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Brief Article

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Synthesis and Resolution of *cis*-(±)-Methyl (1*R*,2*S*/1*S*,2*R*)-2-[(4-Hydroxy-4-phenylpiperidin-1-yl)methyl]-1-(4-methylphenyl)cyclopropanecarboxylate [(±)-PPCC)]: New σ Receptor Ligands with Neuroprotective Effect

Orazio Prezzavento,*[,†] Agata Campisi,[‡] Carmela Parenti,[§] Simone Ronsisvalle,[†] Giuseppina Aricò,[§] Emanuela Arena,[†] Marco Pistolozzi,[∥] Giovanna M. Scoto,[§] Carlo Bertucci,[∥] Angelo Vanella,[‡] and Giuseppe Ronsisvalle[†]

[†]*Department of Pharmaceutical Sciences, Medicinal Chemistry Section,* [‡]*Department of Biological Chemistry, Medical Chemistry, and Molecular Biology, and* [§]*Department of Pharmaceutical Sciences, Pharmacology Section, University of Catania, Viale A. Doria 6, 95125 Catania, Italy, and* [∥]*Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, I-40126 Bologna, Italy*

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The enantiomers of *cis*-(±)-methyl (1*R*,2*S*/1*S*,2*R*)-2-[(4-hydroxy-4-phenylpiperidin-1-yl)methyl]-1-(4-methylphenyl)cyclopropanecarboxylate [**1**, (±)-PPCC], a selective σ ligand, were synthesized. The (+)- and (−)-enantiomers bind predominantly to σ_1 receptors and have a reduced σ_2 affinity. Both individually restore the astroglial oxidative status modified by glutamate, counteracting also transglutaminase-2 overexpression. They exhibited in vivo anti-opioid effects on κ opioid (KOP) receptor-mediated analgesia. Our findings demonstrate that the enantiomers display mainly σ_1 agonist activity and that they have neuroprotective effects.

Introduction

The σ[a] receptors are a distinct class of proteins with widespread distribution in the central nervous system (CNS) and in peripheral tissues.[1] Biochemical and pharmacological studies have suggested that there are at least two types of σ receptors (σ_1 and σ_2)[2] which are involved in numerous physiological and pharmacological functions.[3,4] σ_2 receptor agonists produce transient and sustained increases in intracellular calcium ion concentration $[Ca^{2+}]_i$, and have been implicated in the induction of apoptosis.[4]

The activation of cloned σ_1 receptor,[5] in vitro and in vivo, prevents the neuronal death associated with glutamate excitotoxicity.[3,6] It is well-known that hyperstimulation of glutamate receptors leads to the neuronal loss associated with many neurological diseases.[7] In fact, briefly exposing differentiated astrocytes to glutamate causes an increase in $[Ca^{2+}]_i$, glutathione (GSH) depletion, the activation of several calcium-dependent proteins, mitochondrial impairment, and decreases in ATP concentration and reactive oxygen species (ROS) production, which in concert contribute to neuronal cell death.[8]

It has been demonstrated that the recovery of cellular redox status achieved by antioxidants is accompanied by a concomitant and dose-dependent reduction in glutamate-induced transglutaminase-2 (TG2) up-regulation.[9] TG2 is a calcium-dependent multifunctional protein implicated in several acute and chronic neurodegenerative diseases associated with excitotoxicity.[10]

In previous studies, we reported the synthesis and biological profile of the new σ selective ligand *cis*-(±)-methyl (1*R*,2*S*/1*S*,2*R*)-2-[(4-hydroxy-4-phenylpiperidin-1-yl)methyl]-1-(4-methylphenyl)cyclopropanecarboxylate [**1**, (±)-PPCC][11,12] (Figure 1). We demonstrated that **1** was able to modulate TG2 expression in primary rat astroglial cell cultures.[11]

Herein, we report the synthesis of the corresponding (+)- and (−)-enantiomers in high optical purity and their σ receptor binding affinities. Furthermore, we describe the effects of **1**, its enantiomers, (+)-pentazocine, and ibogaine on primary rat neocortical astroglial cell cultures in the absence or in the presence of glutamate on intracellular oxidative status and on glutamate-induced TG2 overexpression.

