of the human $\sigma_1$ receptor [4]. It is located at the mitochondria-associated endoplasmic reticulum membrane (MAM) where, acting as molecular chaperone, modulates calcium signaling [5-7]. Several reports have documented the involvement of this subtype in many physiological and pathological pathways such as immune reactions [8], neuronal plasticity [9], cognitive capacities [10; 11], pain [12; 13], neuroinflammation [14], psychiatric and neurodegenerative disorders [15-18]. Recent studies have also suggested that activation of $\sigma_1$ receptors is implicated in protection against oxidative stress [19-23]. In this regard, the $\sigma_1$ agonist PRE084 (2-morpholin-4-ylethyl 1-phenylcyclohexane-1-carboxylate) increased the levels of cellular antioxidants by activating the NF-kB pathway [24]. It has been observed that knockdown of $\sigma_1$ receptors is able to promote an accumulation of reactive oxygen species (ROS) [19; 20], while their up-regulation or activation by agonists produces a suppression of oxidative stress [21]. Increased ROS levels and decreased expression of endogenous antioxidant systems were also found in Müller cells harvested from $\sigma_1$ receptors-knockout mice [22]. Even if the mechanism remains to be elucidated, the $\sigma_1$ modulation of calcium release from endoplasmic reticulum (ER) and its chaperoning activities seem to play an important role in counteracting oxidative stress. Moreover, $\sigma_1$ receptors mediate the activation of the antioxidant response elements (ARE) which in turn up-regulates the expression of downstream genes, such as

NAD(P)H:quinone oxidoreductase 1 and superoxide dismutase 1, able to counterbalance oxidative stress [23]. Thus, $\sigma_1$ agonists selective toward the related $\sigma_2$ receptors could represent potential therapeutic agents for the treatment of oxidative stress related neurodegenerative diseases. However, since these disorders mostly involve multiple pathways and are characterized by a multifactorial etiology arising from the interaction of genetic, epigenetic, environmental, and endogenous factors, a multi-target approach is preferred.

Therefore, our purpose is the design of bifunctional ligands composed by a $\sigma_1$ selective portion linked to a well-known antioxidant moiety. We have already reported a set of novel bifunctional antioxidants/$\sigma_1$ ligands (**1** and **2,** Chart **1**) [25].



**1**

$K_i\sigma_1$ = 5.5 ± 0.5 nM
$K_i\sigma_2$ = 2,302 ± 45 nM

**2**

$K_i\sigma_1$ = 1.8 ± 0.2 nM
$K_i\sigma_2$ = 637 ± 15 nM

**Chart 1. $\sigma_1$** Selective ligands containing the (*R*/*S*) lipoyl function.

In the present paper, we designed and synthesized novel compounds obtained by linking the strong natural antioxidant α-lipoic acid (ALA) and substituted phenyl- piperidine or piperazine scaffolds well known as $\sigma_1$ pharmacopores. Furthermore, since (*R*)-α-lipoic acid ((*R*)-ALA) is the only naturally occurring enantiomer, the only active form in biological systems, and possesses a favorable pharmacokinetic profile [26-29] we synthesized (*R*)-**1** and (*R*)-**2** enantiomers. In particular, it has been reported that for animal and human uses the administration of (*R*)-ALA is superior to its racemate inducing a more effective and faster biological response [27]. To determine

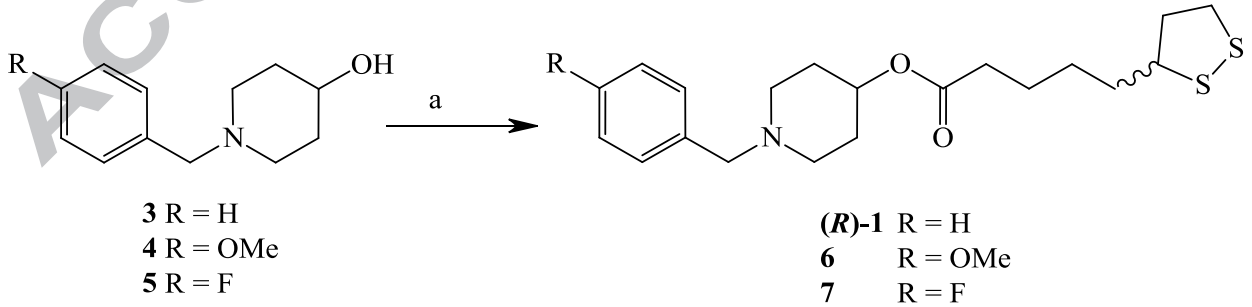
the agonist or antagonist profile of the most interesting compounds in the series, we also performed *in vivo* studies [30].

Herein, we also aimed to assess the cytotoxicity potentials of novel reported compounds in primary rat cerebral cortex neuron cultures in order to establish their potential use in pharmaceutical applications. Cell injury was evaluated by measuring cell viability with methylthiazol tetrazolium (MTT) assay, and by determining the release of intracellular lactate dehydrogenase. Total antioxidant capacity (TAC) and total oxidative stress (TOS) levels were also determined to evaluate the oxidative alterations. Finally, the chemical and enzymatic stabilities of compounds **1** and **2**, and their respective *p*-methoxy and *p*-fluoro substituted analogs were evaluated by *in vitro* pharmacokinetics studies, in rat and human plasma.

## 2. Materials and methods

### *2.1. Chemistry*

The synthetic procedure to compounds (*R*)-**1,** (*R*)-**2**, **6**-**7**, and **11**-**12** is reported in Schemes **1** and **2**. Briefly, condensation of ALA or (*R*)-ALA with the appropriate 1-(4-substitutedbenzyl)piperidin-4-ol (**3-5**) afforded (*R*)-**1**, **6**, and **7**. The reaction was carried out in dry dichloromethane (DCM) with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) as a nucleophilic catalyst (Scheme **1**). Compounds **4** and **5** were synthesized as previously reported [31].

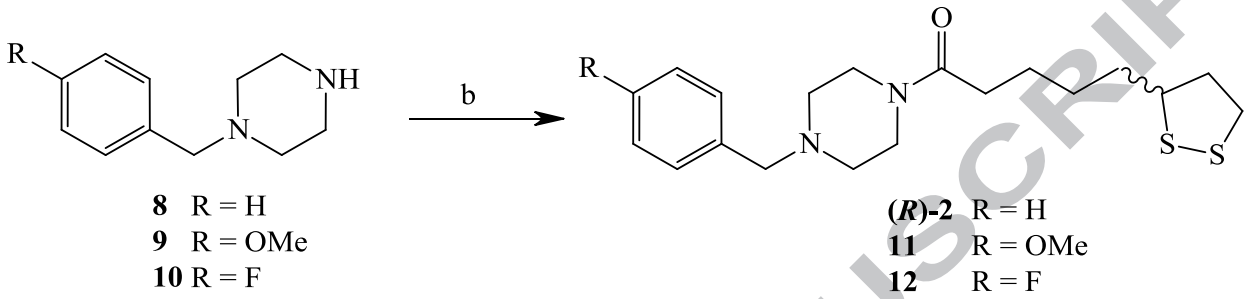



**3** R = H
**4** R = OMe
**5** R = F

**(*R*)-1** R = H
**6** R = OMe
**7** R = F

**Scheme 1.** Reagents and conditions: (a) (*R*)-ALA or ALA, DCC, DCM, DMAP, 3 h at room temperature (rt).

Acylation of commercially available piperazines **8-10** with ALA or (*R*)-ALA using the above reported procedure without using 4-(*N,N*-dimethylamino)pyridine as catalyst gave (*R*)-**2**, **11**, and **12** (Scheme **2**).




