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Chiral arylpyrrolidinols: preparation and biological profile

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Abstract—The preparation and biological evaluation of a new class of arylpyrrolidinols is reported. The antinociceptive activity was evaluated in vivo with the hot plate test (HPT) and formalin test (FT), excluding any involvement on motor coordination with the rota-rod test (RRT). The nociceptive behavior in the late phase of FT (representative of chronic pain) suggests an involvement of the antiinflammatory process and it is clearly influenced by the stereochemical features, being the eutomer of phenylpyrrolidinols, the (2*R*,3*S*) enantiomer. Despite this, a specific mechanism of action is not yet clarified.

1. Introduction

In recent years, we have focused our attention on heterosteroid 17-methyl-17-aza-equilenine analogues, synthesizing structurally related compounds with analgesic activity that interact with the opioidergic system.[1–3] Aryl-1,2-dimethylpyrrolidinols (Fig. 1) have been studied owing to their interesting biological properties, and a SAR study has been performed in order to investigate the relationship between stereoidogenesis and opioid analgesia. All compounds were found to possess interesting analgesic activity,[1–3] being tested in the hot plate test (HPT), but evidenced poor affinity with opioid receptor subtypes in vitro, as evaluated in binding assays. Despite this, the reversal of the antinociceptive activity by the nonspecific opioid antagonist naloxone (NLX) could confirm an indirect involvement of the opioidergic system. The involvement of cholecystokinin or neuropeptide FF analogues could be considered to explain antinociceptive properties of these compounds. Any effect on motor coordination[3] was evidenced by monitoring the locomotory control of the animals with the rota-rod test. All compounds with a different aromatic nucleus showed relevant antinociceptive properties, with exception for the 9-phenantrenyl derivative (2*R*,3*S*/2*S*,3*R*)-**10**.

The most interesting compounds are (2*R*,3*S*/2*S*,3*R*)-**1** (*hit* compound), (2*R*,3*S*/2*S*,3*R*)-**5**, (2*R*,3*S*/2*S*,3*R*)-**6**, and (2*R*,3*S*/2*S*,3*R*)-**8**, so a more deep investigation has been performed with the formalin test in mice, which demonstrated a pain reduction, suggesting their involvement in the antiinflammatory process.

Herein, (2*R*,3*S*)-**1** and (2*R*,3*S*)-**3**–**8** and their enantiomers were prepared according to the synthetic procedure performed for the enantiomers of compound **2**, previously published,[1] in order to investigate the role of stereochemistry on the pharmacological activity. Moreover the aryl-1-methylpyrrolidinols (*R*/*S*)-**17**–**19** (Fig. 2) were also synthesized according to the principles of simplification.

The antinociceptive activity was evaluated in vivo with the hot plate test (HPT) and formalin test (FT), excluding any involvement of motor coordination with the rota-rod test (RRT).

A molecular modeling study of (2*R*,3*S*)-**1**, (2*R*,3*S*)-**5**, and their simplified analogues (*S*)-**17** and (*S*)-**18** has

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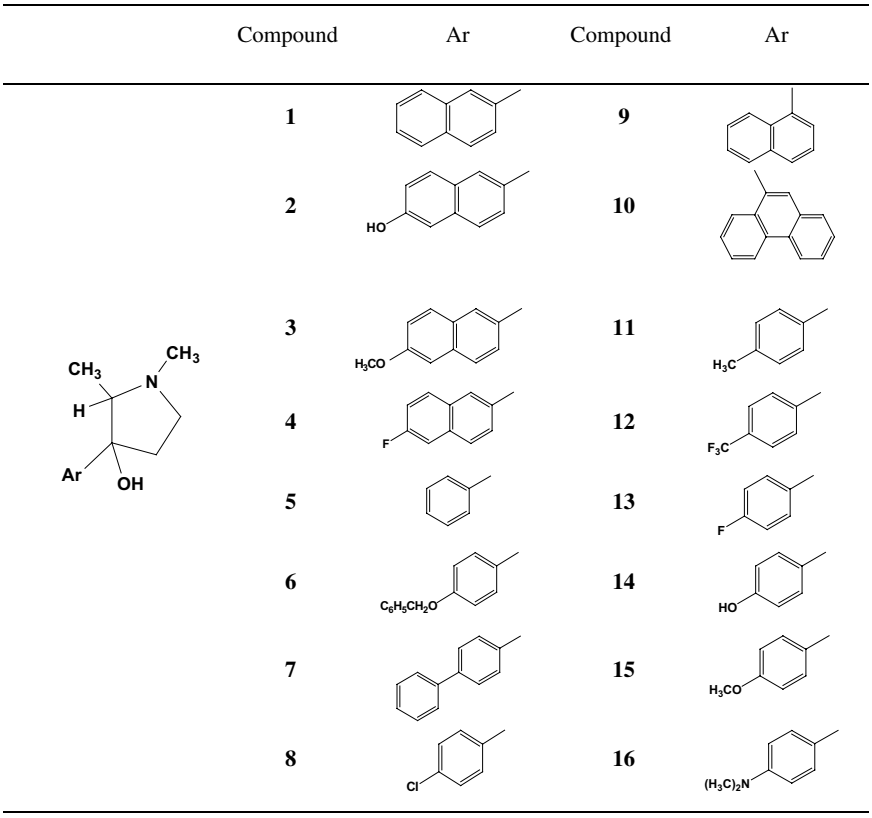


Figure 1. Pyrrolidinols **1**–**16**.

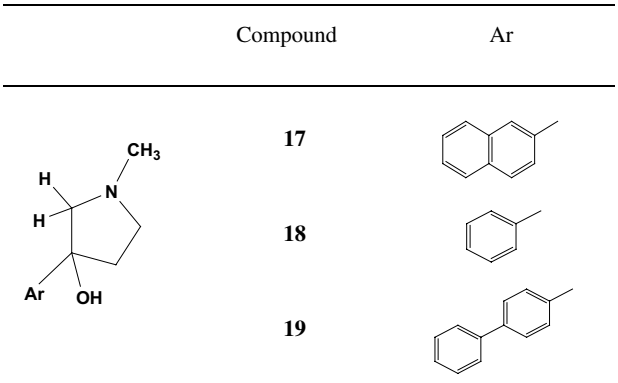


Figure 2. Pyrrolidinols **17**–**19**.

been performed in order to consider the conformational properties of biologically relevant compounds.

2. Results

2.1. Chemistry

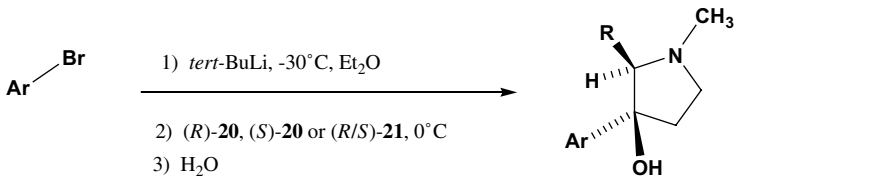
Compounds (2*R*,3*S*)-**1** and (2*R*,3*S*)-**3**–**8**, and their enantiomers were synthesized (Scheme 1) by nucleophylic addition to (*R*)-**20** or (*S*)-**20**[4] (Fig. 3) of the appropriate aromatic anion commercially available, with exception for compound **6** whose precursor, the 1-benzyloxy-4-bromo-benzene, has been prepared according to Balasubramanian et al.[5] Benzilbromide and KOH were added to a solution of 4-bromo-phenol in PEG 400 at room temperature under stirring. After 24 h the reaction mixture was added with water and extracted with diethyl ether. The combined organic phases provided the 1-benzyloxy-4-bromo-benzene, as confirmed by IR and ^{1}H NMR analysis.