Because σ_1 receptor agonists attenuate KOP-induced analgesia while σ_1 receptor antagonists potentiate KOP-induced analgesia,[12−14] we also performed in vivo studies to determine whether the enantiomers were able to modulate the analgesic effect induced by the KOP agonist *trans*-(1*S*,2*S*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide [**2**, (−)-U50,488H][15,16] in rats.

Chemistry

Treatment of *cis*-lactone (±)-**3**, which was prepared as previously described,[11,17] with *R*-(+)-α-methylbenzylamine provided two diastereoisomeric amides, (+)-**4** and (−)-**4** (Scheme 1;

*To whom correspondence should be addressed. Phone: +39-095-738-4205. Fax: +39-095-222-239. E-mail: prezzave@unict.it.

[a] Abbreviations: ATP, adenosine triphosphate; CD, circular dichroism; DCFDA, 2,7-dichlorofluorescein diacetate; DIV, days in vitro; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; DTG, 1,3-di-(2-tolyl)guanidine; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; ee, enantiomeric excess; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GSH, glutathione; GSSG, glutathione disulfide; haloperidol, 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; ibogaine, 12-methoxyibogamine; IgG, immunoglobulin G; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; (+)-pentazocine, (2*S*,6*S*,11*S*)-6,11-dimethyl-3-(3-methylbut-2-en-1-yl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; sc, subcutaneous; SD, standard deviation; SDS, sodium dodecyl sulfate; σ, sigma receptor; TFLs, tail flick latencies; TG2, transglutaminase-2; TLC, thin-layer chromatography; UV, ultraviolet.

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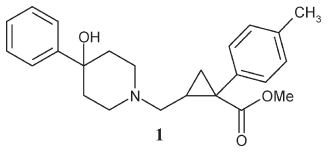
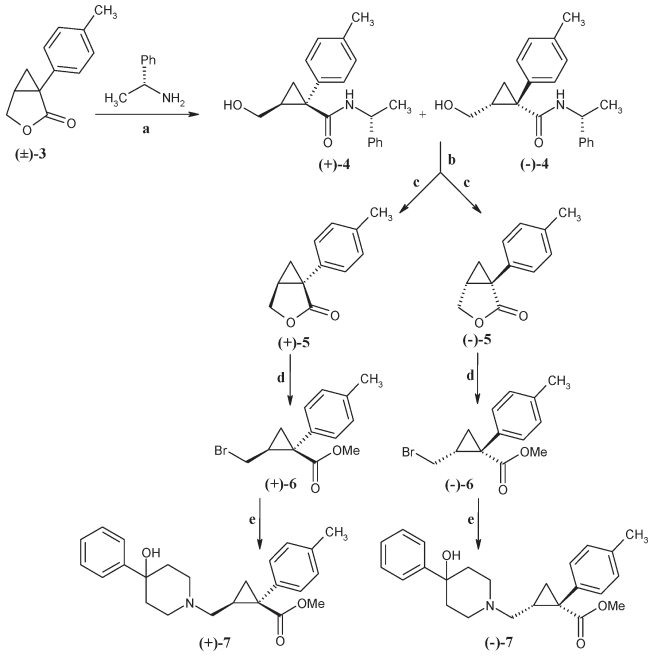


Figure 1

Scheme 1[a]



[a] Reagents and conditions: (a) 2-hydroxypyridine, toluene, 70 °C, 24 h; (b) separation by flash chromatography; (c) 1 N H_2SO_4, dioxane/H_2O (1:1), 5 h, 85 °C; (d) $SOBr_2$, CH_3OH, room temp, 24 h; (e) 4-phenylpiperidin-4-ol, DMF, $NaHCO_3$, 60 °C, 8 h.

also see Supporting Information). Separation by flash chromatography and subsequent hydrolysis of the amides gave lactones (+)-**5** and (−)-**5**. Enantiomeric bromides (+)-**6** and (−)-**6** were obtained by esterification of the intermediate lactones with methanol and thionyl bromide. The alkylation of commercially available 4-phenylpiperidin-4-ol with the appropriate enantiomer of the bromoester gave the two desired enantiomers (+)-**7** and (−)-**7**.

The absolute configuration of the cyclopropane ring was established by comparison with reported ^{1}H NOE NMR data[18] and by X-ray diffraction analysis.[19] Circular dichroism (CD) spectra of (+)-**7** and (−)-**7** are reported in Figure 6 (see Supporting Information).