**8** R = H
**9** R = OMe
**10** R = F

**(*R*)-2** R = H
**11** R = OMe
**12** R = F

**Scheme 2.** Reagents and conditions: (b) (*R*)-ALA or ALA, DCC, DCM, 3 h rt.

*2.1.1. Experimental*

All commercial chemicals used for synthesis were purchased from Sigma-Aldrich Company (Milan, Italy) and utilized as received without any further purification. Reactions were monitored by thin-layer chromatography (TLC) on precoated silica gel 60 $F_{254}$ aluminum sheets (Merck, Darmstadt, Germany) and the spots were visualized under UV light or in an iodine chamber. Flash column chromatography was performed using Merck silica gel 60 (230-400 mesh, Merck). Melting points were determined in open capillary tubes on digital Electrothermal apparatus 9100 (Rochford, UK) and are reported uncorrected. Optical rotations were determined in MeOH solution with a Perkin-Elmer 241 polarimeter. $^{1}$H NMR and $^{13}$C-NMR spectra were recorded with a Varian Inova 200 and 500 MHz spectrometer. Proton chemical shifts were reported in ppm ($\delta$) relative to the internal standard tetramethylsilane (TMS, $\delta$= 0.00 ppm). Carbon chemical shifts were reported in ppm ($\delta$) relative to the residual solvent signal ($CDCl_3$, $\delta$= 77.0 ppm, dimethyl sulfoxide-$d_6$, $\delta$= 39.5 ppm or $CD_3OD$-$d_4$, $\delta$= 49.2 ppm). Gas chromatography-mass spectroscopy (GC-MS) analyses were recorded using a Shimadzu QP500 EI 171 (70 eV). Elemental analyses (C, H, N), performed on an elemental analyser Carlo Erba Model 1106 (Carlo Erba, Milan, Italy), confirmed that the samples had a purity equal to or greater than 95%; the analytical results were within ± 0.4% of the

theoretical values (Table S2). The radioactive materials (+)-*cis*-*N*-2-dimethylallyl-*N*-normetazocine ([$^3$H]-(+)-Pentazocine; specific activity 29 Ci/mmol) and 1,3-di-(2-tolyl)-guanidine ([$^3$H]-DTG) (specific activity 53.3 Ci/mmol) were purchased from Perkin Elmer (Milan, Italy). Compounds **4** and **5** were synthesized following procedures described in the literature [31].

*2.1.2. 1-Benzylpiperidin-4-yl-5-[(3R)-1,2-dithiolan-3-yl]pentanoatechloride ((R)-**1**).*

To a solution of benzylpiperidin-4-ol (**3**) (0.185 g, 0.97 mmol), (*R*)-ALA (0.2 g, 0.97 mmol), and DMAP(11.85 mg, 0.097 mmol) in 2.43 ml of dry DCM at 0 °C was added 0.2 g of DCC (0.97 mmol) under stirring and nitrogen atmosphere. The crude mixture was concentrated and purified by flash chromatography using cyclohexane/EtOAc (6:4) as mobile phase. The free base was converted into corresponding hydrochloride salt with 4 N HCl in dioxane and recrystallized from diethyl ether/methanol. Yield = 43%; $R_f$ = 0.5 (AcOEt/cyclohexane, 8:2); mp = 173 °C dec; MS *m*/*z* $[M]^+$ =379.15; $[\alpha]_D^{25}$ + 62° (*c* 1.0, $CH_3OH$); $^1$H NMR (200 MHz, $CDCl_3$) $\delta_H$ 1.37−1.76 (m, 6H), 1.82−1.93 (m, 4H), 2.17−2.35 (m, 4H), 2.39−2.54 (m, 2H), 2.66-2.71 (m, 2H), 3.05-3.26 (m, 2H), 3.50 (s, 2H), 3.57−3.64 (m, 1H), 4.73-4.86 (m, 1H), 7.26-7.33 (m, 5H);$^{13}$C NMR (500 MHz, $CD_3OD$-$d_4$) δ 174.1, 132.6, 131.5, 130.6, 130.3, 65.3, 61.9, 57.7, 51.6, 41.5, 39.5, 35.8, 35.1, 29.9, 29.3, 28.3, 25.8. Anal. ($C_{20}H_{29}NO_2S_2$·HCl) C, H, N, S.

*2.1.3. 1-(4-Methoxybenzyl)piperidin-4-yl-5-(1,2-dithiolan-3-yl)pentanoate chloride (**6**).*

This compound was synthesized using 1-(4-methoxybenzyl)piperidin-4-ol (**4**) (0.53 g, 2.42 mmol), ALA (0.5 g, 2.42 mmol), DMAP (29.57 mg, 0.242 mmol), and DCC (2.42 mmol) in 6 ml of dry DCM by means of the same procedure above reported for (*R*)-**1**. Purification of the crude compound was performed by flash silica gel chromatography using EtOAc/cyclohexane (5:5) and afforded a yellow oil. The free base was converted into the corresponding hydrochloride salt with 4 N HCl in dioxane. Yield = 44.5%; $R_f$ = 0.6 [EtOAc/EtOH, 8:2]; mp = 176.3-177.6 °C; MS *m*/*z* $[M]^+$ = 410.7; $^1$H NMR (200 MHz, $CDCl_3$) $\delta_H$ 1.37−1.74 (m, 6H), 1.83−1.99 (m, 4H), 2.17−2.34 (m, 4H), 2.39−2.54 (m, 2H), 2.62-2.72 (m, 2H), 3.05-3.25 (m, 2H), 3.44 (s, 2H), 3.50−3.64 (m, 1H), 3.80 (s,

3H), 4.72-5.30 (m, 1H), 6.82-6.90 (m, 2H), 7.20-7.27 (m, 2H).$^{13}$C NMR (200 MHz, $CDCl_3$) δ 171.9, 160.9, 132.9, 119.7, 114.6, 64.3, 60.7, 56.3, 55.3, 47.3, 40.2, 38.5, 34.5, 34.1, 28.7, 26.9, 24.6. Anal. ($C_{21}H_{31}NO_3S_2$·HCl) C, H, N, S.

*2.1.4. 1-(4-Fluorobenzyl)piperidin-4-yl 5-(1,2-dithiolan-3-yl)pentanoate chloride (**7**).*

This compound was prepared through the procedure described for compound **6**, using as starting material, 1-(4-fluorobenzyl)piperidin-4-ol (0.55 g, 2.63 mmol) (**5**) and ALA (0.54 g, 2.63 mmol). The crude compound was purified by flash chromatography on silica gel using EtOAc/cyclohexane (6:4) and afforded a yellow oil. The free base was converted into the corresponding hydrochloride salt with 4 N HCl in dioxane. Yield = 49%; $R_f$ = 0.57 [EtOAc]; mp = 175.9-176.9 °C; MS *m*/*z* $[M]^+$ = 398.1; $^1$H NMR (200 MHz, $CDCl_3$) $\delta_H$1.41−1.76 (m, 6H), 1.82−2.05 (m, 4H), 2.18−2.35 (m, 4H), 2.42−2.51 (m, 2H), 2.61 and 2.76 (m, 2H), 3.10-3.20 (m, 2H), 3.47 (s, 2H), 3.50−3.57 (m, 1H), 4.75-4.81 (m, 1H), 6.94-7.04 (m, 2H), 7.23-7.31 (m, 2H); $^{13}$C NMR (200 MHz, $CDCl_3$) δ 173.1, 162.8, 134.0, 131.2, 115.2, 71.7, 62.1, 56.0, 48.3, 40.1, 38.4, 34.7, 34.5, 34.0, 28.7, 25.0. Anal. ($C_{20}H_{28}FNO_2S_2$·HCl) C, H, N, S.

