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Article

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(+)-Methyl (1R,2S)-2-{[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate [(+)-MR200] Derivatives as Potent and Selective Sigma Receptor Ligands: Stereochemistry and Pharmacological Properties

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ABSTRACT

Methoxycarbonyl-1-phenyl-2-cyclopropylmethyl based derivatives *cis*-(+)-**1a** [*cis*-(+)-MR200], *cis*-(−)-**1a** [*cis*-(−)-MR201], and *trans*-(±)-**1a** [*trans*-(±)-MR204], have been identified as new potent sigma (σ) receptor ligands. In the present paper, novel enantiomerically pure analogues

were synthesized and optimized for their σ receptor affinity and selectivity. Docking studies rationalized the results obtained in the radioligand binding assay. Absolute stereochemistry was unequivocally established by X-ray analysis of precursor *trans*-(+)-**5a** as camphorsulfonyl derivative **9**. The most promising compound, *trans*-(+)-**1d**, showed remarkable selectivity over a panel of more than fifteen receptors as well as good chemical and enzymatic stability in human plasma. An *in vivo* evaluation evidenced that *trans*-(+)-**1d**, in contrast to *trans*-(–)-**1d**, *cis*-(+)-**1d**, or *cis*-(–)-**1d** which behave as σ_1 antagonists, exhibited a σ_1 agonist profile.

These data clearly demonstrated that compound *trans*-(+)-**1d**, due to its σ_1 agonist activity and favorable receptor selectivity and stability, provided an useful tool for the study of σ_1 receptors.

INTRODUCTION

Sigma (σ) receptors, first introduced as subtypes of the opioid receptors, are now considered unique class of proteins classified into two subtypes, sigma-1 (σ_1) and sigma-2 (σ_2) with peculiar structure, biological functions and ligands sensitivity. Indeed, σ_1 binding sites display high affinity for dextro benzomorphan enantiomers like (+)-pentazocine, while opioid receptors bind preferentially the levo isomers.[1-3] The σ_1 and σ_2 receptor subtypes can be distinguished by molecular weight (25 and 18–21.5 kDa, respectively), tissue distribution, subcellular localization, and ligand binding profile.[4,5]

The σ_1 receptor has been classified as a protein chaperone at the endoplasmatic reticulum (ER)-mitochondrion interface that regulates Ca^{2+} signaling and cell survival.[6] Additional important functions have also been well documented. Among these, the σ_1 receptor modulates ion channel activity and ER stress, having thus an important role in neurodegenerative disorders.[7,8] In this perspective, mutations of the σ_1 receptor were linked to the establishment of amyotrophic lateral

sclerosis in humans.[9] In addition, it has been shown a close relationship between σ_1 and opioid receptors in pain modulation, where the σ_1 system acts as antiopioid and deeply influences the sensitivity toward opioid analgesia, since σ_1 antagonists enhance opioid antinociception.[10-12] Ligands that specifically target the σ_1 receptor have shown a plethora of pharmacological effects;[13] in particular, σ_1 antagonists, in addition to the modulation of opioid antinociception, proved their ability in relieving neuropathic and inflammatory pain and to have antiproliferative and antiangiogenic effects, while σ_1 agonists provided an efficacious neuroprotection.[3,14-23] The σ_1 receptor is also involved in other human diseases including schizophrenia, depression, and drug/alcohol addiction.[24,25] The σ_2 receptor is overexpressed in a wide variety of cancer tissues and due to this peculiarity has been used for the generation of biomarker for cancer imaging.[26-28] Moreover, σ_2 receptor ligands are known to cause cancer cell death *in vitro* and *in vivo*.[28-30]

Recently, the σ_2 receptor was purified, revealing its identity as the transmembrane protein 97 (TMEM97), an endoplasmic reticulum-resident transmembrane protein that regulates the sterol transporter Niemann–Pick disease protein (NPC1).[31] Some quantitative structure–activity relationship (QSAR) models for the determination of σ receptors binding were setup.[32-35] Although no σ receptors ligands have been yet marketed as drugs, there are ongoing clinical trials aimed at their evaluation. Thus, σ receptors represent attractive targets useful for neuropathic pain management, neurodegenerative disorders, and as brand new tools for cancer treatment and diagnosis.

Haloperidol, a typical neuroleptic agent, is a promiscuous drug showing high affinity for a panel of targets including the main dopaminergic D_1, D_2, D_3, $D_{4.2}$, serotoninergic 5-HT_{2A}, adrenergic α_1, σ_1 and σ_2 receptors.[36] Haloperidol has already been used for the design of selective σ receptors ligands.[37] Indeed, we previously reported two methoxycarbonyl-1-phenyl-2-

cyclopropylmethyl based compounds, *cis*-(+)-**1a** [*cis*-(+)-MR200] and *cis*-(–)-**1a** [*cis*-(−)-MR201], with a notable improvement of pharmacological *in vitro* σ receptors selectivity with respect to haloperidol. At the same time, we reported the synthesis and the radioligand binding profile of *trans*-(±)-**1a** [*trans*-(±)-MR204] (Figure 1).[36,38]

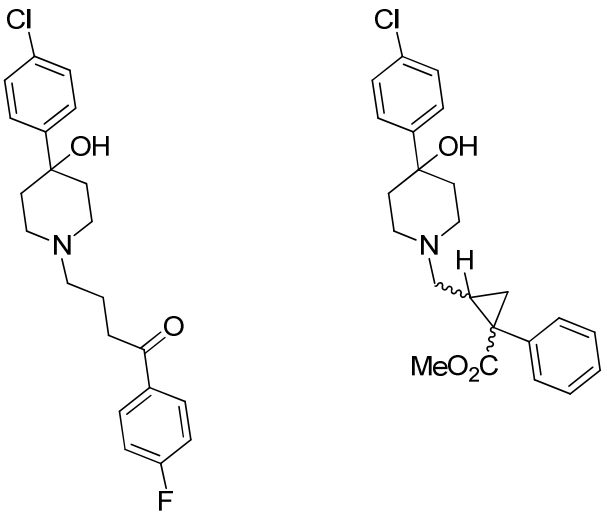


Figure 1. Structures of sigma receptors ligands.

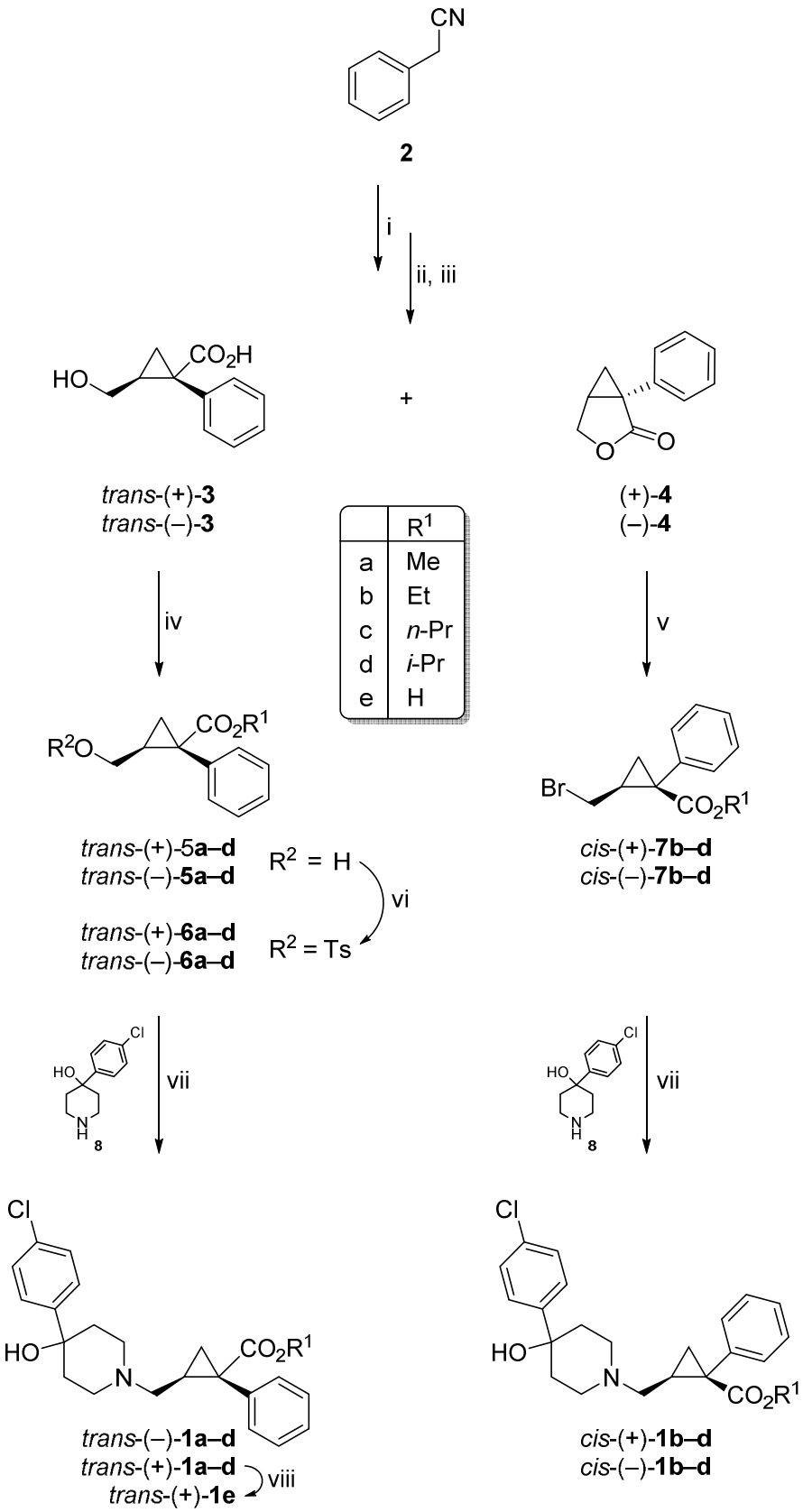
We noted that the *trans*-(±)-**1a** racemic mixture, while keeping a double-digit nanomolar affinity for σ_1 and σ_2 receptors, showed a similar affinity toward dopaminergic D_2 receptor with respect to compound *cis*-(+)-**1a**, but a lower affinity with respect compound *cis*-(–)-**1a**, [K_i = 3230, 378 and 5200 nM for compounds *cis*-(+)-**1a**, *cis*-(–)-**1a**, and *trans*-(±)-**1a**, respectively]. While considering that these molecules share the same 4-(4-chlorophenyl)piperidin-4-ol portion, our rationale is driven by the hypothesis that the fragment *N*-tethered to piperidine nucleus may be a major driver for selectivity towards σ receptors with respect to dopaminergic receptors. Thus, here we report the synthesis and pharmacological characterization of the enantiomerically pure

compounds *trans*-(+)-**1a** and *trans*-(–)-**1a**. Moreover, to evaluate the importance of the ester substituent on σ receptor affinity, we synthesized the corresponding ethyl, *n*-propyl and isopropyl esters of both *cis*-and *trans*-**1a** enantiomerically pure compounds. The designed compounds fit known pharmacophore models for σ receptor recognition.[39,40] Finally, the absolute stereochemistry of synthesized compounds was confirmed by a comparison with a *trans*-(+)-**5a** precursor. Stereochemistry of this precursor has been in turn established by X-ray analysis upon transformation of the corresponding camphorsulfonyl derivative **9** (see supporting information).