This reaction occurs via a nucleophllic attack of the aromatic anion at the prochiral center of the (*R*)-**20** or (*S*)-**20** on the less hindered side, consequently (2*R*,3*S*)- or (2*S*,3*R*)-**1**–**8** enantiomers were obtained, respectively, as evidenced by ^{1}H NMR analysis of the crude products.

The employed synthetic procedure provided compounds with high enantiomeric excess (ee), as reported in Table 1. Liquid chromatographic procedures were attempted using several chiral stationary phases in order to develop a suitable direct method for the evaluation of the enantiomeric excess of all enantiomeric compounds. Baseline separation was successfully achieved on analytical Chiralpak AD, Chiralpak AS, and Chiralcel OD-H columns with good α and R_s values (Table 2).

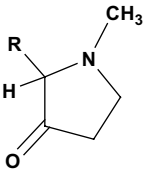
Compounds (*R*/*S*)-**17**–**19** were prepared from ketone **21**, according to the same synthetic procedure employed for compounds **1**–**8** (Scheme 1).

1-Methyl-pyrrolidin-3-one **21** was prepared according to the synthetic procedure described by Cavalla et al.,[6] with suitable modifications (Scheme 2). *N*-Methyl-β-alaninonitrile and anhydrous K_2CO_3 were added with α-Br-acetic acid ethyl ester affording



R = CH_3 (2*R*,3*S*)-**1-8** and enantiomers
R = H (*R*/*S*)-**17-19**

Scheme 1. Synthesis of the enantiomers of compounds **1**–**8** and **17**–**19**.



R = CH_3 20
R = H 21

Figure 3. Compounds **20** and **21**.

Table 1. Optical properties of compounds **1**, **3**–**8**

Compound	[α][a]	Ee %
(2*R*,3*S*)-**1**	+22.5 (589)	99.9
(2*S*,3*R*)-**1**	−22.8 (589)	99.9
(2*R*,3*S*)-**3**	+34.6	99.9
(2*S*,3*R*)-**3**	−33.8	99.7
(2*R*,3*S*)-**4**	+22.9 (365.1)	99.5
(2*S*,3*R*)-**4**	−23.9 (365.1)	99.9
(2*R*,3*S*)-**5**	−24.40	99.9
(2*S*,3*R*)-**5**	+24.69	99.9
(2*R*,3*S*)-**6**	+12.03	99.2
(2*S*,3*R*)-**6**	−12.67	99.7
(2*R*,3*S*)-**7**	+46.88	99.9
(2*S*,3*R*)-**7**	−46.62	99.9
(2*R*,3*S*)-**8**	+38.18	99.9
(2*S*,3*R*)-**8**	−38.46	99.9

[a] Performed on hydrochlorides, *c* 0.5, in MeOH; λ 405 nm.

[(2-cyano-ethyl)-methyl-amino]-acetic acid ethyl ester (intermediate A), and a further alcoholysis gave 3-(ethoxycarbonylmethyl-methyl-amino)-propionic acid ethyl ester (intermediate B). Treatment with sodium ethanoate to obtain intermediate C was unsuccessful. Therefore, a Dieckmann condensation with 60% NaH in anhydrous toluene at 0 °C was performed, obtaining 1-methyl-4-oxo-pyrrolidine-3- carboxylic acid ethyl ester. This not isolated intermediate (C) was hydrolyzed and decarboxylated with HCl 6 N under heating, affording **21** as a yellow oil. The crude product has been purified by distillation under reduced pressure and kept in the dark at −10 °C and in nitrogen atmosphere. GC analysis showed a 99.9% purity of the desired product. All isolated intermediates as well as compound **21** were characterized by IR and ^{1}H NMR analysis.

The results of the elemental analyses, IR and ^{1}H NMR spectra agreed with the assigned structures for all compounds.

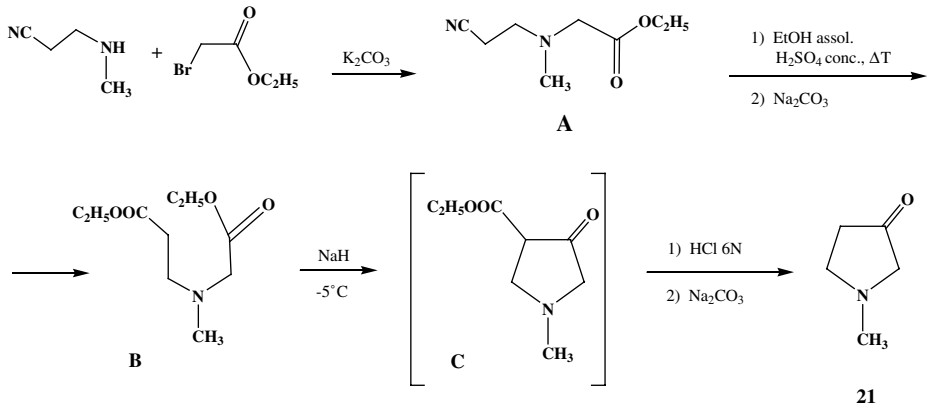
2.2. Molecular mechanics calculations

The computational work was dedicated to the characterization of biologically relevant compounds and to the clarification of the C2 methyl substitution. Stereoisomers of compounds **1**, **5**, **17**, and **18** were considered in this study. The (2*R*,3*S*)-**1** and (2*R*,3*S*)-**5** were modeled by the Monte Carlo (MC) conformational search method applied to torsional angles (see Experimental section).

Table 2. Analytical resolution of compounds **1**, **3**–**8**

Compound	Column[a]	Mobile phase[b]	α	R_s	R_t (min) (config.)
1·HCl	Chiralcel OD-H	90.0/10.0/0.1			6.5 (2*R*,3*S*)
					9.3 (2*S*,3*R*)
3·HCl	Chiralpak AD	98.0/2.0/0.1	1.18	1.47	20.2 (2*S*,3*R*)
					23.1 (2*R*,3*S*)
4·HCl	Chiralpak AD	98.0/2.0/0.1	1.11	2.23	24.1 (2*S*,3*R*)
					26.1 (2*R*,3*S*)
5·HCl	Chiralpak AD	95.0/5.0 EtOH/0.05			18.6 (2*R*,3*S*)
					25.9 (2*S*,3*R*)
6·HCl	Chiralpak AS	98.0/2.0/0.1	1.23	1.54	21.7 (2*S*,3*R*)
					25.1 (2*R*,3*S*)
7·HCl	Chiralpak AD	98.0/2.0/0.1	1.18	2.25	20.1 (2*S*,3*R*)
					23.0 (2*R*,3*S*)
8·HCl	Chiralpak AD	99.0/1.0/0.1	1.15	1.44	25.1
					28.2

[a] 250 × 4.6 mm.
[b] Solvent mixture: *n*-hexane/IPA/DEA (v/v/v); flow rate: 0.5 mL/min; UV detector: λ 273 nm.



Scheme 2. Synthesis of compound **21**.

Table 3. Conformational distribution of stereoisomers **1**, **5**, **17**, and **18**

Compound	Number of conformers within 50 kJ/mol above the global energy minimum	Number of conformers with Boltzmann population > 90%
(2*R*,3*S*)-**1**	16	4
(2*R*,3*S*)-**5**	7	2
(*S*)-**17**	13	4
(*S*)-**18**	7	2

The quality of the MC conformational exploration was considered exhaustive as demonstrated by the consistent average number of duplicates found during the simulations, found always higher than 53.2. The conformational search was repeated also considering the (2*S*,3*R*) stereoisomer of compounds **1** and **5**. As expected, the same conformational distribution was found with specular geometry with respect to the former stereoisomers (data not shown). So the conformational analysis was focused on the (2*R*,3*S*) series. Starting with these stereoisomers, molecular models of simplified compounds (*S*)-**17** and (*S*)-**18** were obtained and the same MC search was applied to these compounds. The conformational distribution of the four stereoisomers is reported in Table 3.