*2.1.5. 1-(4-Benzylpiperazin-1-yl)-5-[(3R)-1,2-dithiolan-3-yl]pentan-1-one chloride ((R)-**2**).*

A solution of (*R*)-ALA (0.2 g, 0.97 mmol) in anhydrous DCM (3.88 ml) was added dropwise to a solution of DCC (0.2 g, 0.97 mmol) in the same solvent (3.88 ml) under stirring and nitrogen atmosphere. After 10 min, a solution of 1-benzylpiperazine (**8**) (0.24 g, 0.97 mmol) in DCM (1.94 ml) was added dropwise, and the resulting mixture was stirred for 3 h. The *N*,*N* -

-glass Büchner funnel, and the filtrate was washed with sodium bicarbonate 5% solution (2 × 10 ml) and brine (2 × 10 ml). After being dried over $Na_2SO_4$, the solvent was removed under reduced pressure. The organic phase was concentrated and the crude product was purified by flash chromatography on silica gel using EtOAc/cyclohexane 7:3 as mobile phase. The free base was converted into the corresponding hydrochloride salt with 4 N HCl in dioxane and recrystallized from diethyl ether/methanol to give (*R*)-**2**. Yield = 43.8%; $R_f$ = 0.7 [dichloromethane/$CH_3OH$, 9:1]; mp = 188.6 °C dec; MS *m*/*z* $[M]^+$ =

364.30; $[\alpha]_D^{25}$ + 26°(c 1.0, $CH_3OH$); $^1H$ NMR (200 MHz, $CDCl_3$) $\delta_H$ 1.42-1.51 (m, 2H), 1.54-1.76 (m, 4H), 1.83-2.00 (m, 1H), 2.28-2.54 (m, 7H), 3.05-3.25 (m, 2H), 3.43-3.65 (m, 7H), 7.32-7.38 (m, 5H). $^{13}C$ NMR (500 MHz, $CD_3OD$-$d_4$) δ 174.1, 132.6, 131.6, 130.6, 130.0, 61.8, 57.8, 52.7, 43.6, 41.5, 39.7, 39.5, 35.9, 33.5, 30.10, 26.0. Anal. ($C_{19}H_{28}N_2OS_2$·HCl) C, H, N, S.

*2.1.6. 5-(1,2-Dithiolan-3-yl)-1-[4-(4-methoxybenzyl)piperazin-1-yl]pentan-1-onechloride (**11**).* This compound was synthesized using ALA (1 g, 4.85 mmol) in DCM (20 ml), a solution of DCC (1.031 g, 5 mmol) in the same solvent (20 ml) and a solution of 1-(4-methoxybenzyl)piperazine (**9**) (1 g, 4.85 mmol) in DCM (10 ml) with the same procedure above reported for (*R*)-**2**. Purification of the crude compound was performed by flash silica gel chromatography using EtOAc as mobile phase. The free base was converted into the corresponding hydrochloride salt with 4 N HCl in dioxane. Yield = 15.5%; $R_f$ = 0.6 [EtOAc /EtOH, 8:2]; mp = 176.6-177.2 °C; MS *m*/*z* $[M]^+$ = 349.7; $^1H$ NMR (200 MHz, $CDCl_3$) $\delta_H$ 1.38−1.54 (m, 2H), 1.58−1.77 (m, 4H), 1.83−2.02 (m, 1H), 2.28−2.54 (m, 7H), 3.05−3.25 (m, 2H), 3.42−3.69 (m, 6H), 3.81 (s, 3H), 4.07-4.18 (m, 1H), 6.83-6.90 (m, 2H), 7.20-7.27 (m, 2H). $^{13}C$ NMR (200 MHz, dimethyl sulfoxide-$d_6$) δ 171.2, 160.5, 133.5, 133.2, 121.6, 114.7, 114.3, 58.5, 56.7, 55.7, 50.3, 42.0, 40.8, 38.5, 34.6, 32.1, 28.8, 24.7. Anal. ($C_{20}H_{30}N_2O_2S_2$·HCl) C, H, N, S.

*2.1.7. 5-(1,2-Dithiolan-3-yl]-1-[4-(4-fluorobenzyl)piperazin-1-yl]pentan-1-one chloride (**12**).* This compound was synthesized using 1-(4-fluorobenzyl)piperazine (**10**) (0.85 g, 4.36 mmol) in according to the synthetic method described for **11**. Purification of the crude compound was performed by flash silica gel chromatography (EtOAc 10, EtOAc/EtOH 9:1) affording a yellow oil, that was converted into the corresponding hydrochloride salt with 4 N HCl in dioxane. Yield = 44%; $R_f$ = 0.64 [EtOAc]; mp = 189-191.7 °C dec; MS *m*/*z* $[M]^+$ = 382.6; $^1H$ NMR (200 MHz, $CDCl_3$) $\delta_H$ 1.44−1.51 (m, 2H), 1.54−1.70 (m, 4H), 1.86-1.96 (m, 1H), 2.28−2.51 (m, 7H), 2.76-2.77 (m, 1H), 3.10−3.20 (m, 1H), 3.43−3.64 (m, 7H), 6.95-7.05 (m, 2H), 7.24-7.31 (m, 2H). $^{13}C$ NMR

(200 MHz, $CDCl_3$) δ 171.0, 161.2, 133.7, 133.5, 123.3, 116.5, 116.3, 60.0, 56.4, 51.4, 51.0, 42.0, 40.2, 38.5, 38.1, 34.5, 32.5, 28.8, 24.5. Anal. ($C_{19}H_{27}FN_2OS_2$·HCl) C, H, N, S.