RESULTS AND DISCUSSION

Chemistry

The synthetic pathway of compounds *cis*-**1b**–**d** and *trans*-**1a**–**d** was pursued by coupling the intermediates *trans*-**6a**–**d**, and *cis*-**7b**–**d**, synthesized following the previously reported procedures (see SI), with 4-(4-chlorophenyl)piperidin-4-ol (**8**) in the appropriate solvent and base under reflux conditions (Scheme 1).[36,38,41-47] The acid derivative compound *trans*-(–)-**1e** was produced by hydrolysis of the corresponding ethyl ester compound *trans*-(–)-**1b** (Scheme 1). All compounds were characterized by means of their ^{1}H- and ^{13}C-NMR spectroscopic data. The optical purity was determined by chiral HPLC and reported as percentage of enantiomeric excess (e.e.).[48] Moreover its absolute stereochemistry was also confirmed by comparison with the configuration of the camphorsulfonyl derivative of **9** (Figure 2) as specified in the Experimental Section.


CN
2
i
ii, iii
CO2H
HO
+
O
O
trans-(+)-3
trans-(–)-3
(+)-4
(–)-4
R1
a Me
b Et
c n-Pr
d i-Pr
e H
iv
v
CO2R1
R2O
Br
CO2R1
trans-(+)-5a–d
trans-(–)-5a–d
R2 = H
vi
trans-(+)-6a–d
trans-(–)-6a–d
R2 = Ts
cis-(+)-7b–d
cis-(–)-7b–d
Cl
HO
N
H
8
vii
vii
Cl
HO
N
CO2R1
trans-(–)-1a–d
trans-(+)-1a–d
trans-(+)-1e
viii
Cl
HO
N
CO2R1
cis-(+)-1b–d
cis-(–)-1b–d

Scheme 1. Synthetic strategy for the preparation of target compounds. Reagents and conditions: (i) $NaNH_2$, (*S*)-(+)- or (*R*)-(–)-epichlorohydrin, benzene, rt, 2 h; (ii) KOH, EtOH, reflux, 15 h; (iii) 150 °C, 30 min at atmospheric pressure, then 45 min at 15 mmHg; (iv) ROH, H_2SO_4, reflux, 2 h; (v) $SOBr_2$, ROH, rt, 24 h; (vi) tosyl chloride, pyridine, CH_2Cl_2, 0 °C, 24 h; (vii) no base or $KHCO_3$ or $NaHCO_3$, ROH or DMF, 75–80 °C, 9 h; (viii) *Trans*-(+)-1b, LiOH, MeOH/H_2O, 60 °C, 3 h.

X-ray Analysis

With the aim of determining the absolute stereochemistry, compound **9** (Figure 2) has been crystallized in the monoclinic P21 space group with one molecule in the asymmetric unit, by using diethyl ether. Colorless block crystals were obtained. Its unit cell refined to a = 6.395Å, b = 16.488 Å, c = 10.488 Å, β = 107.02 °. No residual solvent accessible voids were evidenced as a consequence of a fairly high (67.9%) packing index.[49] Two linear, weak and intermolecular hydrogen bonds are present – namely C2–H2A···O6 and C17–H17C···O6 – both involving O6 in an adjacent molecule (symmetry code x,y,-1+z).[50,51] They contribute to the stabilization of the whole molecular architecture.[52,53]

A ring puckering analysis highlights the presence of two five-membered rings with a typical envelope form – C1–C4–C12–C8–C7 and C1–C4–C12–C14–C9 – as well as a boat form for the six-membered ring – C1–C7–C8–C12–C14–C9 – resulting from the merging of the above mentioned five-membered ones.[54-56]

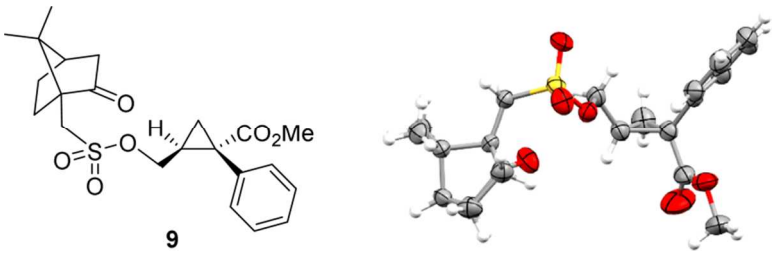


Figure 2. 2D and X-ray ortep structures of compound **9**.

Radioligand Binding and σ_1 and σ_2 Receptor Affinities

The synthesized compounds [*cis*-(+)-**1b–d**, *cis*-(–)-**1b–d**, *trans*-(+)-**1a–d**, and *trans*-(–)-**1a–d**] were evaluated for their affinities for both σ_1 and σ_2 receptors (Table 1 and 2). Moreover, compounds *trans*-(–)-**1a** and *trans*-(+)-**1a**, were also evaluated for their affinity against the dopaminergic receptors D_1 and D_2. All the reported compounds show a high affinity towards σ_1 receptor and a σ_2/σ_1 ratio ranging from 1.1 to 262.4.

Table 1. Radioligand binding assays[a]

Compound	$K_i \pm$ SD (nM)[a]				Selectivity
	σ_1	σ_2	D_1	D_2	σ_2/σ_1
trans-(+)-**1a**	129 ± 4.2	142 ± 4.8	>10,000	4,970 ± 148	1.1
trans-(–)-**1a**	11 ± 0.8	40 ± 3.8	>10,000	5,624 ± 164	3.6
cis-(+)-**1a**[b]	1.51 ± 0.2	21.9 ± 2.4	>10,000	3,230 ± 110	14.5
cis-(–)-**1a**[b]	5.6 ± 0.4	23.4 ± 2.7	>10,000	378 ± 49	4.2
trans-(±)-**1a**[b]	14.6 ± 1.3	61.1 ± 4.5	>10,000	5,200 ± 241	4.2
Haloperidol[b]	2.2 ± 0.5	16 ± 2.7	318 ± 59	2.1 ± 0.3	7.3

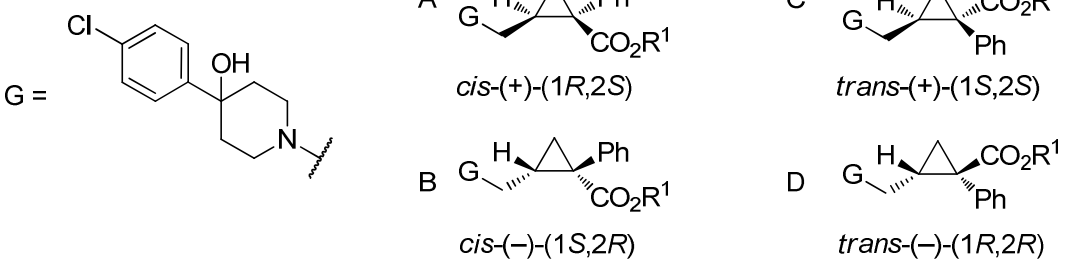
[a]Each value is the mean ± SD of three determinations. [b]Data from following reference.[38]

The methyl esters *trans*-(+)-**1a** and *trans*-(–)-**1a** showed a slightly lower affinity with respect to parent *cis* methyl ester compounds (+)- and (–)-**1a**, with K_i σ_1 of 129 and 11 nM and K_i σ_2 of 142 and 40 nM, respectively (Table 1). The compound *trans*-(–)-**1a** demonstrated a better affinity profile with respect to its racemic mixture *trans*-(±)-**1a**. While the compound *trans*-(+)-**1a**, showed a lower affinity towards both σ receptor subtypes in comparison with *trans*-(±)-**1a** and *trans*-(–)-**1a**. With regard to dopaminergic receptors D_1 and D_2, compounds *trans*-(–)-**1a** and *trans*-(+)-**1a** evidenced a similar affinity to the racemic mixture *trans*-(±)-**1a** [K_i D_1 >10000 nM and K_i D_2 4970, 5624, and 5200 nM for *trans*-(–)-**1a**, *trans*-(+)-**1a**, and *trans*-(±)-**1a**, respectively]. In general, the *trans* methyl ester compounds with respect to the *cis* methyl ester compounds and haloperidol, present a better σ receptors selectivity over dopaminergic receptors.

When the ester modification takes place, *cis* and *trans* derivatives behave differently (Table 2). Within the *cis* series, the elongation or branching of the alkyl chain determined a limited reduction in both σ_1 and σ_2 affinity for both dextrorotatory and levorotatory series (nucleus A and B respectively), with compound *cis*-(+)-**1b** being the most active and selective with K_i σ_1 of 1.35 nM and K_i σ_2 of 57 nM respectively. Thus, dextro *cis* methyl and ethyl ester compounds displayed the best affinity profile, with the ethyl ester compound *cis*-(+)-**1b** showing a better selectivity (σ_2/σ_1 = 14.5 and 42.2 for compounds *cis*-(+)-**1a** and *cis*-(+)-**1b,** respectively). In the *trans* dextro series (nucleus C), the alkyl chain branching gave rise to an optimal affinity; in particular, compound *trans*-(+)-**1d**, bearing an *i*-Pr group, exhibited a K_i σ_1 of 7 nM and K_i σ_2 of 1837 nM, with a notable 262-fold selectivity for σ_1 receptor. Comparable results were observed for the other *trans* compounds, in both dextro (nucleus C) and levo (nucleus D) configurations, showing a good σ_1 and a weaker σ_2 affinity (K_i σ_1 ranging from 12 to 52 nM and K_i σ_2 ranging from 73 to 1300 nM respectively). These results suggest that the alkyl chain bonded to the ester

function may play a pivotal role in discriminating between the sigma receptor subtypes depending on the appropriate stereochemistry of cyclopropyl skeleton.[57] Finally, in order to evaluate the importance of the ester function in the σ receptor binding affinity, we evaluated the acid derivative of the *trans* dextro (nucleus C) series [*trans*-(+)-**1e**]. Results showed that the acid compound *trans*-(+)-**1e** has negligible affinity at both σ receptor subtypes (Table 2).

Table 2. Radioligand binding Assays of enantiomerically pure compounds.[a]



			K_i ±SD (nM)[a]		Selectivity
Compound	Nucleus	R^1	σ_1	σ_2	σ_2/σ_1
cis-(+)-**1b**	A	Et	1.35 ±0.1	57 ±3.7	42.2
cis-(+)-**1c**	A	*n*-Pr	14 ±2.1	146 ±7.0	10.4
cis-(+)-**1d**	A	*i*-Pr	20 ±3.4	209 ±8.7	10.5
cis-(–)-**1b**	B	Et	45 ±2.8	918 ±26.1	20.4
cis-(–)-**1c**	B	*n*-Pr	33 ±4.6	290 ±14.4	8.8
cis-(–)-**1d**	B	*i*-Pr	67 ±5.1	1,638 ±64.3	24.4
trans-(+)-**1b**	C	Et	52 ±4.0	1,300 ±45.7	25.0
trans-(+)-**1c**	C	*n*-Pr	13 ±1.4	171 ±16.9	13.2
trans-(+)-**1d**	C	*i*-Pr	7 ±0.6	1,837 ±49.5	>250
trans-(+)-**1e**	C	H	445 ±21	650 ±31	1.5

trans-(–)-**1b**	D	Et	45 ±2.9	107 ±9.1	2.4
trans-(–)-**1c**	D	*n*-Pr	12 ±0.9	73 ±8.2	6.1
trans-(–)-**1d**	D	*i*-Pr	43 ±4.7	214 ±11.2	5.0

[a]Each value is the mean ±SD of three determinations.