Results and Discussion

The target compounds were evaluated with respect to their $\sigma_{1/2}$ receptor binding affinities. (+)-**7** and (−)-**7** display a high affinity for the σ_1 subtype (K_i = 1.9 ± 0.1 and 13 ± 1.2 nM, respectively). In these experiments **1** gave slightly different K_i values ($K_i(\sigma_1)$ = 1.8 ± 0.08 nM, $K_i(\sigma_2)$ = 20.8 ± 1.8 nM) than in our previous experiments ($K_i(\sigma_1)$ = 1.5 ± 0.06 nM, $K_i(\sigma_2)$ = 50.8 ± 3.0 nM).[11] The (+)-**7** and (−)-**7** exhibit moderate σ_2 binding affinities (K_i = 50.2 ± 5 and 103 ± 4 nM, respectively).

To assess the biological effects of the σ ligands, we used primary rat neocortical astroglial cell cultures that were 14 days in vitro (DIV) in the absence or in the presence of glutamate.[8]

Table 1. GSH and ROS Levels in Astroglial Cell Cultures[a]

treatment	GSH levels (nmol/mg protein)	ROS levels (nmol of dichlorofluoresceine produced per mg protein/30 min)
control	14.61 ± 2.1	3.16 ± 0.41
glutamate	9.08 ± 0.9*	4.45 ± 0.51*
ibogaine	9.95 ± 1.1*	4.99 ± 0.25*
1	11.80 ± 0.5*	4.25 ± 0.31*
glutamate + ibogaine	7.95 ± 1.1*	5.82 ± 0.76*
(+)-pentazocine	14.45 ± 0.3	3.68 ± 0.33
glutamate + (+)-pentazocine	14.15 ± 0.4**	3.46 ± 0.44**
glutamate + **1**	8.05 ± 0.8*	5.51 ± 0.81*
(+)-**7**	14.87 ± 0.9	3.88 ± 0.19
glutamate + (+)-**7**	13.44 ± 0.7**	3.05 ± 0.29**
(−)-**7**	13.99 ± 0.9	3.39 ± 0.24
glutamate + (−)-**7**	14.87 ± 0.2**	3.67 ± 0.31**

[a] (*) $p < 0.05$ vs control; (**) $p < 0.001$ vs control.

Cell cultures were prepared and characterized by immunofluorescent staining for a specific marker, such as glial fibrillary acidic protein (GFAP). About 90−95% of the cells were GFAP-positive, indicating that the cultures were enriched in astrocytes. The effects of racemate and its enantiomers were compared with (+)-pentazocine, a putative σ_1 agonist,[13] and ibogaine, a σ_2 agonist.[20] Specifically, we selected ibogaine because, even though it interacts with numerous biological systems in the CNS, it is known to possess a higher affinity for σ_2 receptors than for any other known neurotransmitter receptors.[21] In preliminary experiments, we established the optimal concentrations of the σ ligands and their optimal exposure times to the cultures in the absence or in the presence of glutamate (500 μM).[8] This set of experiments showed that 25 μM was a nontoxic concentration of the various drugs for the cells and that the optimal exposure time was 24 h (data not shown). It has been reported that ibogaine at concentrations up to 130 μM does not affect glutamate uptake or release by rat astrocyte cultures.[22]

To evaluate the σ ligands' ability to modify the intracellular oxidative status of the astroglial cell cultures, we tested GSH and ROS levels. Significant increases in GSH depletion and ROS production were observed in glutamate-exposed cell cultures when compared with the untreated control cultures (Table 1). Ibogaine had the same effect as glutamate, but its effect was more evident when the cell cultures were co-treated with the neurotransmitter. Compound **1** induced a significant increase in GSH depletion and ROS production with respect to the controls. Nevertheless, the racemate's effect was less pronounced than that of ibogaine or treatment with glutamate. The combination of **1** and glutamate treatment caused a further increase in GSH depletion and ROS production (Table 1). These data demonstrate that **1** and ibogaine induce oxidative stress, which leads to astroglial death by activating the apoptotic pathway, as previous reported.[11,23]

(+)-Pentazocine did not modify GSH or ROS levels in untreated cells. However, it was able to completely counteract the effect of glutamate. Exposure to (+)-**7** or (−)-**7** alone or in glutamate-exposed cells showed a pattern similar to that of (+)-pentazocine (Table 1). These data demonstrate that both enantiomers and (+)-pentazocine are able to restore oxidative status after it has been modified by glutamate.