*2.2. Biology*

*2.2.1. Radioligand Binding Assays.*

*In vitro* σ binding assays were performed as earlier reported [32]. $_1$ Binding assays were carried out on guinea pig brain membranes in accordance with experimental protocol described by DeHaven et al. [33]. In brief, 500 μg of membrane protein were suspended in 50 mM Tris-HCl (pH 7.4) and incubated with 3 nM [$^3$H]-(+)-Pentazocine (29 Ci/mM; the value of the apparent dissociation constant ($K_d$) was 14  0.3 nM, n = 3). Test compounds were added at concentrations ranging from $10^{-5}$ to $10^{-11}$ M. Nonspecific binding was evaluated in the presence of 10 μM of unlabeled haloperidol. The reaction was performed for 150 min at 37 °C and stopped by filtering the solution through Whatman GF/B glass fiber filters, presoaked for 1 h in a 0.5% poly(ethylenimine) solution. Filters were washed twice with 4 ml ice cold incubation buffer. Regarding the $\sigma_2$ binding assays [34], the membranes (360 μg/sample) were incubated with 3 nM [$^3$H]DTG (53.3 Ci/mM; $K_d$ = 11 ±0.8 nM; n = 3) in the presence of 400 nM (+)-SKF10,047 for blocking $_1$ sites [2]. Nonspecific binding was assessed with DTG (5μM). Incubation was carried out in 50 mM Tris-HCl (pH 8.0) for 120 min at rt and terminated by adding ice cold 10 mM Tris-HCl (pH 8.0). Samples were filtered by a millipore filter apparatus onto Whatman GF/B glass fibers filters, presoaked for 1 h in a 0.5% poly(ethylenimine) solution and afterward washed with ice cold buffer (2 x 4 ml). Filters were dried, soaked in 4 ml of "Ultima Gold MV" scintillation cocktail and counted on a Beckman LS 6500 liquid scintillation counter. Inhibition constants ($K_i$ values) were calculated using the EBDA/LIGAND program purchased from Elsevier/Biosoft.

*2.2.2. Cell Cultures*

Primary rat cerebral cortex neuron cultures were prepared using rat fetuses as described previously [35]. Briefly, nine newborn Sprague–Dawley rats were used in the study. The rats were decapitated by making a fracture in the cervical midline, and the cerebral cortex was dissected and removed. The cerebral cortex was placed into 5 ml of Hank's balanced salt solution, which had already been placed in a sterile Petri dish and macromerotomy was performed with two lancets. This composition was pulled into a syringe and treated at 37 °C for 25-30 min as 5 ml Hank's balanced salt solution plus 2 ml Trypsin– ehylenediaminetetraacetic acid (0.25% trypsin–0.02% ehylenediaminetetraacetic acid) and chemical decomposition was achieved. Then 8 µl of DNase type 1 (120 U/ml) was added to this solution, treated for 1-2 min and centrifuged at 800 rpm for 3 min. After having thrown away the supernatant, 31.5 ml of neurobasal medium and 3.5 ml fetal calf serum were added to the residue. The single cell, which was obtained after physical and chemical decomposition, was divided into 3.5 ml samples in each of ten flasks coated with poly-D-lysine formerly dissolved in phosphate buffered saline. The flasks were left in the incubator including 5% $CO_2$ at 37 °C. The flasks were then changed with a fresh medium of half of their volumes every three days until the cells were branched and had reached certain maturity; *in vitro* experiments were performed eight days later. This study was conducted at the Medical Experimental Research Center in Atatürk University (Erzurum, Turkey). The Ethical Committee of Atatürk University approved the study protocol.

*2.2.2.1. Cell Culture Treatments*

ALA, (*R*)-ALA and compounds **1-2, 6-7**, and **11-12** were dissolved in dimethyl sulfoxide (0.5%). The others were dissolved in sterile distilled water. Different aqueous concentrations (1, 10, 25, 50 and 100 µM) of each formulation were added to the cultures. After applications of compounds, the cultures were incubated for 24 h at 37 °C. Mitomycin C ($C_{15}H_{18}N_4O_5$; Sigma-Aldrich, at $10^{-7}$ M) alone added to group was considered as a positive control (control$^{+}$) for cytotoxicity analysis. Likewise, ascorbic acid (10 µM) and hydrogen peroxide (25 µM) added groups were also used as

the control$^{+}$ in TAC and TOS analysis, respectively. Each individual rat cerebral cortex culture without compound was studied as a control group (control). The cell viability and oxidative alterations were carried out in four independent experiments.

*2.2.3. Evaluation of Cell Viability*

*2.2.3.1. MTT Assay*

The viability of cells was assessed by measuring the formation of a formazan from MTT spectrophotometrically via commercially available kits (Cayman Chemical, USA). At the end of the experiment, the cells were incubated with 0.7 mg/ ml MTT for 30 min at 37°C. After washing, the blue formazan was extracted from cells with isopropanol/formic acid (95:5) and photometrically determined at 560 nm. The density of formazan formed in control cells was taken as indicative of 100% viability.

*2.2.3.2. Lactate Dehydrogenase Assay*

Lactate dehydrogenase released from damaged cells in culture medium was quantified by using lactate dehydrogenase assay kit (Cayman Chemical, USA) and 100 µl of cell medium was used for lactate dehydrogenase analysis. Released lactate dehydrogenase catalyzed the oxidation of lactate to pyruvate with simultaneous reduction of $NAD^{+}$ to NADH. The rate of $NAD^{+}$ reduction was measured as an increase in absorbance at 490 nm. The rate of $NAD^{+}$ reduction was directly proportional to lactate dehydrogenase activity in the cell medium.

*2.2.4. TAC and TOS Analyses*

After cells were exposed to each compound for 24 h, the cultures were washed with ice-cold phoshate buffered saline and homogenized with 0.9% normal saline. Following homogenization, intracellular levels of TAC and TOS were determined by using commercially available kits (Rel Assay Diagnostics, Gaziantep, Turkey).

*2.3. In vivo pharmacology*

*2.3.1. Animals*

Male Swiss CB1 mice (Harlan Laboratories, S.Pietro al Natisone (UD)) weighing 25–30 g were housed six to a cage. Animals were kept at a constant rt (25 ± 1 °C) under a 12:12 h light and dark cycle with free access to food and water. Each mouse was used for only one experiment. Experimental procedures were approved by the Local Ethical Committee (IACUC) and conducted in accordance with international guidelines as well as European Communities Council Directive and National Regulations (CEE Council 86/609 and DL 116/92).

*2.3.2. Tail-flick test*

Nociception was evaluated by the radiant heat tail-flick test, as previously reported [36]. Briefly, it consisted of irradiation of the lower third of the tail with an IR source (Ugo Basile, Comerio, Italy). The day before the experiment, mice were habituated to the procedure for measuring nociception threshold. Experiments were performed at rt (25 ± 1 °C). The basal pre-drug latency was established between 3 and 4 s and was calculated as the average of the first three measurements, which were performed at 5 min intervals. A cutoff latency of 15 s was established to minimize damage to the tail. Post-treatment TFLs were determined at 30, 45, 60, and 90 min after intraperitoneally (i.p.) injection. TFLs obtained in succeeding tests, after each pharmacological treatment, were expressed as percentage changes from basal level and we have reported the results as mean areas under the curve (MAUC) over a 90 min testing session. Data are expressed as the mean ± SEM. Intergroup comparisons were assessed by analysis of variance (ANOVA), followed by Post-hoc test (Bonferroni). A p value less than 0.05 ($p < 0.05$) was considered statistically significant

*2.4 Chemico-physical Evaluation*

*2.4.1.* HPLC–*UV Assay*

Analytical HPLC measurements were run on a Waters 600 HPLC pump (Waters Corporation, Milford, MA, USA). The column was a Phenomenex Kinetex RP-C8 (4.6 x 150 mm, 5 µm). The mobile phase was a mixture of $KH_2PO_4$/ $CH_3OH$ (pH 2.5) using isocratic conditions and a flow rate of 1 ml/min. The chromatograms were recorded at 220 nm using the Waters 2996 photodiode array detector. Stock solutions of all compounds were prepared by dissolving them in acetonitrile in order to obtain a final concentration of $10^{-4}$ M.