Finally, we evaluated a ratio for the considered enantiomerically pure compounds.[58] By analyzing the ratios reported in Table 3, it can be noticed that there is a slight preference of the pure enantiomers *cis* dextro and *trans* levo for both receptor subtypes. Within the series, the unique exception is compound *trans*-(+)-**1d**, which shows an opposite profile.

Table 3. Ratios for σ_1 and σ_2 receptor subtypes of enantiomerically pure compounds.

	K_i (nM)			K_i (nM)		Ratio	
Compound	σ_1	σ_2	Compound	σ_1	σ_2	(–/+) σ_1	(–/+) σ_2
cis-(+)-**1a**	1.51	21.9	*cis*-(–)-**1a**	5.6	23.4	3.71	1.07
cis-(+)-**1b**	1.35	57	*cis*-(–)-**1b**	45	918	33.33	16.11
cis-(+)-**1c**	14	146	*cis*-(–)-**1c**	33	290	2.36	1.99
cis-(+)-**1d**	20	209	*cis*-(–)-**1d**	67	1638	3.35	7.84
trans-(+)-**1a**	129	142	*trans*-(–)-**1a**	11	40	0.09	0.28
trans-(+)-**1b**	52	1300	*trans*-(–)-**1b**	45	107	0.87	0.08
trans-(+)-**1c**	13	171	*trans*-(–)-**1c**	12	73	0.92	0.43
trans-(+)-**1d**	7	1837	*trans*-(–)-**1d**	43	214	6.14	0.12

Given the notable profile of compound *trans*-(+)-**1d,** we investigated the binding affinities towards other pharmacological systems, its water solubility, chemical and enzymatic stability.

The σ_1 agonist or antagonist in vivo pharmacological activity for *cis*- and *trans*-**1d** isopropyl esters was also investigated through determination of the modulation of opioid antinociception.

Binding affinity profile

Binding studies to assess the affinities of compound *trans*-(+)-**1d** towards opioid, dopaminergic, serotoninergic, and adrenergic receptors showed its very low affinity for opioid, dopamine (D_1, D_2, D_3, $D_{4.2}$), 5HT, and α_1 receptors compared to Haloperidol and 1,3-di-(2-tolyl)guanidine (DTG). In addition, *trans*-(+)-**1d** did not show any significant affinity for other receptors or transporters tested (see supporting information, Table S2).

Docking studies

A docking study was performed to probe and identify the key molecular interactions involved into the protein/ligand recognition and in order to better understand the observed affinity. To this extent, the crystal structure of the human σ_1 receptor model bound to the high-affinity and selective the σ_1 antagonist PD144418 were used. To allow each ligand to appropriately adapt to the catalytic site we carried out this study utilizing a three-step sequence: i) docking of ligand upon σ_1 receptor; ii) 5 ns of molecular dynamic (MD) simulation of the best pose obtained for ligand-σ_1 receptor complex, in order to accommodate the ligand; iii) re-docking of the complex obtained from the last 3 ns of MD simulation averaged frames. Since compounds **1** possess a tertiary amine functionality, by considering physiological conditions (pH = 7.4), we performed all the studies on the *N*-protonated structures.

The calculated binding energies (ΔG_B) and K_i values to the catalytic site of the σ_1 receptor, for compounds **1a**,**d**, haloperidol, and PD144418 are reported in Table 4. These data show that

compound *trans*-(+)-**1d** has the lowest binding energy and, although all calculated K_i values are overestimated by a factor of roughly 4.5- and 2.5-fold for compounds **1a** and **1d**, respectively, their relative magnitudes are well in accord with the experimental results. Even for haloperidol and PD144418 there is an overestimation of the K_i values comparable to that of compounds **1**.

Table 4. Calculated binding energies (kcal/mol) and inhibition constants (nM) to the binding site of the σ_1 receptor for compounds **1a**,**d**.

Compound	Calculated ΔG_B	Calculated K_i	K_i calcd. – K_i exp.
trans-(+)-**1a**	–8.5	583	454.0
trans-(–)-**1a**	–10.0	46	35.0
cis-(+)-**1a**	–10.1	39	37.5
cis-(–)-**1a**	–9.7	77	71.4
trans-(+)-**1d**	–10.6	17	10.0
trans-(–)-**1d**	–9.5	108	65.0
cis-(+)-**1d**	–9.9	55	35.0
cis-(–)-**1d**	–9.1	212	145.0
Haloperidol	–10.9	10	7.8
PD144418[a]	–10.3	28	23.7

[a]Experimental K_i σ_1 4.3 nM.[59]

Active site analysis for each of the four stereoisomers corresponding to compounds **1a**,**d** showed that, for each of them, the chemical scaffold has the same orientation. Moreover, the position of the protonated piperidine ring, for all ligands, is rather characteristic being near to the carboxylic moiety of Glu172. In the case of the compounds *trans*-(+)-**1a**, *trans*-(–)-**1a**, *trans*-(+)-**1d**, *trans*-(–)-**1d**, and *cis*-(+)-**1d** the piperidinium proton engages a salt bridge interaction with the Glu172 (Figure 3) and, in the case of *trans*-(+)-**1a**, there is also be a hydrogen bond between the

piperidinium hydrogen and the oxygen atom of the Tyr103 residue. However, these interactions are not sufficient to effectively stabilize these poses with respect to the other; indeed, both *cis*-(+)-**1a** and *cis*-(–)-**1** result more active than the *trans*-(+)-**1a**.

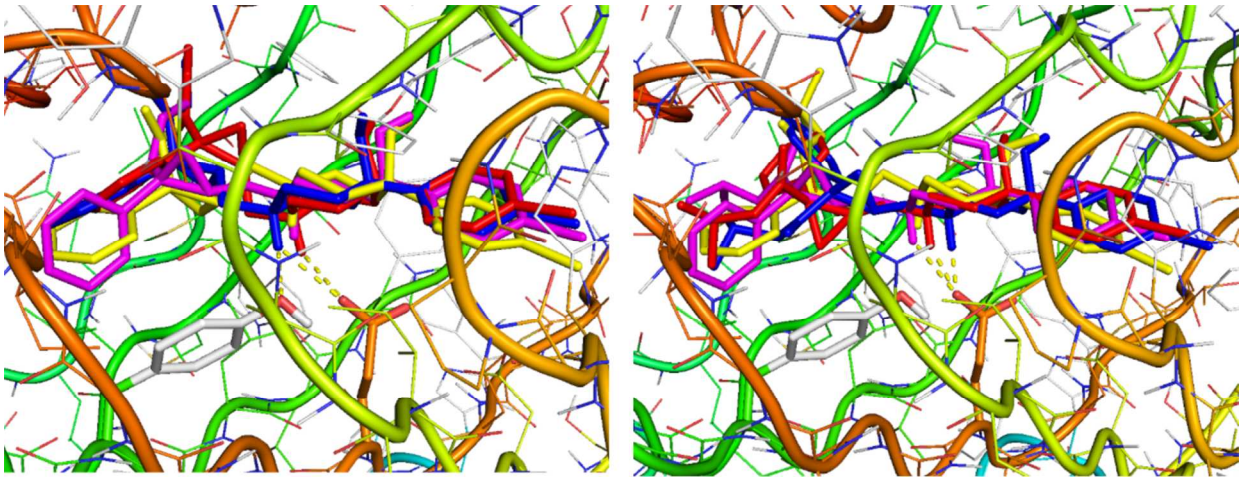


Figure 3. (Left) 3D superposition of the best-docked pose for *trans*-(+)-**1a** (blu) *trans*-(–)-**1a** (red) *cis*-(+)-**1a** (magenta) *cis*-(–)-**1a** (yellow). (Right) The same for *trans*-(+)-**1d** (blu) *trans*-(–)-**1d** (red) *cis*-(+)-**1d** (magenta) *cis*-(–)-**1d** (yellow).

The best-docked pose (*i.e.* the binding pose with the lowest energy in the binding site whose geometry and/or orientation is consistent with that of the original ligand) of *trans*-(+)-**1d**, with amino acidic interactions in the binding pocket of the σ_1 receptor is represented in Figure 4 in both 2D and 3D arrangement (top and bottom).

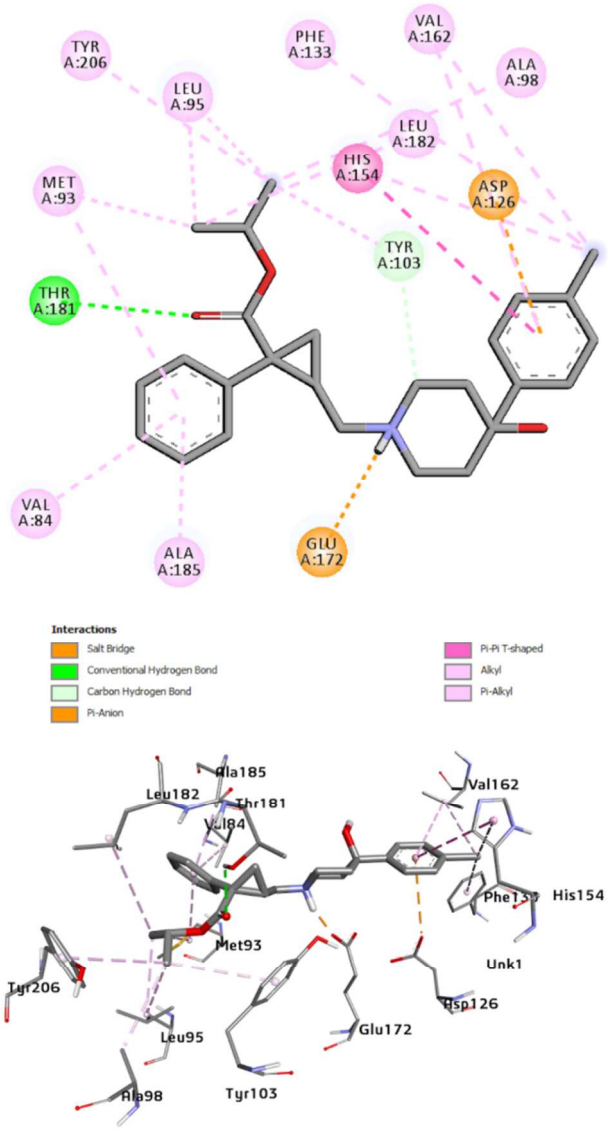


Figure 4. Compound *trans*-(+)-**1d** complexed with σ_1 receptor from docking. 2D Schematic view of hydrophobic and hydrogen bond interactions (top). 3D analog, the ligand is represented as stick whereas amino acids as a small stick (bottom).

Water solubility and chemical and enzymatic stability

The water solubility and chemical stability of *trans*-(+)-**1d** were experimentally determined while LogP was theoretically calculated (Table 4). Compound *trans*-(+)-**1d** displayed moderate water solubility (3.1 mg/mL) and a LogP value below 5 (theoretically calculated logP 4.37). The

chemical stability of *trans*-(+)-**1d** was checked in vitro at 37 ºC in an aqueous buffer solutions at pH 1.3 (non-enzymatic simulated gastric fluid) as well as pH 7.4. The compound showed good stability both at pH 1.3 and 7.4 (84 and 91 hours, respectively). The enzymatic stability of *trans*-(+)-**1d** was also studied at 37 ºC in rat, mouse, and human plasma. As evidenced by data in Table 5, the compound undergoes a rapid hydrolysis in rat plasma, whereas in mouse and human plasma it is fairly stable.