Figures 2 and 3 respectively show immunoblots (A) and densitometric (B) analyses of TG2 expression in astroglial cell cultures exposed to σ ligands in the absence or in the presence of glutamate. A significant increase in the percentage of TG2

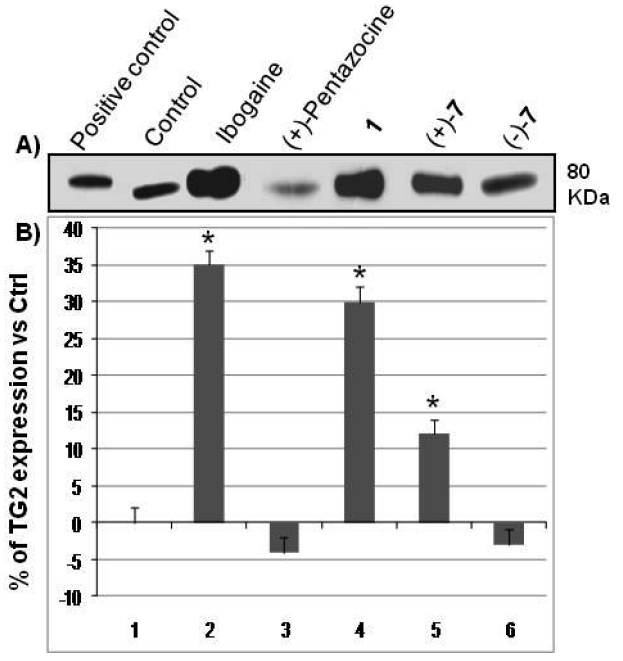


Figure 2. Representative immunoblot (A) and densitometric (B) analyses of TG2 expression in astroglial cell cultures: (*) $p < 0.05$ vs control.

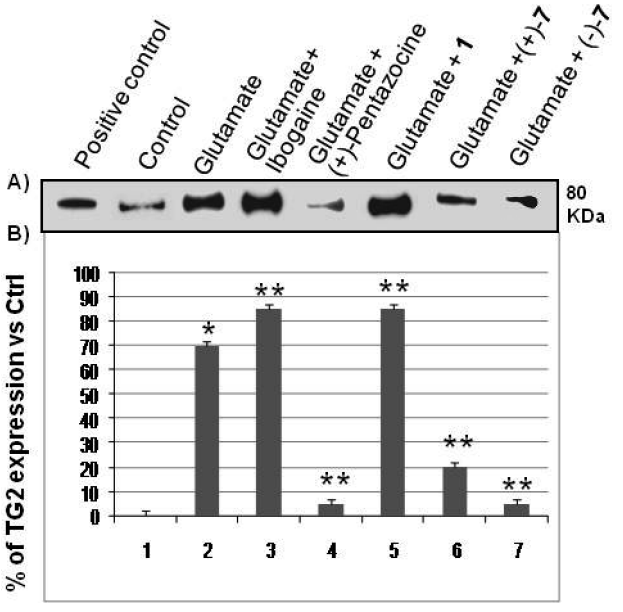


Figure 3. Representative immunoblot (A) and densitometric (B) analyses of TG2 expression in astroglial cell cultures exposed to glutamate: (*) $p < 0.05$ vs control; (**) $p < 0.05$ vs glutamate.