*2.4.2. Kinetics of Chemical Hydrolysis*

Briefly, a 0.02 M HCl buffer at pH 1.3, as non-enzymatic simulated gastric fluid, and a 0.02 M phosphate buffer at pH 7.4 were used for these experiments. Reactions were initiated by adding 1 ml of $10^{-4}$ M stock solution in acetonitrile of the compound to 10 ml of the properly thermostated (37 ± 0.5 °C) aqueous buffer solution, containing 20% acetonitrile. At appropriate time intervals, samples of 20 µL were withdrawn and analyzed by HPLC. The experiments were run in triplicate and the mean values of the rate constants were calculated [37].

*2.4.3. Kinetics of Enzymatic Hydrolysis.*

Human and rat plasma were obtained by centrifugation of blood samples containing 0.3% citric acid at 3000 × g for 15-20 min. Plasma fractions (4 ml) were diluted with 0.02 M phosphate buffer (pH 7.4) to give a final volume of 5 ml (80% plasma). Incubation was performed at 37 ± 0.5 °C using a shaking water bath. The reaction was initiated by adding 200 µL of a stock solution of compound (1 mg/ml in acetonitrile) to 5 ml of preheated plasma. Aliquots (100 µL) were taken at various times and deproteinized by mixing with 200 µL of 0.01 M HCl in $CH_3OH$. After centrifugation for 5 min at 5000 × g, 10 µL of the supernatant layers were analyzed by chromatography as described above. The amounts of remaining intact compound were plotted as a function of incubation time [38].

*2.4.4. Drug Stability Testing in Intestinal Fluids*

To assess enzymatic stability, hydrochloric buffer with pepsin (10 or 40 mg/ml) and phosphate buffer with pancreatin (10 or 40 mg/ml) were used. Buffer solution (250 µL) was preincubated at 37 °C and 50 µL of drug stock solution [50 µM in ethanol and Fasted State Intestinal Fluid 1:9] was added and shaken at 37 °C and 650 rpm. Samples of 100 µL were withdrawn at various times and 100 µL of ice-cold acetonitrile containing 0.5 vol.% formic acid was added to stop the enzymatic activity [38]. The samples were vortexed and centrifuged at 2 °C and 10000 rpm, for 10 min. The drug content in the supernatant was analyzed by HPLC.

*2.4.5. Data Analyses*

The data are expressed as the mean ± standard derivation (SD) of four repetitions. One-way analysis of variance was used to determine the significant differences between the groups followed by a Dunnett's t test for multiple comparisons. A probability < 0.05 was considered as significant. All analyses were performed using SPSS version 15.0 (USA).

## 3. Results and Discussion

Binding affinity of newly synthesized compounds was evaluated at $\sigma_1$ and $\sigma_2$ receptors by radioligand binding assays (Table 1). Compound (*R*)-**1** and (*R*)-**2** when compared to the corresponding racemic analogues showed only a slight decrease in $\sigma_1$ affinities and selectivity towards $\sigma_2$ receptors (Table 1). On the contrary, phenyl-substituted piperidines **6** and **7** when compared to compound **1** showed improved $\sigma_1$ affinity ($K_i$ = 3.1 ± 0.1 and 4.1 ± 0.2 nM, respectively) and selectivity toward $\sigma_2$ receptors ($Ki\sigma2/Ki\sigma1$ = 484 and 1,585, respectively). Thus, the introduction of a substituent on the phenyl ring of compound **1**, even if presenting opposite $\sigma$ and $\pi$ parameters, seems to improve the $\sigma_1$ binding profile. Phenyl-substituted piperazines **11** and **12**, even if they displayed a negligible loss in $\sigma_1$ affinity when compared to **2** ($Ki\sigma1$= 5.7 ± 0.1 and 2.9 ± 0.1 nM, respectively) showed an improvement in selectivity ($Ki\sigma2/Ki\sigma1$ = 432 and 448,

respectively). These binding data suggest that the introduction of a substituent on the phenyl ring of compounds **1** and **2** slightly improved $\sigma_1$ affinity and selectivity. Among synthesized compounds, **7** stands out for its improvement in $\sigma_2/\sigma_1$selectivity ratio ($Ki\sigma2/Ki\sigma1$ = 1,585).

**Table 1. σ**-Binding affinities of novel compounds (*R*)-**1**, (*R*)-**2, 6-7**, **11-12**. Each value is the means ± SD of three determinations.

| **σ Binding affinities ($K_i$, nM ± SD)** | | | |
|---|---|---|---|
| **Compound** | **$\sigma_1$** | **$\sigma_2$** | **Kiσ2/Kiσ1** |
| | **[$^3$H]-Pentazocine** | **[$^3$H]-DTG** | |
| (*R*)-**1** | 17.7 ± 1.5 | 1,870 ± 50 | 105.6 |
| (*R*)-**2** | 6.1 ± 0.4 | 1,245 ± 35 | 204.1 |
| **6** | 3.1 ± 0.1 | 1,500 ± 42 | 484 |
| **7** | 4.1 ± 0.2 | 6,500 ± 127 | 1,585 |
| **11** | 5.7 ± 0.1 | 2,460 ± 85 | 432 |
| **12** | 2.9 ± 0.1 | 1,300 ± 37 | 448 |
| **Haloperidol** | 2.7 ± 0.5 | 17.0 ± 1.5 | 6.3 |

Given the implication of σ receptors in opioid-mediated analgesia [30], we analyzed the ability of compounds **1**, **2**, (*R*)-**1**, **7**, and **12** to modulate the analgesic effect of the systemically injected morphine (M). *In vivo* results showed that **1**, **2**, (*R*)-**1**, **7**, and **12** (1 mg/kg i.p.), (dose used in previous experiments with **1** [25]) did not affect basal tail flick latency (TFL), expressed as MAUC, during the entire time of observation (data not shown). Injection of M (4 mg/kg i.p.) significantly increased the nociceptive latency following thermal stimulation, clearly demonstrating its analgesic effect. Indeed, the percent change from basal level of TFL, compared with the group of saline treated rats, was increased up to 84,54%. Pretreatment with **1**, **2**, (*R*)-**1**, **7**, and **12** (1 mg/kg i.p.) 45 min prior to M (4 mg/kg i.p.) significantly decreased the antinociceptive effect of the opioid agonist (Figure **1**). In particular, the calculated value of MAUC was significantly lower than the corresponding value obtained with M. Similarly to **1** σ agonist profile [25], also evaluated in the

present experimental conditions (Figure **1**), the *in vivo* results confirmed the σ agonist profile also for **2**, (*R*)-**1**, **7**, and **12** compounds.