Table 5. Water solubility, cLogP, chemical and enzymatic stabilities for compound *trans*-(+)-**1d**

		trans-(+)-**1d**		
		$t_{½}$ (h)	k_{obs} (h^{-1})	
Chemical Stability[a]	pH 1.3	83.6 ±3.4	0.009 ±0.001	
	pH 7.4	91.0 ±4.3	0.009 ±0.001	
Enzymatic Stability[a]	Rat plasma	0.38 ±0.004	3.26 ±0.09	
	Mouse plasma	1.14 ±0.009	0.51 ±0.02	
	Human plasma	65.2 ±1.8	0.01 ±0.001	
Water solubility (mg/mL)[b]				3.1 ±0.2
LogP[c]				4.37

[a]Values are means ±SD of three experiments. [b]Value are means ±SD of three experiments. [c]*n*-Octanol/water partition coefficients were theoretically calculated by the ChemAxon program JChem for Excel 14.9.100.809.

In vivo pharmacological activity

Mechanical antinociception induced by the tested drugs was assessed by monitoring the increase in the struggle response latency to the nociceptive mechanical stimulus applied to the hindpaw. Subcutaneous (s.c.) administration of *trans*-(–)-**1d** (32 mg/kg) alone did not alter the struggle response latency to the mechanical stimulus in comparison to control mice treated with HPMC

(Figure 5A). A low dose of s.c. morphine (4 mg/kg) was also unable to alter the behavioral response to the nociceptive stimulation (Figure 5A). However, when we tested the association of morphine with *trans*-(–)-**1d** (16–32 mg/kg), we observed a dose-dependent increase in the response latency, indicating a synergistic antinociceptive effect (Figure 5A). Very similar results were found when testing the effects of *cis*-(+)-**1d** (16–32 mg/kg, s.c.) and *cis*-(–)-**1d** (12-16 mg/kg, s.c.): these compounds were unable to induce any antinociceptive effect when administered alone, but they both markedly enhanced morphine antinociception (Figure 5B and 5C, respectively). These results fully agree with the widely reported effects of σ_1 antagonists on opioid antinociception.[12,60-64] Furthermore they evidenced a σ_1 antagonist profile of the tested compounds. We then studied the effects of the σ_1 agonist PRE-084 (32 mg/kg, s.c.) on the synergistic antinociceptive effect induced by the association of *trans*-(–)-**1d**, *cis*-(+)-**1d** or *cis*-(–)-**1d** with morphine. This dose of PRE-084 has been shown to reverse the effects of prototypical σ_1 antagonists on opioid antinociception.[12,60,61] We found that PRE-084 completely reversed the potentiation of morphine antinociception induced by *trans*-(–)-**1d**, *cis*-(+)-**1d** and *cis*-(–)-**1d**. These results further confirm that the effects induced by these three compounds on morphine antinociception were mediated by antagonism at σ_1 receptor.

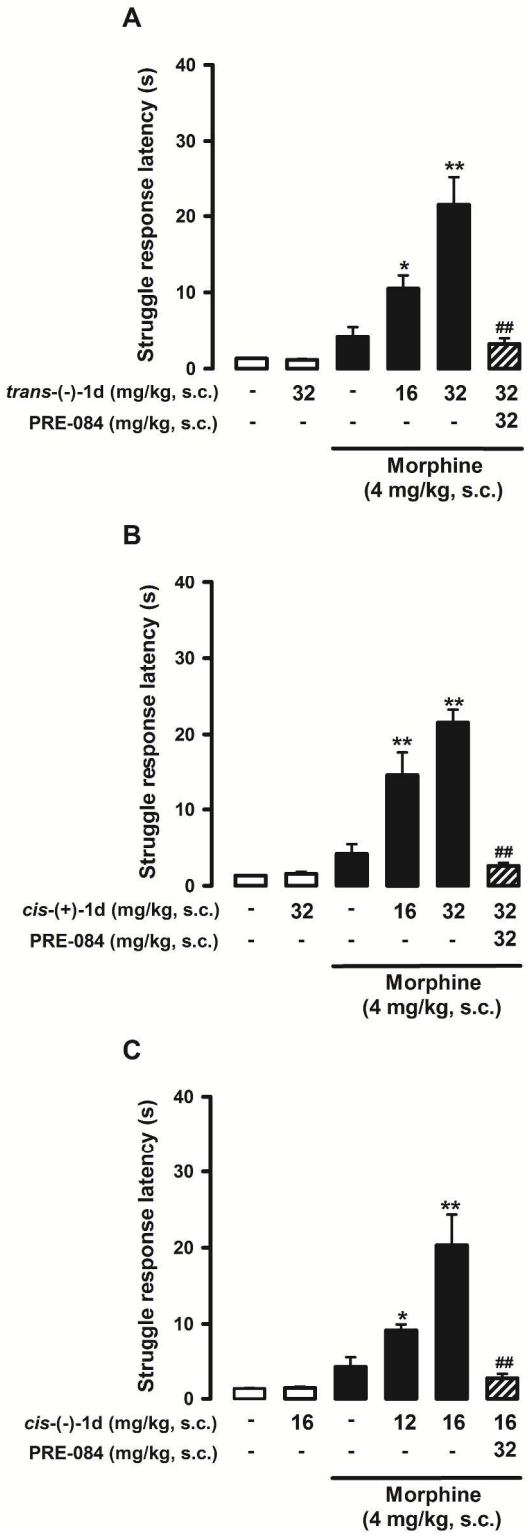


Figure 5. Effects of the administration of *trans*-(–)-**1d**, *cis*-(+)-**1d** and *cis*-(–)-**1d** on mechanical nociception and on the antinociceptive effect induced by morphine. The results represent the

struggle response latency during stimulation of the hindpaws with 450 g pressure. Mice were treated subcutaneously (s.c.) with morphine (4 mg/kg) alone or associated with (A) *trans*-(–)-**1d**, (B) *cis*-(+)-**1d** or (C) *cis*-(–)-**1d**. Animals treated with the association of morphine with *trans*-(–)-**1d**, *cis*-(+)-**1d** or *cis*-(–)-**1d** were administered with the σ_1 agonist PRE-084 (32 mg/kg, s.c.). HPMC was used as the solvent of all drugs tested. Each bar and vertical line represents the mean ± SEM of values obtained at both hindpaws in 6–8 animals. One-way analysis of variance followed by the Bonferroni test was used to determine statistically significant differences between the values obtained in the groups treated with HPMC and the groups treated with morphine associated with *trans*-(–)-1**d**, *cis*-(+)-**1d** and *cis*-(–)-**1d** (*P < 0.05; **P < 0.01) and between the values obtained in mice treated with each of these drug combinations alone or associated with PRE-084 (##P < 0.01).

On the other hand, *trans*-(+)-**1d** (16–32 mg/kg, s.c.) did not potentiate the antinociceptive effect of morphine (Figure 6). These results are in marked contrast to those obtained using *trans*-(–)-**1d**, *cis*-(+)-**1d**, *cis*-(–)-**1d**, and therefore suggesting that *trans*-(+)-**1d** does not exert σ_1 antagonistic actions. We then tested whether this compound might act as a σ_1 receptor agonist by reversing the effects of the prototypical σ_1 antagonist BD-1063 on morphine antinociception. BD-1063 (8 mg/kg, s.c.) enhanced morphine-induced antinociceptive effects (Figure 6), in agreement with previous studies .[60,61] The association of *trans*-(+)-**1d** (32 mg/kg, s.c.) with BD-1063 completely reversed the potentiation of morphine antinociception induced by this known σ_1 receptor antagonist.

Therefore, our results suggest that *trans*-(–)-**1d**, *cis*-(+)-**1d** or *cis*-(–)-**1d** act as σ_1 receptor antagonists, while *trans*-(+)-**1d** acts as a σ_1 receptor agonist.

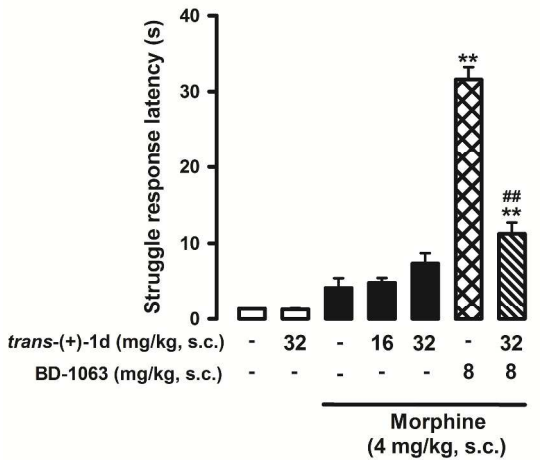


Figure 6. Effects of the administration of *trans*-(+)-**1d** on mechanical nociception and on the antinociceptive effect induced by the association of BD-1063 and morphine. The results represent the struggle response latency during stimulation of the hindpaws with 450 g pressure. Mice were treated subcutaneously (s.c.) with morphine (4 mg/kg) alone or associated with *trans*-(+)-**1d**. Animals treated with the association of morphine with *trans*-(+)-**1d** were administered with the σ_1 antagonist BD-1063 (8 mg/kg, s.c.). HPMC was used as the solvent for all tested drugs. Each bar and vertical line represents the mean ± SEM of values obtained at both hindpaws in 6–8 animals. One-way analysis of variance followed by the Bonferroni test was used to determine statistically significant differences between the values obtained in the groups treated with HPMC and the group treated with morphine associated with *trans*-(+)-**1d** (**$P < 0.01$) and between the values obtained in mice treated with *trans*-(+)-**1d** + morphine associated with BD-1063 (##$P < 0.01$).

CONCLUSIONS

The present paper deals with the synthesis and the optimization of a new series of enantiomerically pure analogues of compound **1a** in which the role of the ester function has been

investigated. The chosen synthetic route afforded four different diastereoisomers in high purity and the absolute stereochemistry was unequivocally established by means of X-ray single crystal diffraction. Molecular docking studies highlighted for each of the four stereoisomers the same scaffold orientation with a characteristic salt bridge interaction with Glu172. In radioligand binding assay compound *cis*-(+)-**1b** revealed to be the most potent but rather unselective over σ_2 receptor (σ_2/σ_1 ratio ≈ 40). Compound *trans*-(+)-**1d**, though maintaining a strong nanomolar affinity for σ_1 receptor, possessed the best selectivity ratio over σ_2 (σ_2/σ_1 ratio >250) and a remarkable selectively against a panel of more than fifteen receptors. In addition, results showed that the acid compound *trans*-(+)-**1e** has negligible affinity at both σ receptor subtypes highlighting the importance of the ester function in the σ receptor binding affinity. Compound *trans*-(+)-**1d**, with the best combined pharmacological profile from the *in vitro* characterization, good chemical and enzymatic stability in human plasma also performed as σ_1 agonist in *in vivo* studies. Conversely, compounds *trans*-(–)-**1d**, *cis*-(+)-**1d**, and *cis*-(–)-**1d**, exhibited σ_1 antagonist profile, providing evidence that stereochemistry on cyclopropane ring drives not only affinity and selectivity but also functional activity profile at σ_1 receptor.

As a whole, these studies provided the evidence that *trans*-(+)-**1a** may provide an important chemical probe which will enable the investigation to further clarify σ_1 receptor function.