expression was observed in glutamate-exposed cells when compared with the control cultures (Figure 3). Ibogaine produced a marked up-regulation of the protein (Figure 2). The presence of glutamate and ibogaine induced a further increase in TG2 expression (Figure 3). Compound **1** alone or in the presence of glutamate also caused a significant up-regulation in TG2 expression (Figures 2 and 3). However, the effect appeared less pronounced than that induced by ibogaine (Figures 2). In contrast, in the presence of glutamate, they showed a similar pattern (Figure 3). (+)-Pentazocine did not induce TG2 expression. In fact, cells exposed to (+)-pentazocine showed lower levels of the protein expression than the control (Figure 2). This σ_1 agonist was able to completely counteract glutamate-induced TG2 up-regulation (Figure 3). A slight enhancement of TG2 expression was caused by (+)-**7**, even though it produced a down-regulation of TG2 in the presence of glutamate (Figures 2 and 3). The other enantiomer, (−)-**7**, did not exert any effect on TG2 expression (Figure 2). However, it was able to totally counteract the effect of glutamate exposure on TG2 expression. Densitometric analysis showed that (−)-**7** had a pattern similar to that of (+)-pentazocine on TG2 expression (Figure 3). Our findings demonstrate that the individual enantiomers of **1** modulate TG2 expression differently in the absence or in the presence of glutamate. In particular, we observed that (−)-**7** has more effect on TG2 expression than (+)-**7**. Taken together, our data suggest that (+)-**7** and (−)-**7**, at least under our experimental conditions, act mainly as σ_1 agonists. The different effects of racemate may be due to a concentration dependent effect or to an interaction with other protein targets. Moreover, given the involvement of σ receptors in different forms of calcium signaling in neuronal cells,[24−26] the obtained results could also be justified by the effects of our σ ligands on $[Ca^{2+}]_i$. However, the pharmacological characterization of the interaction between σ receptors and calcium channels has not yet been well elucidated.[27] In fact, some findings demonstrated that there is no correlation between the potency of the tested compounds and their σ binding affinities,[28] while in other experiments, σ_1 agonists and σ_1 antagonists produced the same effects, which might also be due to the involvement of σ_2 receptors.[29] Furthermore, the selective σ_1 agonists (+)-pentazocine and 2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate (PRE 084) caused opposite effects on the increase in $[Ca^{2+}]_i$.[30] On the basis of these literature data,[27−30] we hypothesize that in our results the racemate or the single enantiomers may differently modulate the $[Ca^{2+}]_i$ and consequently TG2 expression levels and functions.

The in vivo studies were performed with the aim of better understanding the pharmacological profile of the individual enantiomers. Systemic administration of (+)-**7** or (−)-**7** at a dose of 1 mg/kg sc (this dosage was chosen from the previous evaluation of racemate's dose-response curve)[12] did not modify the basal tail flick latency in rats, whereas the enantiomers were able to prevent the analgesic effect induced by the KOP agonist **2**. However, pretreatment with (−)-**7** significantly decreased analgesic effect of **2** about 30 min after opioid administration, while the dextro isomer's effect was significant over the entire observation period (see Supporting Information Figure 4A,B). This anti-opioid effect was prevented, at least at the dose utilized, by pretreatment with the putative σ_1 antagonist haloperidol (data not shown).[14] Because it has been reported that σ_1 agonists are able to antagonize KOP-mediated analgesic response, we have further confirmed the σ_1 agonist activity of (+)-**7** and (−)-**7** through their anti-opioid effect.

In conclusion, our data demonstrate that (+)-**7** and (−)-**7**, different from the racemate, are able to restore GSH and ROS levels to control values in glutamate-exposed astroglial cell cultures. Furthermore, both enantiomers, when administered individually, counteract the glutamate induced TG2 overexpression. Thus, our findings show that (+)-**7** and (−)-**7** possess neuroprotective effects. Studies are now in progress to clarify the astrocytes' different response to the single enantiomers and to the racemate.

Experimental Section

For complete details, refer to the Supporting Information.

General Experimental Details. All commercial reagents used for synthesis were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise specified. Flash column chromatography was carried out on Merck silica gel 60 (230−400 mesh). ^{1}H NMR spectra (δ, ppm) were recorded with a Varian Inova 200 MHz spectrometer (Varian, Leini, Italy). HPLC analysis was performed at room temperature on a Jasco HPLC system using a CHIRALCEL AD, 250 mm × 4.6 mm, 5 μm particle-size HPLC column (Daicel Chemical Industries LTD) and hexane/2-propanol (97:3) containing 0.1% (v/v) diethylamine (Fluka) as an eluent. CD spectra were recorded with a Jasco J-810 spectropolarimeter (Jasco, Tokyo). Elemental analyses (C, H, N) were determined on an elemental analyzer Carlo Erba model 1106 (Carlo Erba, Milan, Italy) and were within ±0.4% of the theoretical values. Purities of tested compounds were determined by elemental analysis and HPLC and were ≥99%.