The superimposition of four of the most populated conformers of (2*R*,3*S*)-**1** and (*S*)-**17** revealed a perfect match of the pyrrole ring atoms, not affected at all by the methyl substitution in C2. In few conformations, where this moiety interacts directly to the Ar group, the rotatable bond C3–Ar, pertinent to the orientation of this substituent with respect to the five-member ring, seems to be fairly influenced. A moderate effect is also observed with the hydroxyl rotamer, orientating the alcoholic hydrogen toward the nitrogen to establish an

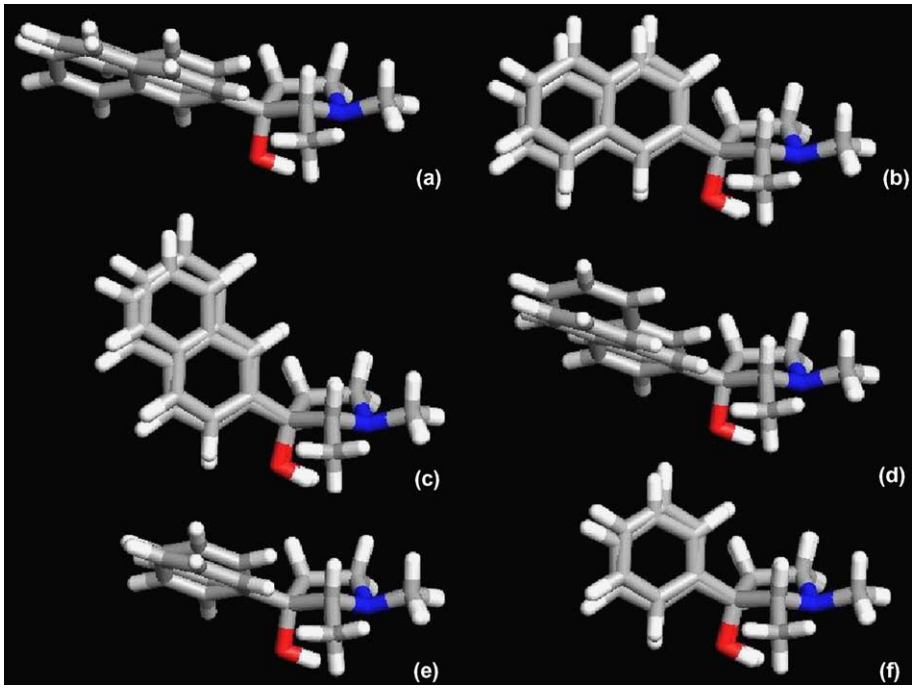


Figure 4. Comparison between the four most populated conformers of compounds **1** and **17** (a–d) and the two of compounds **5** and **18** (e–f) superimposed onto the pyrrole ring.

intramolecular hydrogen bond, as found in the most populated conformers (Fig. 4). Similar considerations can be done by the superimpositions of the two (2*R*,3*S*)-**5** and (*S*)-**18** most populated conformers (Fig. 4).

2.3. Biological evaluation

The synthesized compounds were evaluated as antinociceptive agents by the in vivo HPT and FT assays (see Experimental section). The influence on the locomotory activity was also determined with the RRT to exclude motor impairment associated with the analgesic effect.

3. Discussion

Racemic arylpyrrolidinols possess interesting analgesic activity in the HPT,[2,3] with a potency similar or superior to that of morphine (AD_{50} values between 0.10 and 4.82 mg/kg). Although the biological effect is reversed by naloxone, the mechanism of action of these compounds is still to be determined, since binding affinity for opioid receptors (K_i, μM) is too low to account for the opioid analgesia.

After a wide, but unsuccessful in vitro screening of the *hit* with several receptors[7] (cannabinoid, angiotensin type, benzodiazepinic, cholecystokinic, GABAergic, hystaminergic, dopaminergic, adrenergic, muscarinic, serotoninergic and ionotropic glutamate receptors, and Ca^{2+} channel) we focused our attention on the in vivo profile of novel compounds.

Therefore, we performed an investigation with the formalin test, an experimental model suitable to assess the animal's response both to acute and continuous pain. As known, the response to formalin is biphasic: the early phase (phase I) is related to an intense pain comparable to that obtained by thermal stimulus in HPT and the late phase (phase II) is related to a moderate and continuous pain caused by damaged tissue.

In this paper we have investigated the role of the stereogenic center C2 of the heterocyclic moiety preparing both enantiopure compounds **1**, **3**–**8** and the C2 unsubstituted analogs **17**–**19**. First of all, compounds were tested on mice with HPT (Table 4) and FT by subcutaneous administration of 2, 4, 8 mg/kg. At the doses employed in our study, no motor impairment was evidenced with RRT (mice remaining on the rota-rod: $\geqslant 67\%$).

In HPT, the dose of 4 mg/kg (Fig. 5), comparable with the AD_{50} of morphine (4.18 mg/kg), was the most significative ideal one for almost all compounds, being 2 mg/kg sometimes not active and 8 mg/kg too high to assess for the analgesic activity. In the FT (Fig. 6) the most significative response was obtained at 8 mg/kg.

Regarding the acute pain, experimental data (Figs. 5 and 6) clearly evidence that in both assays there are no significative differences ($p > 0.05$) in the activity of the enantiomers for all the examined compounds.

Table 4. Effect (in s) in the HPT of compounds administered at doses of 2, 4, and 8 mg/kg

Compounds	*E* (s) (2 mg/kg)	*E* (s) (4 mg/kg)	*E* (s) (8 mg/kg)
(2*R*,3*S*/2*S*,3*R*)-**1**	31.2 ± 3.1	35.3 ± 6.8	41.6 ± 5.6
(2*R*,3*S*)-**1**	37.2 ± 6.4	44.5 ± 5.5	34.8 ± 4.2
(2*S*,3*R*)-**1**	34.6 ± 7.2	39.4 ± 5.6	47.4 ± 4.7
(2*R*,3*S*/2*S*,3*R*)-**2**	25.4 ± 4.2	27.0 ± 5.3	37.4 ± 6.8
(2*R*,3*S*)-**2**	23.5 ± 4.6	23.6 ± 5.7	34.3 ± 5.3
(2*S*,3*R*)-**2**	25.9 ± 3.2	27.3 ± 5.1	35.8 ± 5.9
(2*R*,3*S*/2*S*,3*R*)-**3**	28.7 ± 3.3	34.3 ± 4.5	50.2 ± 3.3
(2*R*,3*S*)-**3**	45.8 ± 3.9	49.6 ± 3.3	51.2 ± 4.2
(2*S*,3*R*)-**3**	30.8 ± 4.9	48.5 ± 4.9	52.5 ± 4.3
(2*R*,3*S*/2*S*,3*R*)-**4**	26.2 ± 4.3	28.5 ± 4.3	47.1 ± 4.6
(2*R*,3*S*)-**4**	n.d.	28.1 ± 6.0	35.0 ± 6.9
(2*S*,3*R*)-**4**	n.d.	26.3 ± 4.8	45.4 ± 5.3
(2*R*,3*S*/2*S*,3*R*)-**5**	29.4 ± 4.5	35.0 ± 3.5	40.5 ± 4.9
(2*R*,3*S*)-**5**	38.1 ± 4.7	45.9 ± 4.6	38.5 ± 5.2
(2*S*,3*R*)-**5**	38.2 ± 8.3	45.7 ± 6.1	35.9 ± 5.3
(2*R*,3*S*/2*S*,3*R*)-**6**	43.6 ± 7.1	54.4 ± 3.7	49.6 ± 4.7
(2*R*,3*S*)-**6**	29.4 ± 5.8	35.2 ± 5.0	40.8 ± 4.0
(2*S*,3*R*)-**6**	39.1 ± 5.8	39.7 ± 5.6	39.2 ± 5.3
(2*R*,3*S*/2*S*,3*R*)-**7**	36.3 ± 4.8	41.8 ± 4.6	48.2 ± 4.6
(2*R*,3*S*)-**7**	29.5 ± 4.3	38.8 ± 4.0	47.5 ± 4.1
(2*S*,3*R*)-**7**	39.3 ± 4.7	44.1 ± 5.0	31.8 ± 4.0
(2*R*,3*S*/2*S*,3*R*)-**8**	49.6 ± 4.0	43.2 ± 4.4	37.1 ± 4.5
(2*R*,3*S*)-**8**	36.5 ± 3.6	42.7 ± 4.7	45.5 ± 5.9
(2*S*,3*R*)-**8**	46.5 ± 4.2	48.6 ± 4.6	45.8 ± 5.1
(*R*/*S*)-**17**	45.1 ± 5.7	45.3 ± 5.4	42.7 ± 4.9
(*R*/*S*)-**18**	34.1 ± 6.1	43.6 ± 6.2	38.1 ± 5.8
(*R*/*S*)-**19**	30.6 ± 4.5	36.8 ± 4.1	36.3 ± 5.6