EXPERIMENTAL SECTION

Chemistry

General Details. Reagents used for synthesis were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise specified. The reaction was monitored by thin layer chromatography (TLC) on precoated silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany). Visualization of

TLC spots was performed under ultraviolet (UV) light or in an iodine chamber. Merck silica gel 60, 230-400 mesh, was used for flash column chromatography. Melting points were obtained in open capillary tubes with a Büchi 530 apparatus (Büchi Italia, Assago, Italy) and are uncorrected. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Varian Inova 200 MHz spectrometer (Varian, Leini, Italy). Chemical shifts are reported in δ values (ppm) relative to an internal standard of tetramethylsilane. Optical rotations were determined in MeOH or CH_2Cl_2 (c = 1) with a Perkin-Elmer 241 polarimeter. Elemental analyses (C, H, N) were determined on an elemental analyzer, Carlo Erba model 1106 (Carlo Erba, Milan, Italy), and the results were within 0.4% of the theoretical values. The optical purity was determined by chiral HPLC analysis performed on a Perkin Elmer Flexar FX-10 UHPLC System (software Analyst 1.6.1) with a Daicel chemical industries chiral column Chiralcel-OJ, 0.46x250 mm, using a single-wavelength UV–visible detector. Compounds nomenclature were generated with ChemBioDraw Ultra version 16.0.0.82. **Radioactive materials**. [^{3}H]-(+)-Pentazocine (45 Ci/mmol), [^{3}H]-DTG (31 Ci/mmol), [^{3}H]-SCH23390 (75.5 Ci/mmol), [^{3}H]-Spiperone (20 Ci/mmol), [^{3}H]-QNB (42 Ci/mmol), [^{3}H]-Ketanserin (63.3 Ci/mmol), [^{3}H]-5HT (80 Ci/mmol), [^{3}H]-Prazosin (83.8 Ci/mmol), [^{3}H]-Pyrilamine (20 Ci/mmol), [^{3}H]-CGP39653 (40 Ci/mmol), [^{3}H]-WIN 35,428 (86 Ci/mmol), [^{3}H]-MK801 (30 Ci/mmol), [^{3}H]-7OH-DPAT (139 Ci/mmol), [^{3}H]-Mesulergine (86 Ci/mmol), [^{3}H]-LY278584 (84 Ci/mmol), [^{3}H]-GR113808 (81 Ci/mmol), [^{3}H]-Citalopram (85 Ci/mmol), [^{3}H]-RX821002 (49 Ci/mmol), and [^{3}H]-Naloxone (55.5 Ci/mmol) were obtained from Perkin-Elmer.

General procedure for compounds *trans*-(+)-1a, *trans*-(–)-1a: Procedure B. A mixture of anhydrous MeOH (10 mL), 4-(4-chlorophenyl)piperidin-4-ol (2.07 mmol), *trans* tosyl-ester (0.83 mmol), and $NaHCO_3$ (0.83mmol) was kept under magnetic stirring for 9 hours at 80 °C under

argon atmosphere in a sealed tube (Ace pressure tube). The tube content was transferred in a round bottom flask and the solvent evaporated under vacuum. The crude was dissolved in CH_2Cl_2, filtered over filter paper and washed with a saturated solution of $NaHCO_3$ and brine. The organic solvent was dried over Na_2SO_4 and removed in vacuum. The crude product was purified via silica gel chromatography. After purification the pure product was dissolved in diethyl ether and a solution of oxalic acid in diethyl ether was added dropwise to obtain the desired product as oxalic acid salt.

Methyl-(1*S*,2*S*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(+)-1a]: yield 47%; $[\alpha]_D^{20}$ +38° (*c* 1, MeOH); white solid; mp 180–182 °C. According to general procedure B. Compound *trans*-(+)-**1a** was synthesized starting from the tosyl derivative *trans*-(+)-**6a** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, DMSO-d_6) (free base) δ 1.58–1.80 (m, 4H), 1.85–2.05 (m, 1H), 2.05–2.30 (m, 2H), 3.00–3.42 (m, 6H), 3.55 (s, 3H), 5.58 (bs, 5H), 7.22–7.50 (m, 9H).^{13}C-NMR (200 MHz, DMSO-d_6) (free base) δ 20.92, 20.99, 32.38, 34.92, 47.80, 48.24, 52.52, 56.34, 67.91, 126.70, 127.63, 128.04, 128.27, 131.06, 131.46, 134.26, 146.99, 164.51, 173.19. Anal. calcd for: $C_{23}H_{26}ClNO_3$ $H_2C_2O_4 \cdot 0.8$ H_2O: C, 59.53; H, 5.92; N, 2.78. Found: C, 59.53; H, 6.03; N, 2.86.

Methyl-(1*R*,2*R*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(–)-1a]: yield 51%; $[\alpha]_D^{20}$ –40° (*c* 1, MeOH); white solid; mp 180–182 °C. According to general procedure B. Compound *trans*-(–)-**1a** was synthesized starting from the tosyl derivative *trans*-(–)-**6a** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane^{1}H-NMR (200 MHz, DMSO-d_6) (free base) δ 1.58–1.80 (m, 4H), 1.85–2.05 (m, 1H), 2.05–2.30 (m, 2H), 3.00–3.42 (m, 6H), 3.55 (s,

3H), 5.58 (bs, 5H), 7.22–7.50 (m, 9H).^{13}C-NMR (200 MHz, DMSO-d_6) (free base) δ 20.93, 21.09, 32.36, 34.99, 47.82, 48.27, 52.50, 56.14, 67.95, 126.70, 127.61, 128.04, 128.26, 131.07, 131.44, 134.30, 147.04, 164.56, 173.21. Anal. calcd for: $C_{23}H_{26}ClNO_3$ $H_2C_2O_4 \cdot 0.8$ H_2O: C, 59.53; H, 5.92; N, 2.78. Found: C, 59.83; H, 6.13; N, 2.96.

Compound Synthesis. General procedure for compounds *cis*-(+)-1b–d, *cis*-(–)-1b–d: Procedure A. A mixture of the anhydrous alcohol (10 mL), 4-(4-chlorophenyl)piperidin-4-ol (328 mg, 1.55 mmol) and the bromo-ester (0.70 mmol), was kept under magnetic stirring for 9 hours at 80 °C under argon atmosphere in a sealed tube (Ace pressure tube). The tube content was transferred into a round bottom flask and the solvent evaporated under vacuum. The crude was dissolved in CH_2Cl_2 and washed with a saturated solution of $NaHCO_3$ and brine. The organic solvent was dried over Na_2SO_4 and removed in vacuum. The crude product was purified via silica gel chromatography. After purification the pure product was dissolved in diethyl ether and a solution of oxalic acid in diethyl ether was added dropwise to obtain the desired product as oxalate. According to this procedure the following compounds were obtained.

Ethyl-(1*R*,2*S*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*cis*-(+)-1b]: yield 78%; $[\alpha]_D^{20}$ +26° (*c* 1, MeOH); light yellow sticky solid. Compound *cis*-(+)-**1b** was synthesized starting from the bromo derivative *cis*-(+)-**7b** in anhydrous EtOH and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 1.16 (t, 3H, *J* = 7.0 Hz), 1.34 (dd, 1H, *J* = 4.2, 8.8 Hz), 1.65 (dd, 1H, *J* = 4.2, 7.2 Hz), 1.68–1.90 (m, 3H), 1.95–2.32 (m, 3H), 2.40–3.10 (m, 6H), 4.04 (dq, 2H, *J* = 7.2, 10.8 Hz), 7.10–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 14.18, 20.09, 26.36, 33.90, 38.29, 38.34, 48.89, 49.32, 55.56, 60.96, 70.77,

126.08, 126.95, 128.06, 128.30, 129.94, 132.61, 140.59, 146.97, 172.2. Anal. calcd for: $C_{24}H_{28}ClNO_3 \cdot H_2C_2O_4 \cdot 0.3\ H_2O$: C, 61.31; H, 6.06; N, 2.75. Found: C, 61.56; H, 5.71; N, 2.89.

Ethyl-(1*S*,2*R*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*cis*-(–)-1b]: yield 72%; $[\alpha]_D^{20}$ –26° (*c* 1, MeOH); light yellow sticky solid. Compound *cis*-(–)-**1b** was synthesized starting from the bromo derivative *cis*-(–)-**7b** in anhydrous EtOH and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 1.16 (t, 3H, *J* = 7.0 Hz), 1.34 (dd, 1H, *J* = 4.2, 8.8 Hz), 1.65 (dd, 1H, *J* = 4.2, 7.2 Hz), 1.68–1.90 (m, 3H), 1.95–2.32 (m, 3H), 2.40–3.10 (m, 6H), 4.04 (dq, 2H, *J* = 7.2, 10.8 Hz), 7.10–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 14.18, 20.09, 26.36, 33.90, 38.29, 38.34, 48.89, 49.32, 55.56, 60.96, 70.77, 126.08, 126.95, 128.06, 128.30, 129.94, 132.61, 140.59, 146.97, 172.25. Anal. calcd for: $C_{24}H_{28}ClNO_3 \cdot H_2C_2O_4 \cdot 0.3\ H_2O$: C, 61.31; H, 6.06; N, 2.75. Found: C, 60. 65; H, 5.90; N, 2.56.

Propyl-(1*R*,2*S*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*cis*-(+)-1c]: yield 90%; $[\alpha]_D^{20}$ +26° (*c* 1,MeOH); light yellow sticky solid**.** Compound *cis*-(+)-**1c** was synthesized starting from the bromo derivative *cis*-(+)-**7c** in anhydrous *n*-PrOH and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 0.80 (t, 3H, *J* = 7.6 Hz), 1.35 (dd, 1H, *J* = 4.2, 8.8 Hz), 1.48 (t, 2H, *J* = 7.2), 1.64–1.90 (m, 4H), 2.10–2.35 (m, 3H), 2.40–3.10 (m, 6H), 7.10–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 10.33, 20.06, 21.85, 26.49, 33.99, 38.37, 38.43, 48.95, 49.35, 56.58, 66.55, 70.86, 126.10, 126.95, 128.05, 128.33, 129.98, 132.66, 140.67, 146.96, 172.32. Anal. calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.2\ H_2O$: C, 62.17; H, 62.17; N, 2.69. Found: C, 62.11; H, 5.92; N, 2.35.

Propyl-(1*S*,2*R*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*cis*-(–)-1c]: yield 91%; $[\alpha]_D^{20}$ –24° (*c* 1, MeOH); light yellow sticky solid. Compound *cis*-(–)-**1c** was synthesized starting from the bromo derivative *cis*-(–)-**7c** in anhydrous *n*-PrOH and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 0.80 (t, 3H, *J* = 7.6 Hz), 1.35 (dd, 1H, *J* = 4.2, 8.8 Hz), 1.48 (t, 2H, *J* = 7.2), 1.64–1.90 (m, 4H), 2.10–2.35 (m, 3H), 2.40–3.10 (m, 6H), 7.10–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 10.33, 20.06, 21.85, 26.49, 33.99, 38.37, 38.43, 48.95, 49.35, 56.58, 66.55, 70.86, 126.10, 126.95, 128.05, 128.33, 129.98, 132.66, 140.67, 146.96, 172.32. Anal. calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.4\ H_2O$: C, 61.75; H, 6.29; N, 2.67. Found: C, 61.74; H, 5.90; N, 2.32.