(+)-Methyl (1*R*,2*S*)-2-[(4-Hydroxy-4-phenylpiperidin-1-yl)-methyl]-1-(4-nitrophenyl)cyclopropanecarboxylate Oxalate [(+)-7]. A stirred mixture of 4-phenylpiperidin-4-ol (170 mg, 0.96 mmol), (+)-methyl (1*R*,2*S*)-2-(bromomethyl)-1-(4-methylphenyl)cyclopropanecarboxylate (+)-**6** (300 mg, 1.05 mmol), and $NaHCO_3$ (162 mg, 1.92 mmol) in dry DMF (10 mL) was heated to 60 °C under a nitrogen atmosphere for 8 h. After cooling, the mixture was evaporated to dryness and CH_2Cl_2 was added to the residue. The organic phase was washed with aqueous $NaHCO_3$ (5%), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was purified on a silica gel column (cyclohexane/ethyl acetate, 6:3) to afford a colorless oil in 80% yield, which was converted to its oxalate salt. The enantiomeric excess was determined to be 99.5% on a chiral HPLC with a Chiralpak AD chiral column (25 cm × 0.46 cm i.d.) using hexane/2-propanol/triethylamine (97:3:0.1, 1 mL/min) as the eluent. Mp 162−163 °C (dec). ^{1}H NMR ($CDCl_3$): δ 1.25 (t, 1H, J = 7.0 Hz), 1.33 (dd, 1H, J = 4.4, 9.0 Hz,), 1.66 (dd, 1H, J = 4.4, 7.0 Hz), 1.79 (m, 2H), 2.22 (m, 2H), 2.33 (s, 3H), 2.59 (m, 2H), 2.78 (m, 2H), 2.96 (m, 2H), 3.63 (s, 3H), 7.09−7.55 (m, 9H). R_f = 0.30 (cyclohexane:ethyl acetate, 6:3). $[\alpha]_D^{20}$ +44° (1% p/v, MeOH). Anal. ($C_{24}H_{29}NO_3\ C_2H_2O_4$) C, H, N.

(−)-Methyl (1*S*,2*R*)-2-[(4-Hydroxy-4-phenylpiperidin-1-yl)-methyl]-1-(4-nitrophenyl)cyclopropanecarboxylate Oxalate [(−)-7]. This compound was prepared from 4-phenylpiperidin-4-ol (110 mg, 0.64 mmol) and (−)-methyl (1*S*,2*R*)-2-(bromomethyl)-1-(4-methylphenyl)cyclopropanecarboxylate (−)-**6** (200 mg, 0.70 mmol) via the procedure described above for (+)-**7**. After purification by flash chromatography with cyclohexane/ethyl acetate (6:3), the free base (80%) was converted to its oxalate salt. Enantiomeric excess = 97.4%. Mp 156−157 °C (dec). ^{1}H NMR ($CDCl_3$): δ 1.26 (t, 1H, J = 7.0 Hz), 1.33 (dd, 1H, J = 4.4, 9.0 Hz,), 1.67 (dd, 1H, J = 4.4, 7.0 Hz), 1.78 (m, 2H), 2.22 (m, 2H), 2.33 (s, 3H), 2.59 (m, 2H), 2.78 (m, 2H), 2.96 (m, 2H), 3.63 (s, 3H), 7.07−7.55 (m, 9H). R_f = 0.29 (cyclohexane/ethyl acetate, 6:3); $[\alpha]_D^{20}$ −46°, (1% p/v, MeOH). Anal. ($C_{24}H_{29}NO_3 \cdot C_2H_2O_4$) C, H, N.

The purity of (+)-**7** and (−)-**7** was determined by reversed-phase HPLC assays. The mobile phase consisted of Tris-HCl (50 mM, pH 7.4)/acetonitrile (60:40, v/v). Flow rate of 2 mL/min was employed. These chromatographic conditions allowed eluting and separating all the impurities contained into the samples within 10 min. Chromatograms were recorded setting the detector at 205 nm. Stock solutions were prepared as 1 mg/mL in acetonitrile and diluted to 0.5 mg/mL with Tris-HCl buffer before the injection. An amount of 20 μL of the solutions was injected for each run. The retention time for (+)-**7** and (−)-**7** free bases were approximately 2.5 min, while oxalate appeared at the solvent front. The purity was calculated as the percentage of the main peak area over the sum of the areas of all the peaks in the chromatograms. The two samples showed a purity of ≥99%.

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Supporting Information Available: Analgesic effects, elemental analysis results, chiral HPLC traces, CD and UV spectra, and details of compound synthesis and biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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