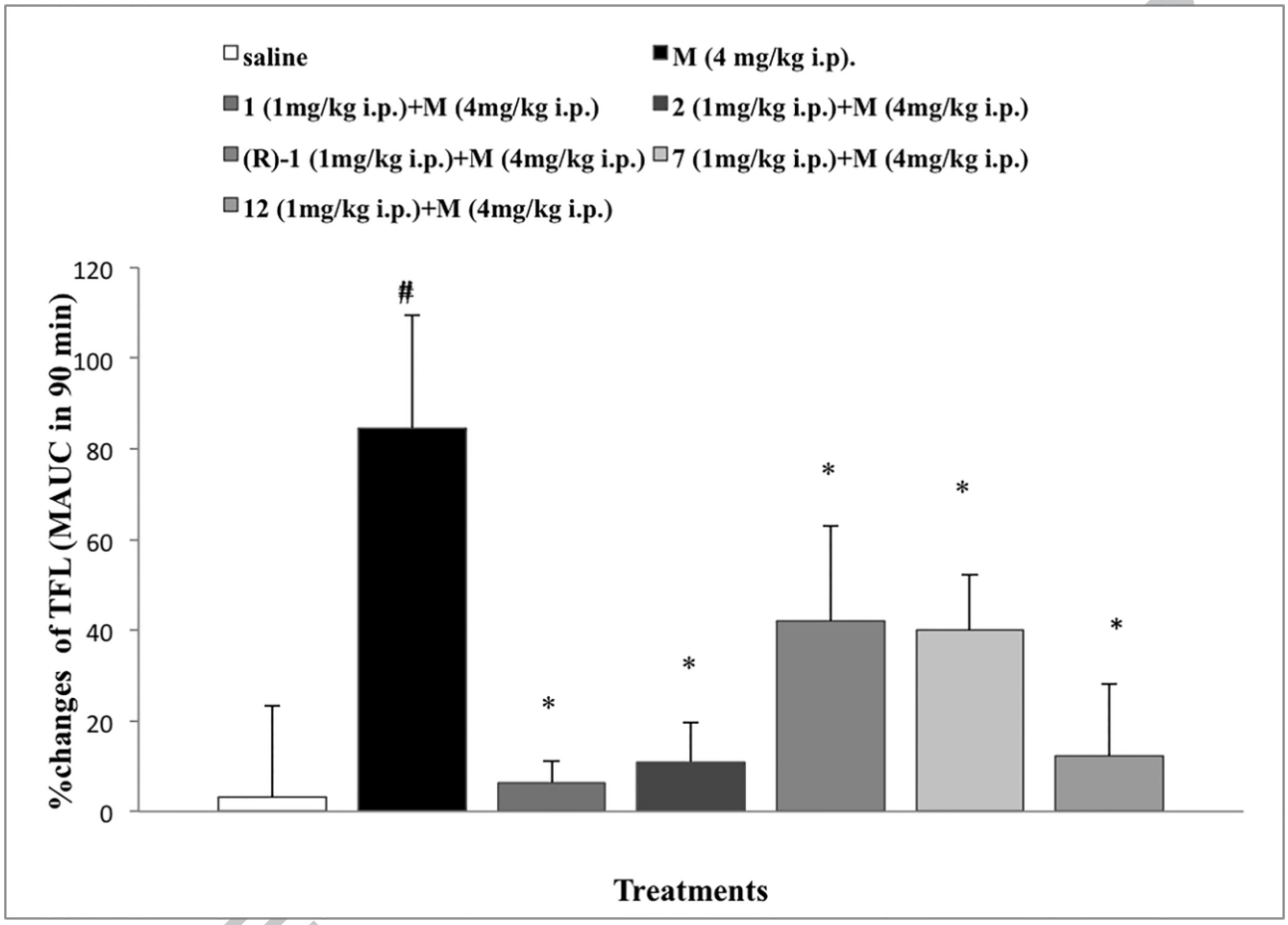



**Figure 1**. Effect of **1**, **2**, (*R*)-**1**, **7**, **12** (1 mg/kg i.p.) on morphine (M) (4 mg/kg i.p.) analgesia. Results are expressed as MAUC (after the last injection) over 90 min testing period. Columns represent the mean ± SD; #, $p < 0.05$ vs saline-treated rats (n = 8-10); *, $p < 0.05$ vs (M) treated rats (n = 8-10).

In order to assess the potential cytotoxicity of our compounds, ALA, and (*R*)-ALA, we evaluated cell injury in primary rat cerebral cortex neuron cultures. Thus, we measured cell viability with MTT assay and determined the release of intracellular lactate dehydrogenase (Table S1, supporting information). MTT absorbance's value was 46.1 ± 2.9% when the rat cerebral cortex cells were

exposed to mitomycin C (10 μM), indicating that mitomycin C caused cell death. Likewise, mitomycin C-induced neurological damage was clearly evidenced by seven fold increases in the activity of lactate dehydrogenase (2.23 ± 0.18) compared with the observations of control groups (Table S1). On the contrary, cultured primary rat cerebral cortical neurons treated with synthesized compounds did not show any significant alterations in cell viability. MTT and lactate dehydrogenase assays were performed at different concentrations (0.1, 1, 10, 25, 50 and 100 μM) of the tested compounds (Table S1). Only the highest concentrations of **6** (100 μM) and **11** (50 and 100 μM) caused cell death or injury cytotoxicity (Table S1). Furthermore, TAC and TOS parameters were determined in rat cerebral cortex cell cultures to assess the $H_2O_2$-induced oxidative alterations (Table 2). As shown in Table 2, all treatments with *p*-methoxy derivatives **6** and **11**, and compound (*R*)-**1** did not alter TAC and TOS levels, while the other tested derivatives increased TAC levels without changing TOS levels, likewise to ALA and (*R*)-ALA (Table 2).

**Table 2.** Total antioxidant capacity (TAC) and total oxidative stress (TOS) levels in rat cerebral cortex cells maintained 24 h in the presence of tested compounds *in vitro*. Values are means ± standard deviation (n = 4);* symbol represents a statistically significant difference from control (p< 0.05); ▲triangles indicate statistically important increases of TAC or TOS levels.