Isopropyl-(1*R*,2*S*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*cis*-(+)-1d]: yield 77%; $[\alpha]_D^{20}$ +22° (*c* 1, MeOH); white solid; mp 191–193 °C. Compound *cis*-(+)-**1d** was synthesized starting from the bromo derivative *cis*-(+)-**7d** in anhydrous *i*-PrOH and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 1.10 (d, 3H, *J* = 6.2 Hz), 1.16 (d, 3H, *J* = 6.2 Hz), 1.35 (dd, 1H, *J* = 4.6, 9.2 Hz), 1.63 (dd, 1H, *J* = 4.6, 7.0 Hz),1.68–2.35 (m, 6H), 2.45–3.09 (m, 6H), 4.96 (ept, 1H, *J* = 6.2 Hz), 7.10–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 19.86, 21.60, 21.81, 26.15, 34.08, 38.42, 49.00, 49.40, 56.73, 68.41, 70.90, 126.10, 126.84, 128.02, 128.34, 129.84, 132.68, 140.80, 146.94, 171.72. Anal. calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.5\ H_2O$: C, 61.53; H, 6.31; N, 2.66. Found: C, 61.51; H, 6.01; N, 2.88.

Isopropyl-(1*S*,2*R*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*cis*-(–)-1d]: yield 85%; $[\alpha]_D^{20}$ –24° (*c* 1, MeOH); light yellow solid; mp 192–194 °C. Compound *cis*-(–)-**1d** was synthesized starting from the bromo

derivative *cis*-(–)-**7d** in anhydrous *i*-PrOH and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 1.10 (d, 3H, *J* = 6.2 Hz), 1.16 (d, 3H, *J* = 6.2 Hz), 1.35 (dd, 1H, *J* = 4.6, 9.2 Hz), 1.63 (dd, 1H, *J* = 4.6, 7.0 Hz), 1.68–2.35 (m, 6H), 2.45–3.09 (m, 6H), 4.96 (ept, 1H, *J* = 6.2 Hz), 7.10–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 19.86, 21.60, 21.81, 26.15, 34.08, 38.42, 49.00, 49.40, 56.73, 68.41, 70.90, 126.10, 126.84, 128.02, 128.34, 129.84, 132.68, 140.80, 146.94, 171.72. Anal. calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.5\ H_2O$: C, 61.53; H, 6.31; N, 2.66. Found: C, 61.49; H, 6.25; N, 2.45.

General procedure for compounds *trans*-(+)-1b–d, *trans*-(–)-1b–d: procedure C. A mixture of anhydrous DMF (5 mL), 4-(4-chlorophenyl)piperidin-4-ol (0.69 mmol) and *trans* tosyl-ester (0.32 mmol) and $KHCO_3$ (0.64), was kept under magnetic stirring for 9 hours at 75 °C under argon atmosphere in a sealed tube (Ace pressure tube). The tube content was transferred in a round bottom flask and the solvent evaporated under vacuum. The crude was dissolved in CH_2Cl_2, filtered over filter paper and washed with a saturated solution of $NaHCO_3$ and brine. The organic solvent was dried over Na_2SO_4 and removed in vacuum. The crude product was purified via silica gel chromatography. After purification the pure product was dissolved in diethyl ether and a solution of oxalic acid in diethyl ether was added dropwise to obtain the desired product as oxalic acid salt.

Ethyl-(1*S*,2*S*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(+)-1b]: yield 66%; $[\alpha]_D^{20}$ +44° (*c* 1, MeOH); white solid; mp 185–187 °C. According to general procedure C. Compound *trans*-(+)-**1b** was synthesized starting from the tosyl derivative *trans*-(+)-**6b** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free

base) δ 1.10 (t, 3H, *J* = 7.0 Hz), 1.55 (dd, 1H, *J* = 4.6, 7.5 Hz), 1.70 (dd, 1H, *J* = 4.6, 8.5 Hz), 1.77–2.20 (m, 1H), 2.30–2.92 (m, 4H), 3.18–3.70 (m, 6H), 3.90 (bs, 1H), 4.04 (dq, 2H, *J* = 7.0, 10.8 Hz), 6.70–7.42 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 13.95, 20.61, 21.80, 33.33, 38.27, 51.66, 55.66, 61.93, 71.00, 124.90, 127.51, 128.50, 129.15, 136.00, 140.85, 144.48, 171.56. Anal. calcd for: $C_{24}H_{28}ClNO_3$ $H_2C_2O_4 \cdot 0.2$ H_2O: C, 61.52; H, 6.04; N, 2.76. Found: C, 61.73; H, 5.94; N, 3.01.

Ethyl-(1*R*,2*R*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(–)-1b]: yield 59%; $[\alpha]_D^{20}$ –42° (*c* 1, MeOH); light yellow solid; mp 186–189 °C. According to general procedure C. Compound *trans*-(–)-**1b** was synthesized starting from the tosyl derivative *trans*-(–)-**6b** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 1.10 (t, 3H, *J* = 7.0 Hz), 1.55 (dd, 1H, *J* = 4.6, 7.5 Hz), 1.70 (dd, 1H, *J* = 4.6, 8.5 Hz), 1.77–2.20 (m, 1H), 2.30–2.92 (m, 4H), 3.18–3.70 (m, 6H), 3.90 (br s, 1H), 4.04 (dq, 2H, *J* = 7.0, 10.8 Hz), 6.70–7.42 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 13.95, 20.61, 21.80, 33.33, 38.27, 51.66, 55.66, 61.93, 71.00, 124.90, 127.51, 128.50, 129.15, 136.00, 140.85, 144.48, 171.56. Anal. calcd for: $C_{24}H_{28}ClNO_3 \cdot H_2C_2O_4 \cdot 0.3$ H_2O: C, 61.31; H, 6.06; N, 2.75. Found: C, 61.20; H, 6.35; N, 3.07.

Propyl-(1*S*,2*S*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(+)-1c]: yield 76%; $[\alpha]_D^{20}$ +42° (*c* 1, MeOH); white solid; mp 180–183 °C. According to general procedure C. Compound *trans*-(+)-**1c** was synthesized starting from the tosyl derivative *trans*-(+)-**6c** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 0.98 (t, 3H, *J* = 7.4 Hz), 1.43 (t, 2H, *J* = 7.4), 1.65–2.35 (m, 7H), 2.22–2.75 (m, 6H),

3.50 (br s, 1H) 3.70–4.15 (m, 2H), 6.70–7.50 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 10.15, 20.14, 21.84, 22.42, 31.36, 38.38, 51.66, 55.63, 67.88, 70.33, 124.86, 127.58, 128.20, 128.90, 130.03, 136.33, 140.87, 145.40, 170.68. Anal. calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.2\ H_2O$: C, 62.17; H, 6.26; N, 2.69. Found: C, 61.52; H, 5.99; N, 3.09.

Propyl-(1*R*,2*R*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(–)-1c]: yield 75%; $[\alpha]_D^{20}$ –40° (*c* 1, MeOH); white solid; mp 183–185 °C**.** According to general procedure C. Compound *trans*-(–)-**1c** was synthesized starting from the tosyl derivative *trans*-(–)-**6c** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 0.98 (t, 3H, *J* = 7.4 Hz); 1.43 (st, 2H, *J* = 7.4); 1.65–2.35 (m, 7H); 2.22–2.75 (m, 6H); 3.50 (br s, 1H) 3.70–4.15 (m, 2H); 6.70–7.50 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 10.15; 20.14; 21.84; 22.42; 31.36; 38.38; 51.66; 55.63; 67.88; 70.33; 124.86; 127.58; 128.20; 128.90; 130.03; 136.33; 140.87; 145.40; 170.68. Anal. calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.4\ H_2O$: C, 61.75; H, 6.29; N, 2.67. Found: C, 61.90; H, 5.98; N, 3.04.

Isopropyl-(1*S*,2*S*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(+)-1d]: yield 65%; $[\alpha]_D^{20}$ +36° (*c* 1, MeOH); white solid; mp 176–178 °C. According to general procedure C. Compound *trans*-(+)-**1d** was synthesized starting from the tosyl derivative *trans*-(+)-**6d** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 1.10 (d, 3H, *J* = 6.2 Hz), 1.13 (d, 3H, *J* = 6.2 Hz), 1.50–2.50 (m, 4H), 2.60–3.05 (m, 3H), 3.20–3.60 (m, 6H), 3.80 (br s, 1H), 4.83 (ept, 1H, *J* = 6.2 Hz), 6.65–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 19.50, 19.65, 20.84, 21.48, 33.45, 38.40, 51.61, 56.65, 69.83, 70.12, 124.87, 127.52, 128.63, 129.00, 130.21, 134.87, 141.86, 146.32, 171.99. Anal.

calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.3\ H_2O$: C, 61.96; H, 6.28; N, 2.68. Found: C, 62.20; H, 6.07; N, 3.06.

Isopropyl-(1*R*,2*R*)-2-((4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methyl)-1-phenylcyclopropane-1-carboxylate [*trans*-(–)-1d]: yield 64%; $[\alpha]_D^{20}$ –34° (*c* 1, MeOH); white solid; mp 178–180 °C. According to general procedure C. Compound *trans*-(–)-**1d** was synthesized starting from the tosyl derivative *trans*-(–)-**6d** and purified via silica gel chromatography, eluting with 90% EtOAc in cyclohexane. ^{1}H-NMR (200 MHz, $CDCl_3$) (free base) δ 1.10 (d, 3H, *J* = 6.2 Hz), 1.13 (d, 3H, *J* = 6.2 Hz), 1.50–2.50 (m, 4H), 2.60–3.05 (m, 3H), 3.20–3.60 (m, 6H), 3.80 (bs, 1H), 4.83 (ept, 1H, *J* = 6.2 Hz), 6.65–7.60 (m, 9H). ^{13}C-NMR (200 MHz, $CDCl_3$) (free base) δ 19.50, 19.65, 20.84, 21.48, 33.45, 38.40, 51.61, 56.65, 69.83, 70.12, 124.87, 127.52, 128.63, 129.00, 130.21, 134.87, 141.86, 146.32, 171.99. Anal. calcd for: $C_{25}H_{30}ClNO_3 \cdot H_2C_2O_4 \cdot 0.2\ H_2O$: C, 62.17; H, 6.26; N, 2.69. Found: C, 61.96; H, 6.56; N, 3.08.

1-(((1S,2S)-2-carboxy-2-phenylcyclopropyl)methyl)-4-(4-chlorophenyl)-4-hydroxypiperidin-1-ium chloride [*trans*-(+)-1e]: yield 97%; $[\alpha]_D^{20}$ –41° (*c* 1, MeOH); white solid; mp 195–197 °C. A H_2O (3 mL) solution of LiOH (2.42 mmol) was added to a MeOH (3 mL) solution of the ester c*ompound trans*-(+)-**1b** (0.48 mmol) and stirred at 60 °C for 3 h. After the reaction was complete, MeOH was removed under vacuum and pH adjusted with a solution of HCl 1 N for neutralizing the excess of LiOH. The desired compound was extracted with $CHCl_3$ (3 x 10 mL) as HCl salt and used as it for further analytical and biological purposes. ^{1}H-NMR (500 MHz, CD_3OD) (HCl salt) δ 1.14 (dd, 1H, *J* = 4.0 Hz, *J* = 6.0 Hz), 1.36 (dd, 1H, *J* = 10.0 Hz, *J* = 13.0 Hz), 1.63 (dd, 1H, *J* = 4.0 Hz, *J* = 8.5 Hz), 1.69–1.72 (m, 2H), 1.95–2.01 (m, 1H), 2.05–2.10 (m, 2H), 2.40–2.50 (m, 2H), 2.65 (dd, 1H, *J* = 3.0 Hz, *J* = 12.5 Hz), 2.75–2.81 (m, 1H), 2.87–2.92 (m, 1H), 7.15–7.52 (m, 9H). ^{13}C-NMR (500 MHz, CD_3OD) (HCl salt) δ

21.14, 23.72, 36.45, 38.78, 38.82, 50.39, 50.77, 61.67, 71.58, 127.29, 127.71, 128.84, 129.28, 132.35, 133.57, 140.98, 149.30, 182.03. Anal. calcd for: $C_{22}H_{24}ClNO_3 \cdot HCl \cdot 0.2\ H_2O$: C, 62.04; H, 6.01; N, 3.29. Found: C, 61.81; H, 6.02; N, 3.30.