n.d.: not determined.

Conversely, the nociceptive behavior in the late phase of FT is clearly influenced by the stereochemical features. The eutomer of compounds bearing the phenyl group (**5**–**8**) is the (2*R*,3*S*) enantiomer, which significatively reduced the nociceptive response ($p < 0.01$).

In order to confirm the relevance of the stereogenic center C2 in the analgesic process, compounds (*R*/*S*)-**17**–**19** were prepared and tested by HPT and FT. Experimental data did not show noticeable differences of activity in the acute pain if compared to the corresponding racemic analogs (2*R*,3*S*/2*S*,3*R*)-**1**, (2*R*,3*S*/2*S*,3*R*)-**5**, and (2*R*,3*S*/2*S*,3*R*)-**7**.

Nevertheless, the antinociceptive activity in the continuous pain seems to be strictly related to the asymmetry at C2, as suggested by the fact that compounds **17**–**19** did not reduce the nociceptive response in phase II of formalin test.

To better understand these findings, a molecular modeling study was performed to establish the role of the conformational distribution in the biological activity.

The molecular mechanics experiments carried out with the biologically relevant compounds revealed that the presence of the methyl moiety in C2 did not change significantly the conformational profiles of compounds **1** and **5** with respect to the simplified **17** and **18**. For instance, distances between the Ar ring centroid and the *N*-methyl group are not effected by the modification

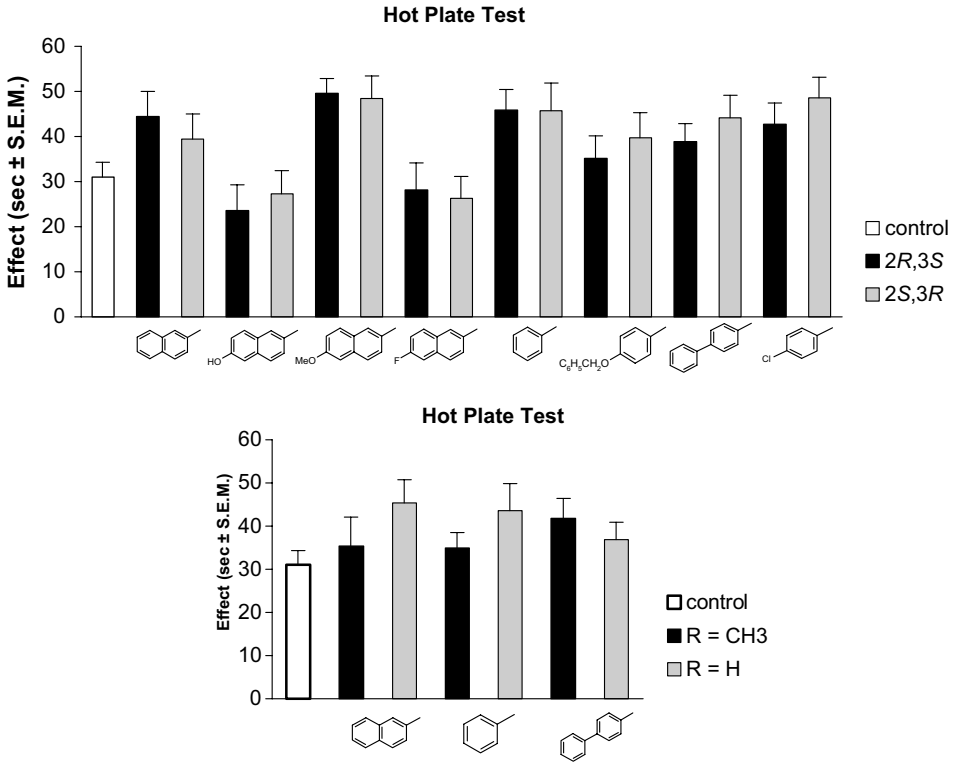


Figure 5. HPT (4 mg/kg dose).

on C2. Minor effects have been observed in the rotatable bonds C3–Ar and C3–OH, due to the Van der Waals repulsion induced by the methyl in C2 of compounds **1** and **5**. Unfortunately these small differences cannot explain the different biological response of these compounds with respect to the simplified **17** and **18**.

The different biological effect should be related to the stereochemistry of the C2/C3 chiral centers of the methylated analogues, even though conformational differences between the two series are not significantly appreciable.

Among the investigated compounds, worth noting is (2*S*,3*R*)-**6** whose activity resides only in phase II of cronic analgesia and which is suggested to be the best compound for a more detailed pharmacological study to elucidate the mechanism of action. Consequently, a rationalization of the biological results will be possible using molecular modeling and QSAR techniques.

4. Experimental

4.1. General methods

All reagents and solvents were purchased from commercial suppliers and employed without further purification. Analytical TLC was performed using precoated glass-backed plates (Fluka Kieselgel 60 F_{254}) and visualized by ultra-violet radiation, acidic ammonium molybdate(IV) or potassium permanganate. Melting points were measured on SMP3 Stuart Scientific apparatus. Elemental analyses were performed on a Carlo Erba 1106 C, H, N analyzer.

IR spectra were recorded on a Perkin–Elmer FT-IR 1605 spectrophotometer; only noteworthy absorptions are given.

^{1}H NMR spectra were performed at 9.4 T (TMS as internal standard $\delta = 0$) with an ADVANCE spectrometer at 400 MHz, mod. Bruker Germany and a BB1 5 mm probe; chemical shifts are given in ppm.

Gas chromatography (GC) analyses were performed on a DANI 3800 apparatus equipped with PVT and FID. HPLC analyses were performed on a Jasco system consisting of a PU-1580 pump, a Reodyne 7125 injector (20 μL sample loop) and a MD-1510 Diode Array Detector (wavelength 273 nm). Experimental data were acquired and interpreted with Borwin PDA and Borwin chromatograph software. Chiral HPLC analyses were performed on Chiralpak AD column (Daicel) (250 × 4.6 mm, 10 μm), Chiralpak AS column (Daicel) (250 × 4.6 mm, 10 μm), and Chiralcel OD-H column (250 × 4.6 mm, 5 μm); elution was carried out using *n*-hexane, isopropyl alcohol (IPA), ethyl alcohol (EtOH), and diethylamine (DEA). All the solvents were purchased by Carlo Erba and are HPLC grade. [α] measurements were recorded at room temperature on a Jasco DIP 1000 polarimeter.