| **Groups** | | **TAC (mmolTrolox Equiv./l)** | **TOS (μmol $H_2O_2$ Equiv./l)** | **Groups** | | **TAC (mmolTrolox Equiv./l)** | **TOS (μmol $H_2O_2$ Equiv./l)** |
|---|---|---|---|---|---|---|---|
| Control | | 10.7±2.1 | 0.5±0.1 | Control | | 10.7±2.1 | 0.5±0.1 |
| Ascorbic acid 10 μM | | 26.9±2.8*▲ | -- | Ascorbic acid 10 μM | | 26.9±2.8*▲ | -- |
| $H_2O_2$ 25 μM | | -- | 2.3±0.3*▲ | $H_2O_2$ 25 μM | | -- | 2.3±0.3*▲ |
| **ALA** | 0,1 μM | 10.7±2.4 | 0.4±0.1 | **(*R*)-ALA** | 0,1 μM | 10.7±2.4 | 0.4±0.1 |
| | 1 μM | 10.5±2.1 | 0.5±0.1 | | 1 μM | 10.9±1.9 | 0.5±0.1 |
| | 10 μM | 10.9±2.6 | 0.4±0.1 | | 10 μM | 11.0±2.4 | 0.4±0.1 |
| | 25 μM | 11.8±2.0*▲ | 0.5±0.1 | | 25 μM | 11.2±2.2 | 0.4±0.1 |
| | 50 μM | 12.1±2.5*▲ | 0.5±0.1 | | 50 μM | 12.7±2.4*▲ | 0.5±0.1 |
| | 100 μM | 14.2±2.6*▲ | 0.5±0.1 | | 100 μM | 13.5±2.3*▲ | 0.5±0.1 |
| **1** | 0,1 μM | 10.8±2.4 | 0.5±0.1 | **(*R*)-1** | 0,1 μM | 10.9±2.7 | 0.4±0.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 μM | 10.9±2.2 | 0.5±0.1 | | 1 μM | 10.7±2.1 | 0.4±0.1 |
| | 10 μM | 11.4±3.0 | 0.4±0.1 | | 10 μM | 10.9±2.5 | 0.4±0.1 |
| | 25 μM | 13.8±2.7*▲ | 0.5±0.1 | | 25 μM | 11.3±2.8 | 0.5±0.1 |
| | 50 μM | 13.6±2.6*▲ | 0.4±0.1 | | 50 μM | 10.9±2.6 | 0.5±0.1 |
| | 100 μM | 12.5±2.4*▲ | 0.5±0.1 | | 100 μM | 11.0±2.7 | 0.5±0.1 |
| **2** | 0,1 μM | 10.4±2.0 | 0.5±0.1 | **(*R*)-2** | 0,1 μM | 10.2±2.6 | 0.5±0.1 |
| | 1 μM | 11.1±2.4 | 0.5±0.1 | | 1 μM | 10.5±2.8 | 0.5±0.1 |
| | 10 μM | 12.6±2.7*▲ | 0.4±0.1 | | 10 μM | 10.8±2.3 | 0.4±0.1 |
| | 25 μM | 12.9±3.0*▲ | 0.5±0.1 | | 25 μM | 13.0±2.6*▲ | 0.4±0.1 |
| | 50 μM | 14.8±2.8*▲ | 0.5±0.1 | | 50 μM | 12.8±2.1*▲ | 0.4±0.1 |
| | 100 μM | 14.6±2.5*▲ | 0.5±0.1 | | 100 μM | 11.2±2.4 | 0.5±0.1 |
| **6** | 0,1 μM | 10.8±3.1 | 0.5±0.1 | **11** | 0,1 μM | 10.5±2.9 | 0.4±0.1 |
| | 1 μM | 11.3±2.5 | 0.4±0.1 | | 1 μM | 11.2±2.9 | 0.5±0.1 |
| | 10 μM | 11.0±3.0 | 0.4±0.1 | | 10 μM | 10.9±3.2 | 0.5±0.1 |
| | 25 μM | 11.4±2.9 | 0.5±0.1 | | 25 μM | 11.3±2.7 | 0.5±0.1 |
| | 50 μM | 10.9±3.1 | 0.5±0.1 | | 50 μM | 11.0±2.6 | 0.5±0.1 |
| | 100 μM | 10.7±2.7 | 0.5±0.1 | | 100 μM | 10.8±2.9 | 0.5±0.1 |
| **7** | 0,1 μM | 11.0±3.1 | 0.4±0.1 | **12** | 0,1 μM | 10.9±3.1 | 0.4±0.1 |
| | 1 μM | 11.2±2.7 | 0.4±0.1 | | 1 μM | 11.2±2.8 | 0.5±0.1 |
| | 10 μM | 11.1±3.2 | 0.4±0.1 | | 10 μM | 12.6±3.1*▲ | 0.4±0.1 |
| | 25 μM | 12.6±2.7*▲ | 0.4±0.1 | | 25 μM | 13.9±3.4*▲ | 0.5±0.1 |
| | 50 μM | 13.5±2.8*▲ | 0.5±0.1 | | 50 μM | 11.4±2.7 | 0.5±0.1 |
| | 100 μM | 12.4±3.0*▲ | 0.5±0.1 | | 100 μM | 11.1±3.0 | 0.5±0.1 |

Finally, we decided to investigate the chemico-physical profile of compounds **1**, **2**, and the correspondent *p*-methoxy and *p*-fluoro-substituted analogs (**6**-**7**, **11**-**12**). We evaluated their chemical and enzymatic stabilities in human and rat plasma, using two buffers (pH 1.3 and 7.4), in simulated gastric and intestinal fluid with different concentrations (10 mg/ml and 40 mg/ml) of enzymes (pepsin and pancreatin) [37; 39]. As reported in Table 3, all compounds are quite stable at pH 1.3 ($t_{½}$ > 47 h) and pH 7.4 ($t_{½}$ > 37 h). In the presence of increasing concentrations of enzymes, the rate of hydrolysis of all compounds was significantly faster at pH 7.4 than 1.3; however, in the presence of pepsin (10 and 40 mg/ml) at pH 1.3, the most stable compounds were the amides **2**, **11** and **12** with $t_{½}$ superior to 23 h. The high stability of these compounds at low pH is an advantage for oral administration since they may remain for a longer period in the stomach without undergoing metabolism. On the other hand, in the presence of 40 mg/ml of pancreatin at pH 7.4, ester derivative **7** was immediately hydrolyzed, while **1** and **6** degraded with $t_{½}$ of 0.09 and 2.83 h, respectively. In the same experimental conditions, derivatives **2**, **11** and **12** resulted in more stable than the

corresponding ester ones ($t_{½}$ >7 h). Outcomes also suggested that compound **2** was 2.8-fold (pH 1.3) and 105-fold (pH 7.4) more stable than ester **1** in the presence of 40 mg/ml of pepsin or pancreatin, respectively. A similar behavior was observed for the couple of compounds **6**, **11** and **7**, **12**. Compounds **1** and **2** were immediately degraded in rat plasma. The most stable compound was **11** ($t_{½}$ = 4.13 h) followed by fluoro derivative **12**. The rate of hydrolysis in 80% human plasma was slower when compared to rat plasma, thus suggesting that the latter may have more selective enzymes for hydrolysis.

**Table 3.** Chemico-physical data at 37 °C of compounds **1**, **2**, *p*-methoxy (**6-11**) and *p*-fluoro derivatives (**7-12**). Values are means of three experiments; standard deviation is given in parentheses.