Preparation of Protein and Ligands. The crystal structure of the human σ_1 receptor model bound to PD144418 (PDB ID: 5HK1) was retrieved from the PDB_REDO databank as fully optimized one. The PDB_REDO procedure combines re-refinement and rebuilding within a unique decision-making framework to improve structures, using a variety of existing and custom-built software modules to choose an optimal refinement protocol (e.g. anisotropic, isotropic or overall B-factor refinement, TLS model) and to optimize the geometry versus data-refinement weights. Next, it proceeds to rebuild side chains and peptide planes before a final optimization round.[65] From the refined structure were retained only the A chain. Only for docking procedure purposes, the 3D structure of the PD144418 ligand was extracted from the PDB file whereas all other were built using Gabedit (2.4.8) software[66] and all geometries were fully optimized, in the same software, with the semi-empirical PM6[67] Hamiltonian implemented in MOPAC2016 (17.019W).[68]

Molecular Dynamics Simulations. The molecular dynamics simulations of the mature human σ_1 receptor/ligand complexes (based on the PDBs prepared as described above) were performed with the YASARA Structure package (17.4.17).[69] A periodic simulation cell with boundaries extending 10 Å from the surface of the complex was employed. The box was filled with water, with a maximum sum of all bumps per water of 1.0 Å, and a density of 0.997 g/mL with explicit solvent. YASARA's pK_a utility was used to assign pK_a values at pH 7.4,[70] and the cell was neutralized with NaCl (0.9% by mass); in these conditions ligands **1** result protonated at pyperidinic nitrogen atom. Water molecules were deleted to readjust the solvent density to 0.997

g/mL. The AMBER14 force field was used with long-range electrostatic potentials calculated with the Particle Mesh Ewald (PME) method, with a cutoff of 8.0 Å.[71-73] The ligand force field parameters were generated with the AutoSMILES utility,[74] which employs semiempirical AM1 geometry optimization and assignment of charges, followed by the assignment of the AM1BCC atom and bond types with refinement using the RESP charges, and finally the assignments of general AMBER force field atom types. Optimization of the hydrogen bond network of the various enzyme-ligand complexes was obtained using the method established by Hooft *et al*.,[75] in order to address ambiguities arising from multiple side chain conformations and protonation states that are not well resolved in the electron density.[76] A short MD was run on the solvent only. The entire system was then energy minimized using first a steepest descent minimization to remove conformational stress, followed by a simulated annealing minimization until convergence (<0.01 kcal/mol Å). The MD simulation was then initiated, using the NVT ensemble at 298 K, and integration time steps for intramolecular and intermolecular forces every 1.25 fs and 2.5 fs, respectively. The MD simulation was stopped after 5 ns and, on the averaged structure of the last 3 ns frames, a second cycle of energy minimization, identical to the first, was applied.

Docking Protocol. Macromolecules and ligands, as obtained after MD simulation and energy minimization, were prepared with Vega ZZ[77] (3.1.1) assigning Gasteiger charges to protein and AM1BCC ones to the ligand. Docking was performed with AutoDock Vina (1.1.2) software.[78] Since no water molecules are directly involved in complex stabilization, they were not considered in the docking process. All protein amino acidic residues were kept rigid whereas all single bonds of ligands were treated as full flexible. The ligand box was centered at x = –4.43, y

= –0.03 and z = 0.67 coordinates with a grid size of 18×14×18 Å and the exhaustiveness parameter related to the Lamarckian genetic algorithm was set to 15.

X-ray Crystallography. A well-grown compound **9** single crystal was chosen and mounted on a Siemens P4 diffractometer, where a full sphere of X-ray diffraction data were collected with Mo Kα at 293 K. These data were processed using XScans (Siemens) including unit cell refinement. The structures were solved and refined with SHELXS 97 (Release 97-2, University of Göttingen, Germany).[79] All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were visible in the difference Fourier map and were treated in the usual manner;[80] while those attached to carbon atoms were initially refined with soft restraints on the bond lengths, angles and temperature factors, after which a riding model was used. The search for possible solvent accessible voids was performed using the VOID algorithm setting a grid of 0.20 Å and a probe radius of 1.20 Å.[81]

The absolute configuration, assigned from the known configuration of the camphorsulfonyl derivative of **9**, was confirmed by the refinement of the Flack parameter which gave values of 0.00(7).[82-84] The final R-indices for the data were R_1 = 2.71% and wR_2 = 7.75%.

Full structural data were submitted to the CCDC as number 1443954. These data can also be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/data_request/cif.

Radioligand Binding Assays. In vitro σ-receptors binding experiments were carried out as previously reported.[85] For σ_1 receptor binding assays, guinea pig brain membranes (500 μg/sample) were incubated with 3 nM [^{3}H]-(+)-Pentazocine (K_d = 1.6 ± 0.1 nM) in binding buffer composed of 50 mM Tris-HCl (pH 7.4). Test compounds were added in concentration ranging from 10^{-5} to 10^{-11} M. Nonspecific binding was assessed in the presence of 10 μM of

unlabeled haloperidol. The reaction was performed for 150 min at 37 °C and terminated by filtering the solution through Whatman GF/B glass fiber filters which were presoaked for 1 h in a 0.5% poly(ethylenimine) solution. Filters were washed with ice-cold buffer (2 × 4 mL). Regarding σ_2 receptor binding assays, the membranes (360 μg/sample) were incubated with 3 nM [^{3}H]-DTG (K_d = 20.0 ± 0.8 nM) in the presence of σ_1 receptor masking agent (+)-SKF10,047 (400 nM). Nonspecific binding was evaluated with DTG (5 μM). Incubation was carried out in 10 mM Tris-HCl buffer (pH 8.0) for 120 min at rt, and assays were terminated by the addition of ice-cold 10 mM Tris-HCl (pH 8.0). Each sample was filtered through Whatman GF/B glass fibers filters, which were presoaked for 1 hour in a 0.5% poly(ethylenimine) solution, using a Millipore filter apparatus. Filters were washed with ice cold buffer (2 × 4 mL).

Various rat brain regions were dissected to assess the following binding assays; rat striatum for D_1 and D_2 receptors and dopamine transporter (DAT); rat olfactory tubercle for D_3 receptors; rat cortex for muscarinic, 5-HT_{2A}, 5-HT_3, α_1, α_2, H_1, and *N*-methyl-D-aspartate (NMDA) receptors and serotonin transporter (SERT); guinea pig cortex for 5-HT_4 receptors. 5-HT_{2C} binding assays were carried out on pig brains (frontal cerebral cortex) obtained from a local slaughterhouse and immediately placed on ice until use. Binding experiments on 5-HT_6 receptors were performed using a rat-cloned serotonin receptor subtype 6 in HEK-293 cells, as reported by Boess et al.[86] Tissue preparations and binding assays were carried out according to Gobbi et al.[87] and Mennini et al.[88] After incubation, the samples were filtered through Whatman GF/B or GF/C glass fiber filters using a Brandel apparatus (model M-48R) or Millipore filter apparatus. The filters were washed three times with 4 mL of ice-cold buffer.

Radioactivity was counted in 4 mL of "Ultima Gold MV" or Filter Count Cocktail (Packard) in a DSA 1409 (Wallac) or 1414 Winspectral Perkin-Elmer Wallac liquid scintillation counter.[85]

Total opioid receptor binding assays were performed using [^{3}H]-Naloxone (K_d = 6.6 ± 0.7 nM), and rat brain membranes were prepared as previously reported. Nonspecific binding was evaluated in the presence of 10 μM Naloxone. For inhibition experiments, the drugs were added to the binding mixture from seven to nine different concentrations. For all binding experiments, GraphPad Prism (GraphPad Software, San Diego, CA, USA) OR EBDA/LIGAND program (Elsevier/Biosoft) was used so as to calculate inhibitory constants (K_i values).

Experimental animals. Female CD1 mice (Charles River, Barcelona, Spain) weighing 25–30 g were used in the behavioral experiments. They were housed in colony cages with free access to food and water prior to the experiments and were maintained in temperature- and light-controlled rooms (22 ±2 °C, lights on at 08.00 h and off at 20.00 h). The experiments were performed during the light phase (from 9:00 AM to 3:00 PM). Animal care was provided in accordance with institutional (Research Ethics Committee of the University of Granada, Granada, Spain), regional (Junta de Andalucía, Spain) and international standards (European Communities Council directive 2010/63).

Evaluation of mechanical nociception (paw pressure test). The antinociceptive effect of the drugs was evaluated with a pressure analgesimeter (Model 37215, Ugo-Basile, Varese, Italy) as previously described.[12,60,61] In brief, mice were gently pincer grasped and then a blunt cone-shaped paw-presser exerting a pressure of 450 g was applied to the dorsal surface of the hindpaw, until the animal showed a struggle response. The response latency (in seconds) was recorded. The evaluations were done twice alternately to each hindpaw at intervals of 1 min between each stimulation. The opioid morphine (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health) or its solvent (hydroxypropylmethylcellulose, HPMC) was subcutaneously (s.c.) administered 30 min before the behavioral evaluation. The prototypical σ_1

receptor agonist PRE-084 ([2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate) hydrochloride]) (Tocris Cookson Ltd., Bristol, United Kingdom), the prototypical σ_1 receptor antagonist BD-1063 ([1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride]) (Tocris Cookson Ltd., Bristol, United Kingdom), and the test compounds *trans*-(–)-**1d**, *cis*-(+)-**1d**, *cis*-(–)-**1d**, *trans*-(+)-**1d**, or their solvent (HPMC) were all s.c. administered 5 min before the injection of morphine or its vehicle.

ASSOCIATED CONTENT

Supporting Information.

Synthesis of intermediates *trans*-(–)-**3**, *trans*-(+)-**3**, (+)-**4**, (–)-**4**, *trans*-(+)-**5a–d**, *trans*-(–)-**5a–d**, *trans*-(+)-**6a–d**, *trans*-(–)-**6a–d**, *cis*-(+)-**7b–d**, and *cis*-(–)-**7b–d**, and compound **9**, elemental analysis results, binding affinity data and molecular formula strings.

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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.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ER, endoplasmatic reticulum; TMEM97, transmembrane protein 97; NPC1, Niemann–Pick disease protein; DTG, 1,3-di-(2-tolyl)guanidine; HPMC, hydroxypropylmethylcellulose.

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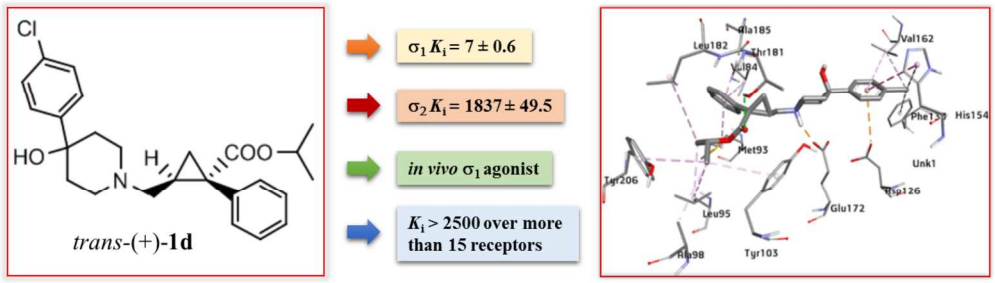

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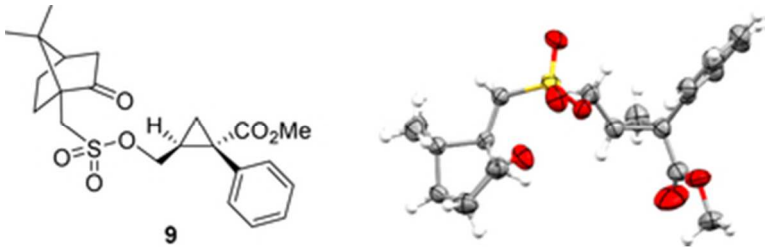

Figure 2. 2D and X-ray ortep structures of compound 9.