4.2. General procedure for the preparation of 1-methyl-pyrrolidin-3-one [21]

4.2.1. Intermediate A. *N*-Methyl-β-alaninonitrile (30.3 g, 360 mmol) and anhydrous K_2CO_3 (49.6 g, 360 mmol) were refluxed in 100 mL of 2-butanone under stirring and slowly added with α-Br-acetic acid ethyl ester

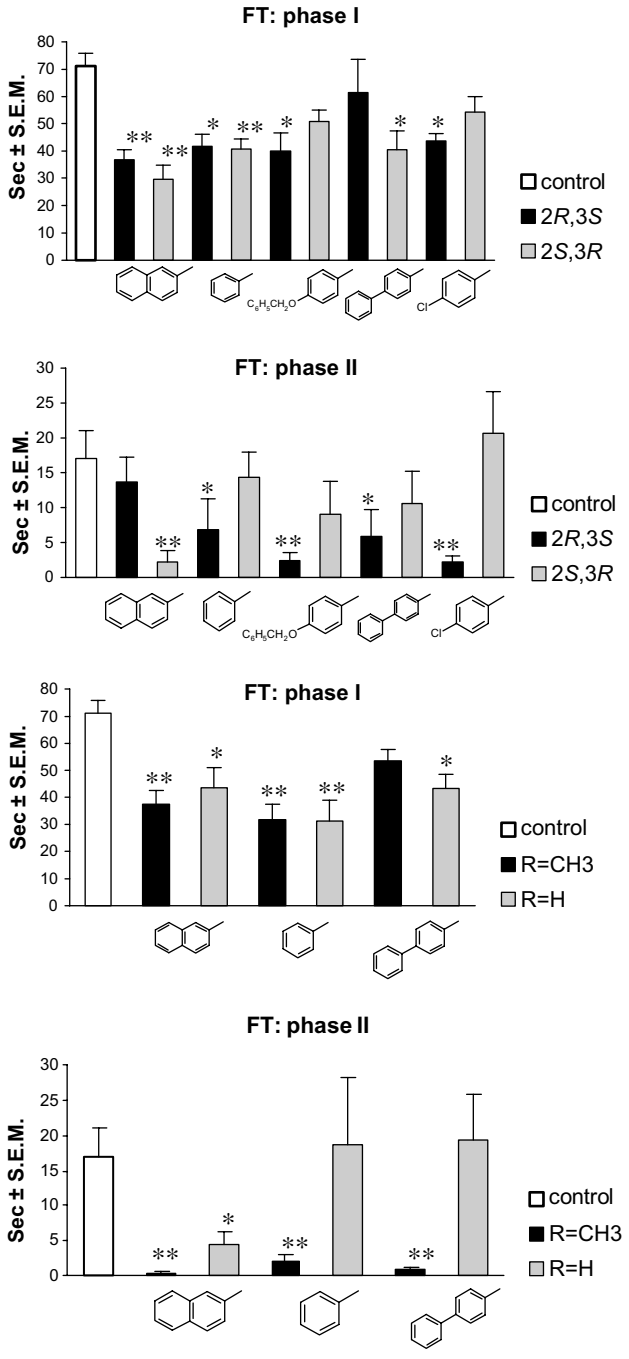


Figure 6. FT (8 mg/kg dose).

(60 g, 360 mmol). The reaction mixture was refluxed for 2 h. After cooling and filtration, the evaporation of the solvent under vacuum afforded a yellowish oil. The crude product was added with water (100 mL) and $NaHCO_3$ until pH = 8, and the aqueous phase was extracted with diethyl ether (6 × 100 mL). The combined organic phases, dried with anhydrous Na_2SO_4 and evaporated under vacuum, provided the desired product as a yellow oil. Yield 54.1 g (88.9%). TLC (*n*-Hex 60/AcOEt 40), $R_f = 0.35$, purity = 99% (GC method: CBWX-15N capillary column; flow rate 45–48 cm/s. Oven temperature: 70 °C for 4 min then 180 °C for 8 min. Injection volume: 1 μL). IR (Nujol): cm^{-1} 3619, 2247, 1737, 1189, 1063, 1031, 972, 847. ^1H NMR (CD_3OD): δ 1.28 (t, 3H, CH_3CH_2, $J = 7.0$ Hz), 2.44 (s, 3H, CH_3–N), 2.63 (t, 2H, CH_2–CH_2N, $J = 6.8$ Hz), 2.90 (t, 2H, CH_2–CH_2N, $J = 6.8$ Hz), 3.34 (s, 2H, $COCH_2$–N), 4.18 (q, 2H, CH_3CH_2, $J = 7.0$ Hz).

4.2.2. Intermediate B. A solution of [(2-cyano-ethyl)-methyl-amino]-acetic acid ethyl ester (54.1 g, 320 mmol) in absolute ethanol (270 mL) under stirring was slowly added with H_2SO_4 96% (76 mL). The reaction mixture was refluxed for 48 h. After cooling, the mixture was diluted with water (500 mL), added with $NaHCO_3$ until pH = 9 and extracted with diethyl ether (5 × 200 mL). The combined organic phases were dried with anhydrous Na_2SO_4 and evaporated under vacuum, affording the crude product as an orange oil, purified by fractional distillation under reduced pressure (bp_{9mmHg}=144–145 °C). Yield 38.3 g (54.0%). TLC (*n*-Hex 60/AcOEt 40), $R_f = 0.51$, purity = 99% (GC). IR (Nujol): cm^{-1} 1736, 1459, 1374, 1298, 1183, 1051, 854, 794. ^1H NMR (CD_3OD): δ 1.24 (m, 6H, CH_3–CH_2O), 2.37 (s, 3H, CH_3–N), 2.47 (t, 2H, $COCH_2CH_2$, $J = 3.3$ Hz), 2.85 (t, 2H, CH_2CH_2–N, $J = 7.3$ Hz), 3.27 (s, 2H, $COCH_2$–N), 3.70 (q, 2H, CH_3CH_2O, $J = 7.0$ Hz), 4.13 (m, 2H, CH_3CH_2O).

4.2.3. 1-Methyl-pyrrolidin-3-one [21]. NaH (60% in mineral oil 5.5 g, 138 mmol) in anhydrous toluene (120 mL) was slowly added under stirring in nitrogen atmosphere at −5 °C with a solution of intermediate B (15 g, 690 mmol) in anhydrous toluene (120 mL). The reaction mixture was kept at the same temperature for 3 h and then added with water (30 mL) and HCl 6 N (50 mL). The aqueous and the organic layers were separated and washed with toluene (1 × 80 mL) and HCl 6 N (2 × 40 mL), respectively. The combined aqueous phases were stirred at room temperature for 24 h and then refluxed for 4 h. The reaction mixture was added with Na_2CO_3 until pH > 9 and extracted with dichloromethane (5 × 40 mL). The combined organic phases were dried with anhydrous Na_2SO_4 and evaporated under vacuum, affording a brown oil. The crude product was purified by distillation in nitrogen atmosphere under reduced pressure, affording a colorless oil (bp_{28mmHg} = 45 °C). Yield 3.15 g (46%). TLC (*n*-Hex 60/AcOEt 40), $R_f = 0.48$. IR (Nujol): cm^{-1} 1751, 1441, 1250, 1183, 1116, 850. ^1H NMR (CD_3OD): δ 2.40 (t, 2H, CH_2CH_2–N, $J = 3.3$ Hz), 2.44 (s, 3H, CH_3–N), 2.86 (t, 2H, CH_2CH_2–N, $J = 7.3$ Hz), 2.91 (s, 2H, $COCH_2$–N).