| **Compounds** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Condition** | | **1** | **2** | **6** | **7** | **11** | **12** |
| **pH 1.3** | $t_{½}$ **(h)** | 58.71 (± 2.29) | 92.97 (± 4.56) | 52 (± 1) | 47 (± 1) | 240 (± 5) | 88 (± 2) |
| | $K_{obs}$ **($h^{-1}$)** | 0.012 (± 0.001) | 0.007 (± 0.001) | 0.013 (± 0.001) | 0.015 (± 0.001) | 0.003 (± 0.001) | 0.008 (± 0.001) |
| **pH 7.4** | $t_{½}$ **(h)** | 40.45 (± 1.25) | 53.80 (± 2.21) | 37 (± 1) | 38 (± 1) | 238 (± 6) | 81 (± 2) |
| | $K_{obs}$ **($h^{-1}$)** | 0.017 (± 0.001) | 0.013 (± 0.001) | 0.019 (±0.001) | 0.019 (± 0.001) | 0.003 (± 0.001) | 0.009 (± 0.001) |
| **pH 1.3 Pepsin 10mg/ml** | $t_{½}$ **(h)** | 24.51 (± 0.74) | 69 (± 2) | 39.15 (± 0.94) | 3.77(± 0.09) | 112 (± 2) | 106.89 (± 3.10) |
| | $K_{obs}$ **($h^{-1}$)** | 0.028 (± 0.001) | 0.010 (± 0.001) | 0.018 (± 0.001) | 0.184 (± 0.005) | 0.006 (± 0.001) | 0.007 (± 0.001) |
| **pH 1.3 Pepsin 40mg/ml** | $t_{½}$ **(h)** | 23.06 (± 0.97) | 66.16 (± 2.32) | 22.16 (± 0.53) | 0.68 (± 0.02) | 23.68 (± 0.66) | 37.19 (± 1.15) |
| | $k_{obs}$ **($h^{-1}$)** | 0.028 (± 0.001) | 0.010 (± 0.001) | 0.031 (± 0.001) | 1.019 (± 0.023) | 0.029 (± 0.001) | 0.019 (± 0.001) |
| **pH 7.4 Pancreatin 10mg/ml** | $t_{½}$ **(h)** | 0.29 (± 0.01) | 22.23 (± 0.82) | 2.78 (± 0.06) | immediate hydrolysis | 60.87 (± 1.71) | 75.08 (± 1.88) |
| | $k_{obs}$**($h^{-1}$)** | 2.389 (± 0.062) | 0.031 (± 0.012) | 0.249(± 0.006) | - | 0.011 (± 0.001) | 0.009 (± 0.001) |
| **pH 7.4 Pancreatin 40mg/ml** | $t_{½}$ **(h)** | 0.09 (± 0.01) | 9.58 (± 0.33) | 2.83 (± 0.07) | immediate hydrolysis | 41.64 (± 0.96) | 32.31 (± 0.81) |
| | $k_{obs}$**($h^{-1}$)** | 7.615 (± 0.223) | 0.072 (± 0.013) | 0.245 (± 0.005) | - | 0.017 (± 0.001) | 0.021 (± 0.001) |
| **Human plasma** | $t_{½}$ **(h)** | 0.43 (± 0.02) | 1.76 (± 0.05) | 0.61 (± 0.01) | 0.81 (± 0.02) | 5.28 (± 0.14) | 4.21 (± 0.11) |
| | $k_{obs}$ **($h^{-1}$)** | 1.61 (± 0.06) | 0.39 (± 0.01) | 1.14 (± 0.03) | 0.856 (± 0.03) | 0.13 (± 0.01) | 0.165 (± 0.004) |
| **Rat plasma** | $t_{½}$ **(h)** | immediate hydrolysis | immediate hydrolysis | 0.46 (± 0.01) | 0.56 (± 0.01) | 4.13 (± 0.12) | 2.92 (± 0.05) |
| | $k_{obs}$ **($h^{-1}$)** | - | - | 1.506 (± 0.042) | 1.238 (± 0.034) | 0.168(± 0.004) | 0.237 (± 0.006) |

In particular, **11** and **12** displayed a better $t_{½}$ ($t_{½}$(h) 5.28 ± 0.14 h and 4.21 ± 0.11, respectively) in contrast to compound **2** ($t_{½}$ = 1.76 ± 0.05 h).

Taken together, these results suggest that the ester derivatives are more susceptible to hydrolysis both in acidic and physiological environments compared to the amide derivatives. Moreover, the faster hydrolyses of ester derivatives, observed in plasma samples, confirmed previously reported data suggesting that well known action of plasma esterases [34]. Notably, **11** and **12** are the most stable compounds in all experimental tested conditions.

## 4. Conclusions

In the present paper, we reported the synthesis of the *p*-methoxy- and *p*-fluoro-substituted analogs of compounds **1** and **2** and the corresponding (*R*)-**1** and (*R*)-**2** enantiomers. Our results point out that *p*-methoxy (**6** and **11**) and *p*-fluoro (**7** and **12**) derivatives showed an improvement of $\sigma_1$ selectivity towards $\sigma_2$ related receptors. Moreover, *in vivo* studies displayed $\sigma_1$ agonist profile for compounds **1**, **2**, (*R*)-**1**, **7**, and **12**. Derivatives **7** and **12** also displayed antioxidant properties by increasing the TAC. Chemico-physical studies revealed that compound **2** was found to be more stable than corresponding ester **1** under acid and physiological conditions, in simulated gastric fluid and simulated intestinal fluid and, even if it is degraded in human plasma, its hydrolysis is slower than **1**. Further improvement of the stability at physiological pH in simulated gastric and intestinal fluid was shown by compounds **11** and **12**.

In conclusion, our results highlight that the presence of *p*-substituents has an important role on $\sigma_1$ selectivity, TAC, chemical and enzymatic stabilities. In particular, our data suggest that compounds **7** and **12** are promising tools to investigate the disorders related to $\sigma_1$ receptors and

oxidative stress whereas they can be converted into the corresponding [$^{18}$F] radiotracers for PET studies. Preclinical *in vivo* studies are in progress and very promising results will be guaranteed.

**Supporting Information**: MTT and LDH studies, their detailed experimental procedures, and elemental analysis results.

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**Conflict of interest**

The authors declare no conflict of interest.

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**Legends**

**Chart 1.** $\sigma_1$ Selective ligands containing the (*R*/*S*) lipoyl function.

**Scheme 1.** Reagents and conditions: (a) (*R*)-ALA or ALA, *N*,*N*′-dicyclohexylcarbodiimide, dichloromethane, 4-(*N,N*-dimethylamino)pyridine, 3 h rt.

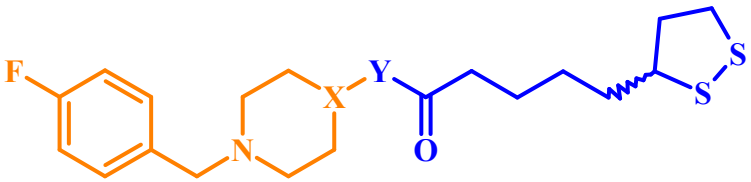
**Scheme 2.** Reagents and conditions: (b) (*R*)-ALA or ALA, *N*,*N*′-dicyclohexylcarbodiimide, dichloromethane, 3 h rt.

**Figure 1**. Effect of **1**, **2**, (*R*)-**1**, **7**, **12** (1 mg/kg i.p.) on morphine (M) (4 mg/kg i.p.) analgesia. Results are expressed as MAUC (after the last injection) over 90 min testing period. Columns represent the mean ±SD; #, $p < 0.05$ vs saline-treated rats (n = 8-10); *, $p < 0.05$ vs (M) treated rats (n = 8-10).

**Table 1.** **σ**-Binding affinities of novel compounds (*R*)-**1**, (*R*)-**2, 6**-**7**, **11**-**12**. Each value is the means ± SD of three determinations.

**Table 2.** Total antioxidant capacity (TAC) and total oxidative stress (TOS) levels in rat cerebral cortex cells maintained 24 h in the presence of tested compounds *in vitro*. Values are means ± standard deviation (n = 4);* symbol represents a statistically significant difference from control$^-$ ($p < 0.05$); ▲triangles indicate statistically important increases of TAC or TOS levels.

**Table 3.** Chemico-physical data at 37 °C of compounds **1**, **2**, *p*-methoxy (**6**-**11**) and *p*-fluoro derivatives (**7**-**12**). Values are means of three experiments; standard deviation is given in parentheses.




$\sigma_1$ Receptor pharmacophore  Natural antioxidant ALA or (*R*)-ALA

**7** X = C;  Y = O;  $K_i\sigma_1$ = 4.1 ± 0.2 nM  $K_i\sigma_2$ = 6,500 ± 127 nM

**12** X = N;  Y = --;  $K_i\sigma_1$ = 2.9 ± 0.1 nM  $K_i\sigma_2$ = 1,300 ± 37 nM