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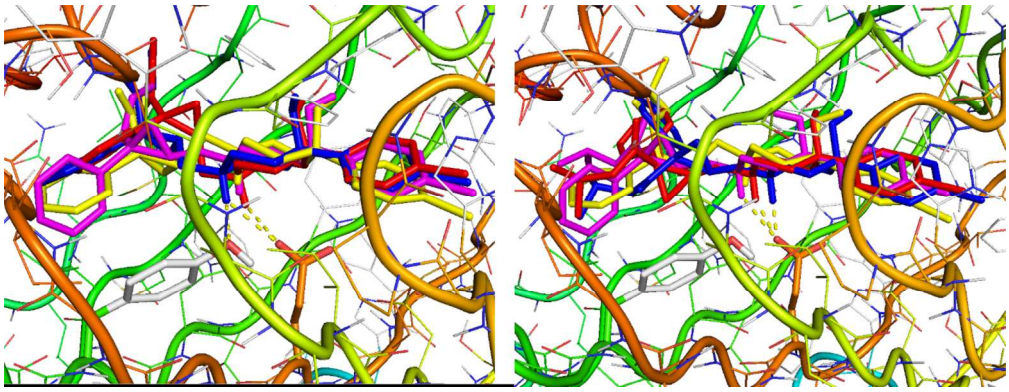

Figure 3. (Left) 3D superposition of the best-docked pose for trans-(+)-1a (blu) trans-(−)-1a (red) cis-(+)-1a (magenta) cis-(−)-1a (yellow). (Right) The same for trans-(+)-1d (blu) trans-(−)-1d (red) cis-(+)-1d (magenta) cis-(−)-1d (yellow).

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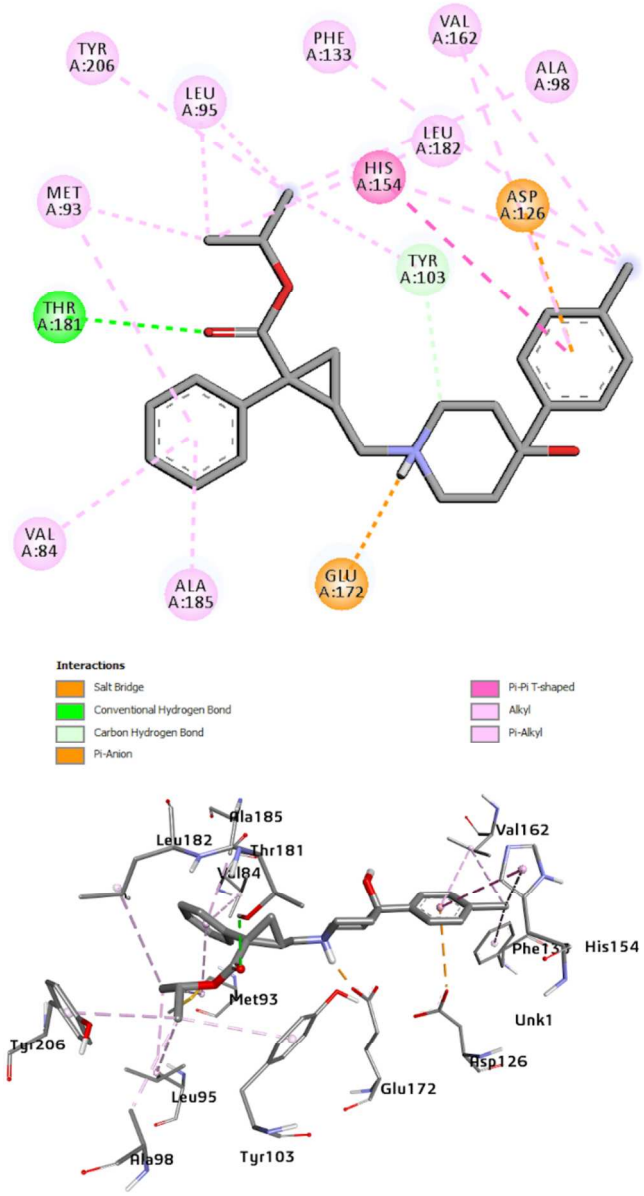

Figure 4. Compound trans-(+)-1d complexed with σ1 receptor from docking. 2D Schematic view of hydrophobic and hydrogen bond interactions (top). 3D analog, the ligand is represented as stick whereas amino acids as a small stick (bottom).

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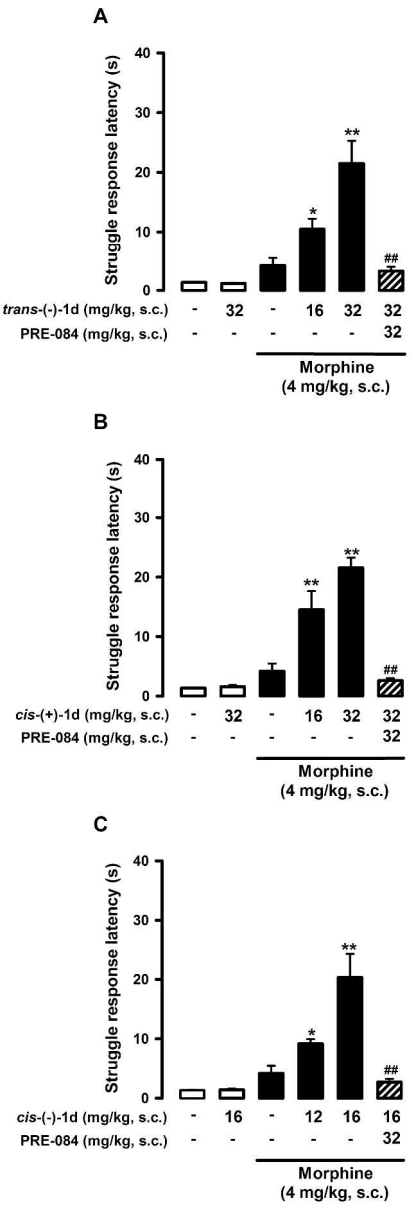

Figure 5. Effects of the administration of trans-(−)-1d, cis-(+)-1d and cis-(−)-1d on mechanical nociception and on the antinociceptive effect induced by morphine. The results represent the struggle response latency during stimulation of the hindpaws with 450 g pressure. Mice were treated subcutaneously (s.c.) with morphine (4 mg/kg) alone or associated with (A) trans-(−)-1d, (B) cis-(+)-1d or (C) cis-(−)-1d. Animals treated with the association of morphine with trans-(−)-1d, cis-(+)-1d or cis-(−)-1d were administered with the σ1 agonist PRE-084 (32 mg/kg, s.c.). HPMC was used as the solvent of all drugs tested. Each bar and vertical line represents the mean ± SEM of values obtained at both hindpaws in 6–8 animals. One-way analysis of variance followed by the Bonferroni test was used to determine statistically significant differences between the values obtained in the groups treated with HPMC and the groups treated with morphine associated with trans-(−)-1d, cis-(+)-1d and cis-(−)-1d (*P < 0.05; **P < 0.01) and between the values obtained in mice treated with each of these drug combinations alone or associated with PRE-084 (##P < 0.01).

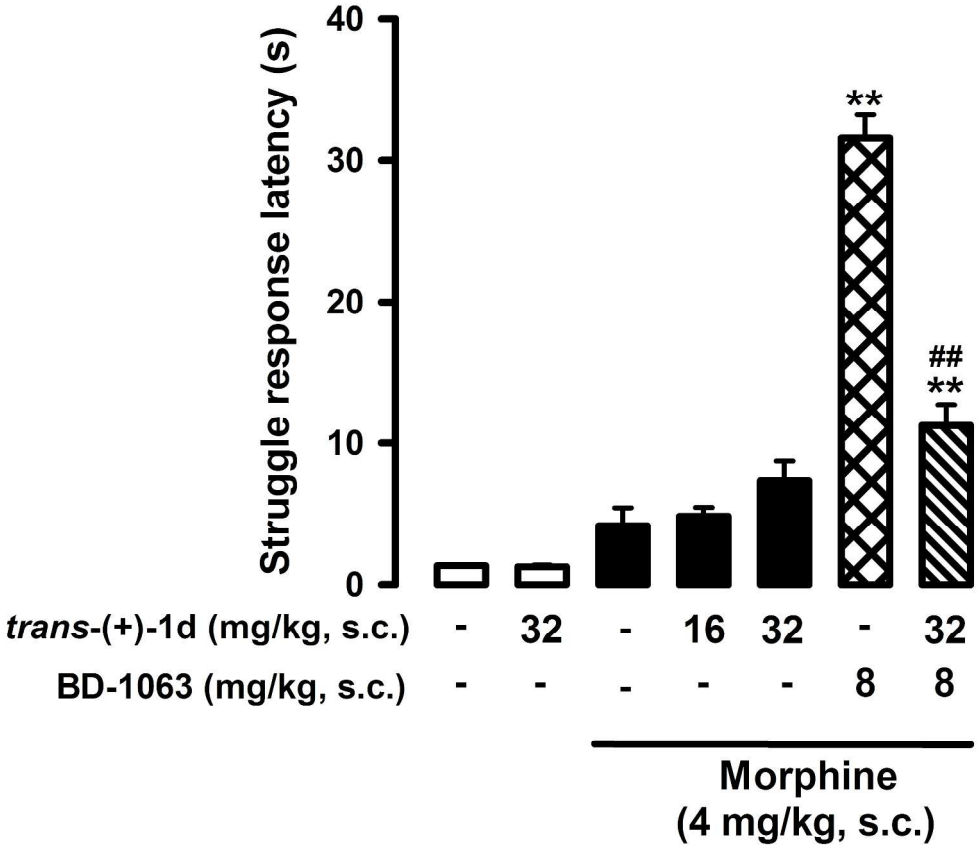

Figure 6. Effects of the administration of trans-(+)-1d on mechanical nociception and on the antinociceptive effect induced by the association of BD-1063 and morphine. The results represent the struggle response latency during stimulation of the hindpaws with 450 g pressure. Mice were treated subcutaneously (s.c.) with morphine (4 mg/kg) alone or associated with trans-(+)-1d. Animals treated with the association of morphine with trans-(+)-1d were administered with the σ1 antagonist BD-1063 (8 mg/kg, s.c.). HPMC was used as the solvent for all tested drugs. Each bar and vertical line represents the mean ± SEM of values obtained at both hindpaws in 6–8 animals. One-way analysis of variance followed by the Bonferroni test was used to determine statistically significant differences between the values obtained in the groups treated with HPMC and the group treated with morphine associated with trans-(+)-1d (**P < 0.01) and between the values obtained in mice treated with trans-(+)-1d + morphine associated with BD-1063 (##P < 0.01).