4.3. General procedure for the preparation of (2*R*,3*S*) or (2*S*,3*R*)-1–8·HCl and (*R*/*S*)-17–19·DL-tartrates

The synthesis of enantiomers of **1** and **3**–**8** was essentially accomplished according to the procedure already described for the preparation of the racemic compounds. A 1.7 M solution of *t*-BuLi in pentane (49.9 mL, 84.8 mmol) in anhydrous diethyl ether was added under stirring in nitrogen atmosphere at −30 °C to a solution of the appropriate aromatic precursor (42.4 mmol). After stirring at −40 °C for 1 h, the temperature was allowed to warm to 0 °C and then a solution of (*R*)-**20** {$[\alpha]_D^{25} = +49.9$ (*c* 0.5, MeOH)}, (*S*)-**20**

{$[\alpha]_D^{25} = -54.0$ (c 0.5, MeOH)} or **21**, respectively, (35.3 mmol) in anhydrous diethyl ether (20 mL) was added dropwise. After stirring for 3 h at 0 °C, the reaction mixture was quenched with water (120 mL) and, after an acid–base work up, the combined organic phases were evaporated under vacuum. The resulting crude product was added with a 10% solution of HCl (15 mL), with exception for (*R*/*S*)-**17**–**19**, which were treated with DL-tartaric acid. The corresponding hydrochlorides or DL-tartrates, obtained as white solids, were crystallized from an appropriate solvent.

4.3.1. (2*R*,3*S*)-1,2-Dimethyl-3-hydroxy-3-naphthalen-2-yl-pyrrolidine·HCl [(2*R*,3*S*)-1·HCl]. Yield 49.4%; white crystals (IPA 9/H_2O 1, v/v), mp 168–170 °C. For [α] and ee% values see Table 1. IR (Nujol): cm^{-1} 3300, 2680, 1602, 1505, 1112, 1075, 960, 900, 819, 750. ^1H NMR (CD_3OD): δ 1.15 (d, 3H, C*H*$_3$–CH, J = 6.5 Hz), 2.28 (ddd, 1H, HC*H*–CH_2N, J_{gem} = 14.0 Hz, J_{vic} = 3.5–8.7 Hz), 2.78 (ddd, 1H, *H*CH–CH_2N, J_{gem} = 14.0 Hz, J_{vic} = 8.3–10.5 Hz), 2.97 (s, 3H, C*H*$_3$–N), 3.45 (dt, 1H, HC*H*–N, J_{gem} = 11.0 Hz, J_{vic} = 3.4 Hz), 3.75 (q, 1H, CH_3–C*H*, J = 6.5 Hz), 3.91 (dt, 1H, *H*CH–N, J_{gem} = 11.0 Hz, J_{vic} = 8.0 Hz), 7.45 (m, 2H, aromatic), 7.61 (dd, 1H, aromatic), 7.83–8.01 (s and m, 4H, aromatic). Elemental analysis: $C_{16}H_{20}NOCl$ requires C, 69.18; H, 7.26; N, 5.04. Found: C, 69.29; H, 7.56; N, 4.88.

4.3.2. (2*S*,3*R*)-1,2-Dimethyl-3-hydroxy-3-naphthalen-2-yl-pyrrolidine·HCl [(2*S*,3*R*)-1·HCl]. Yield 50.9%; white crystals (IPA 9/H_2O 1, v/v), mp 170–171 °C. For [α] and ee% values see Table 1. IR and ^1H NMR spectra are identical to that of the corresponding enantiomer. Elemental analysis: $C_{16}H_{20}NOCl$ requires C, 69.18; H, 7.26; N, 5.04. Found: C, 69.32; H, 7.31; N, 4.96.

4.3.3. (2*R*,3*S*)-1,2-Dimethyl-3-hydroxy-3-(6-methoxy-naphthalen-2-yl)-pyrrolidine·HCl [(2*R*,3*S*)-3·HCl]. Yield 20.7%; white crystals (IPA 9/H_2O 1, v/v), mp 215–217 °C. For [α] and ee% values see Table 1. IR (Nujol): cm^{-1} 3220, 2662, 1630, 1605, 1200, 1028, 962, 905, 850, 812. ^1H NMR (CD_3OD): δ 1.15 (d, 3H, C*H*$_3$–CH, J = 6.5 Hz), 2.28 (ddd, 1H, HC*H*–CH_2N, J_{gem} = 13.5 Hz, J_{vic} = 3.5–8.5 Hz), 2.74 (ddd, 1H, *H*CH–CH_2N, J_{gem} = 13.5 Hz, J_{vic} = 8.6–11.5 Hz), 2.95 (s, 3H, C*H*$_3$–N), 3.42 (dt, 1H, HC*H*–N, J_{gem} = 11.3 Hz, J_{vic} = 3.5 Hz), 3.68 (q, 1H, CH_3–C*H*, J = 6.5 Hz), 3.85 (s, 3H, OC*H*$_3$), 3.89 (dt, 1H, *H*CH–N, J_{gem} = 11.3 Hz, J_{vic} = 8.5 Hz), 7.10 (dd, 1H, aromatic), 7.19 (dt, 1H, aromatic), 7.53 (dd, 1H, aromatic), 7.73 (d, 1H, aromatic), 7.77 (d, 1H, aromatic), 7.91 (s, 1H, aromatic). Elemental analysis: $C_{17}H_{22}NO_2Cl$ requires C, 66.33; H, 7.20; N, 4.55. Found: C, 66.36; H, 7.41; N, 4.68.

4.3.4. (2*S*,3*R*)-1,2-Dimethyl-3-hydroxy-3-(6-methoxy-naphthalen-2-yl)-pyrrolidine·HCl [(2*S*,3*R*)-3·HCl]. Yield 21.3%; white crystals (IPA 9/H_2O 1, v/v), mp 214–217 °C. For [α] and ee% values see Table 1. IR and ^1H NMR spectra are identical to that of the corresponding enantiomer. Elemental analysis: $C_{17}H_{22}NO_2Cl$ requires C, 66.33; H, 7.20; N, 4.55. Found: C, 66.10; H, 7.32; N, 4.89.

4.3.5. (2*R*,3*S*)-1,2-Dimethyl-3-hydroxy-3-(6-fluoronaphthalen-2-yl)-pyrrolidine·HCl [(2*R*,3*S*)-4·HCl]. Yield 71.8%; white crystals (IPA 8/H_2O 2, v/v), mp 210–212 °C. For [α] and ee% values see Table 1. IR (Nujol): cm^{-1} 3250, 2675, 1610, 1510, 1407, 1190, 1103, 969, 949, 897, 802. ^1H NMR (CD_3OD): δ 1.15 (d, 3H, C*H*$_3$–CH, J = 6.5 Hz), 2.28 (ddd, 1H, HC*H*–CH_2N, J_{gem} = 13.7 Hz, J_{vic} = 3.4–8.6 Hz), 2.75 (ddd, 1H, *H*CH–CH_2N, J_{gem} = 13.7 Hz, J_{vic} = 8.6–11.6 Hz), 3.42 (t, 1H, HC*H*–N, J_{gem} = 11.3 Hz, J_{vic} = 4 Hz), 3.70 (q, 1H, CH_3–C*H*, J = 6.5 Hz), 3.90 (dt, 1H, *H*CH–N, J_{gem} = 11.3 Hz, J_{vic} = 8.4–8.4 Hz), 7.28 (dt, 1H, aromatic), 7.49 (d, 1H, aromatic), 7.62 (d, 1H, aromatic), 7.85 (d, 1H, aromatic), 7.90 (dd, 1H, aromatic), 8.03 (s, 1H, aromatic). Elemental analysis: $C_{16}H_{19}NOFCl$ requires C, 64.97; H, 6.47; N, 4.74. Found: C, 64.95; H, 6.59; N, 4.99.

4.3.6. (2*S*,3*R*)-1,2-Dimethyl-3-hydroxy-3-(6-fluoronaphthalen-2-yl)-pyrrolidine·HCl [(2*S*,3*R*)-4·HCl]. Yield 70.5%; white crystals (IPA 8/H_2O 2, v/v), mp 210–211 °C. For [α] and ee% values see Table 1. IR and ^1H NMR spectra are identical to that of the corresponding enantiomer. Elemental analysis: $C_{16}H_{19}NOFCl$ requires C, 64.97; H, 6.47; N, 4.74. Found: C, 65.08; H, 6.34; N, 4.80.

4.3.7. (2*R*,3*S*)-1,2-Dimethyl-3-hydroxy-3-phenyl-pyrrolidine·HCl [(2*R*,3*S*)-5·HCl]. Yield 23%; white crystals (CH_3COCH_3 15/H_2O 1, v/v), mp 233–235 °C. For [α] and ee% values see Table 1. IR (Nujol): cm^{-1} 3223, 2923, 1895, 1599, 1317, 1230, 973, 754. ^1H NMR (CD_3OD): δ 1.16 (d, 3H, C*H*$_3$–CH, J = 6.6 Hz), 2.27 (ddd, 1H, HC*H*–CH_2N, J_{gem} = 12.7 Hz, J_{vic} = 3.5–8.5 Hz), 2.69 (ddd, 1H, *H*CH–CH_2N, J_{gem} = 12.7 Hz, J_{vic} = 2.2–8.5 Hz), 2.99 (s, 3H, N–CH_3), 3.46 (dt, 1H, HC*H*–N, J_{gem} = 12.0 Hz, J_{vic} = 3.5 Hz), 3.68 (q, 1H, C*H*–CH_3, J = 6.6 Hz), 3.92 (dt, 1H, *H*CH–N, J_{gem} = 12.0 Hz, J_{vic} = 2.83–8.12 Hz), 7.30 (t, 1H, aromatic), 7.40 (m, 2H, aromatic), 7.57 (m, 2H, aromatic). Elemental analysis: $C_{12}H_{18}NOCl$ requires C, 63.29; H, 7.97; N, 6.15. Found: C, 63.38; H, 7.85; N, 6.20.

4.3.8. (2*S*,3*R*)-1,2-Dimethyl-3-hydroxy-3-phenyl-pyrrolidine·HCl [(2*S*,3*R*)-5·HCl]. Yield 22%; white crystals (CH_3COCH_3 15/H_2O 1, v/v), mp 234–235 °C. For [α] and ee% values see Table 1. IR and ^1H NMR spectra are identical to that of the corresponding enantiomer. Elemental analysis: $C_{12}H_{18}NOCl$ requires C, 63.29; H, 7.97; N, 6.15. Found: C, 63.11; H, 8.16; N, 5.98.

4.3.9. (2*R*,3*S*)-1,2-Dimethyl-3-hydroxy-3-(4-benzyloxy-phenyl)-pyrrolidine·HCl [(2*R*,3*S*)-6·HCl]. Yield 35%; white crystals (CH_3COCH_3), mp 215–219 °C. For [α] and ee% values see Table 1. IR (Nujol): cm^{-1} 3353, 1511, 1459, 1179, 1074, 1001, 944, 724. ^1H NMR (CD_3OD): δ 1.19 (d, 3H, C*H*$_3$–CH, J = 6.4 Hz), 2.25 (ddd, 1H, HC*H*–CH_2N, J_{gem} = 11.3 Hz, J_{vic} = 3.2–8.1 Hz), 2.66 (ddd, 1H, *H*CH–CH_2N, J_{gem} = 11.3 Hz, J_{vic} = 2.8–9.2 Hz), 2.97 (s, 3H, CH_3–N), 3.40 (dt, 1H, HC*H*–N, J_{gem} = 10.6 Hz, J_{vic} = 3.9 Hz), 3.55 (q, 1H, C*H*–CH_3, J = 6.4 Hz), 3.89 (dt, 1H, *H*CH–N,

J_{gem} = 10.6 Hz, J_{vic} = 3.5 Hz), 5.12 (s, 2H, CH_2–O), 7.05 (m, 2H, aromatic), 7.33 (m, 1H, aromatic), 7.38 (m, 2H, aromatic), 7.45 (m, 4H, aromatic). Elemental analysis: $C_{19}H_{24}NO_2Cl$ requires C, 68.36; H, 7.25; N, 4.20. Found: C, 68.01; H, 7.40; N, 3.98.

4.3.10. (2*S*,3*R*)-1,2-Dimethyl-3-hydroxy-3-(4-benzyloxyphenyl)-pyrrolidine·HCl [(2*S*,3*R*)-6·HCl]. Yield 39%; white crystals (CH_3COCH_3), mp 215–218 °C. For [α] and ee% values see Table 1. IR and ^{1}H NMR spectra are identical to that of the corresponding enantiomer. Elemental analysis: $C_{19}H_{24}NO_2Cl$ requires C, 68.36; H, 7.25; N, 4.20. Found: C, 68.74; H, 7.33; N, 4.04.

4.3.11. (2*R*,3*S*)-1,2-Dimethyl-3-hydroxy-3-(4-biphenyl)-pyrrolidine·HCl [(2*R*,3*S*)-7·HCl]. Yield 35%; white crystals [$(CH_3)_2CHOH$ 10/H_2O 1, v/v], mp 260–261 °C. For [α] and ee% values see Table 1. IR (Nujol): cm^{-1} 3197, 2652, 1230, 1166, 1068, 949, 839, 729. ^{1}H NMR (CD_3OD): δ 1.91 (d, 3H, C*H*$_3$–CH, J = 6.4 Hz), 2.29 (ddd, 1H, HC*H*–CH_2N, J_{gem} = 11.3 Hz, J_{vic} = 3.2–8.1 Hz), 2.73 (ddd, 1H, *H*CH–CH_2N, J_{gem} = 11.3 Hz, J_{vic} = 2.9–9.2 Hz), 2.98 (s, 3H, CH_3–N), 3.45 (dt, 1H, HC*H*–N, J_{gem} = 10.6 Hz, J_{vic} = 3.9 Hz), 3.69 (q, 1H, C*H*–CH_3, J = 6.4 Hz), 3.92 (dt, 1H, *H*CH–N, J_{gem} = 10.6 Hz, J_{vic} = 3.5 Hz), 7.32 (m, 1H, aromatic), 7.42 (m, 2H, aromatic), 7.60 (m, 2H, aromatic), 7.65 (m, 4H, aromatic). Elemental analysis: $C_{18}H_{22}NOCl$ requires C, 71.16; H, 7.30; N, 4.61. Found: C, 71.22; H, 7.02; N, 4.87.

4.3.12. (2*S*,3*R*)-1,2-Dimethyl-3-hydroxy-3-(4-biphenyl)-pyrrolidine·HCl [(2*S*,3*R*)-7·HCl]. Yield 34%; white crystals [$(CH_3)_2CHOH$ 10/H_2O 1, v/v], mp 261–262 °C. For [α] and ee% values see Table 1. IR and ^{1}H NMR spectra are identical to that of the corresponding enantiomer. Elemental analysis: $C_{18}H_{22}NOCl$ requires C, 71.16; H, 7.30; N, 4.61. Found: C, 70.82; H, 7.45; N, 4.22.

4.3.13. (2*R*,3*S*)-1,2-Dimethyl-3-hydroxy-3-(4-chlorophenyl)-pyrrolidine·DL-tartrate [(2*R*,3*S*)-8·DL-tartrate]. Yield 56%; white solid, hygroscopic. For [α] and ee% values see Table 1. IR (Nujol): cm^{-1} 3317, 2679, 2360, 1162, 1082, 976, 946, 721. ^{1}H NMR (CD_3OD): δ 1.19 (d, 3H, C*H*$_3$–CH, J = 6.4 Hz), 2.29 (ddd, 1H, HC*H*–CH_2N, J_{gem} = 12.7 Hz, J_{vic} = 3.4–8.8 Hz), 2.70 (ddd, 1H, *H*CH–CH_2N, J_{gem} = 12.7 Hz, J_{vic} = 2.4–8.8 Hz), 2.99 (s, 3H, CH_3–N), 3.45 (dt, 1H, HC*H*–N, J_{gem} = 10.8 Hz, J_{vic} = 2.5 Hz), 3.63 (q, 1H, C*H*–CH_3, J = 6.4 Hz), 3.93 (dt, 1H, *H*CH–N, J_{gem} = 10.8 Hz, J_{vic} = 2.9 Hz), 7.44 (m, 2H, aromatic), 7.57 (m, 2H, aromatic). Elemental analysis: $C_{12}H_{17}NOCl_2$ requires C, 54.97; H, 6.54; N, 5.34. Found: C, 54.90; H, 6.33; N, 5.12.

4.3.14. (2*S*,3*R*)-1,2-Dimethyl-3-hydroxy-3-(4-chlorophenyl)-pyrrolidine·DL-tartrate [(2*S*,3*R*)-8·DL-tartrate]. Yield 58.5%; white solid, hygroscopic. For [α] and ee% values see Table 1. IR and ^{1}H NMR spectra are identical to that of the corresponding enantiomer. Elemental analysis: $C_{12}H_{17}NOCl_2$ requires C, 54.97; H, 6.54; N, 5.34. Found: C, 55.07; H, 6.26; N, 5.62.

4.3.15. (*R*/*S*)-1-Methyl-3-hydroxy-3-naphthalen-2-yl-pyrrolidine·DL-tartrate [(*R*/*S*)-17·DL-tartrate]. Yield 79%; white solid, hygroscopic. IR (Nujol): cm^{-1} 3300, 3053, 2789, 1475, 1271, 1152, 947, 747. ^{1}H NMR (CD_3OD): δ 2.40 (dd, 1H, HC*H*–CH_2N, J = 9.9 Hz), 2.70 (dd, 1H, *H*CH–CH_2N, J = 9.9 Hz), 3.05 (s, 3H, CH_3), 3.62 (dt, 1H, HC*H*–N, J = 11.3 Hz), 3.77 (dt, 1H, *H*CH–N, J = 8.1 Hz), 4.47 (s, 2H, C*H*$_2$–COH), 7.47 (m, 2H, aromatic), 7.63 (d, 1H, aromatic), 7.83 (t, 1H, aromatic), 7.87 (m, 2H, aromatic), 8.02 (s, 1H, aromatic). Elemental analysis: $C_{19}H_{23}NO_7$ requires C, 60.47; H, 6.14; N, 3.71. Found: C, 60.30; H, 6.53; N, 4.08.

4.3.16. (*R*/*S*)-1-Methyl-3-hydroxy-3-phenyl-pyrrolidine·DL-tartrate [(*R*/*S*)-18·DL-tartrate]. Yield 33%; white solid, hygroscopic. IR (Nujol): cm^{-1} 3200, 2945, 2787, 1493, 1343, 1154, 962, 762. ^{1}H NMR (CD_3OD): δ 2.33 (dd, 1H, HC*H*–CH_2N, J = 9.9 Hz), 2.58 (ddd, 1H, *H*CH–CH_2N, J = 9.9 Hz), 3.01 (s, 3H, CH_3), 3.55 (dt, 1H, HC*H*–N, J = 11.3 Hz), 3.70 (dt, 1H, *H*CH–N, J = 11.3 Hz), 4.44 (s, 2H, C*H*$_2$–CHOH), 7.29 (t, 1H, aromatic), 7.37 (m, 2H, aromatic), 7.54 (m, 2H, aromatic). Elemental analysis: $C_{15}H_{21}NO_7$ requires C, 55.04; H, 6.47; N, 4.28. Found: C, 55.27; H, 6.81; N, 4.01.

4.3.17. (*R*/*S*)-1-Methyl-3-hydroxy-3-(4-biphenyl)-pyrrolidine·DL-tartrate [(*R*/*S*)-19·DL-tartrate]. Yield 86%; white solid, hygroscopic. IR (Nujol): cm^{-1} 3300, 1290, 1237, 1153, 1035, 961, 842, 730. ^{1}H NMR (CD_3OD): δ 2.25 (dd, 1H, HC*H*–CH_2N, J = 9.9 Hz), 2.52 (dd, 1H, *H*CH–CH_2N, J = 9.9 Hz), 2.93 (s, 3H, CH_3), 3.47 (dt, 1H, HC*H*–N, J = 11.3 Hz), 3.62 (dt, 1H, *H*CH–N J = 11.3 Hz), 4.35 (s, 2H, C*H*$_2$–COH), 7.21 (m, 1H, aromatic), 7.31 (m, 2H, aromatic), 7.49 (m, 2H, aromatic), 7.52 (m, 4H, aromatic). Elemental analysis: $C_{21}H_{25}NO_7$ requires C, 62.52; H, 6.25; N, 3.47. Found: C, 62.56; H, 6.60; N, 3.76.

4.4. Molecular mechanics calculations

The molecular mechanics experiments were performed using the MacroModel package 7.2[8] implemented under Linux operating systems. All calculations were carried out on a Intel Xeon biprocessor 3.0 GHz with 1 GB of RAM. The Maestro Graphical User Interface was used for assembling the starting geometries of **1**, **5**, **17**, and **18** stereoisomers. The conformational search was carried out by a 1000 iteration MC exploration of torsional internal degrees of freedom set adding manually the other dihedral angles not selected by the automatic set-up procedure. This operation detected only the hydroxyl in C3. The flexibility of the five-member ring was considered adding a closure bond defined by the atoms C3–C4–C5–N1 and two dihedral angles, respectively, rotating the N1–C2 and C2–C3 bonds. The Ar–C3 bond was also added to the list of the rotatable bonds, but for the symmetric phenyl moiety of compounds **5** and **18**, atom equivalencies were considered in order to avoid conformational duplications. Compounds **1** and **5** required the chirality check to be activated for chiral carbon atoms C2 and C3. Only this last atom was considered for the simplified compounds. Specular geometries were

obtained inverting the sign of the Z atomic coordinates using a module of the MOLINE program.[9] Another module of this tool was considered for computing the Boltzmann population of each conformer at room temperature. The force field considered was the MMFFs[10] and the water environment was simulated by the GB/SA implicit model solvation.[11] During the MC simulations the deduplication protocol between two generic conformations was set to the default values, that is, RMS deviation lower than 0.25 Å and energy difference lower than 4.184 kJ/mol. The average number of duplicates was considered to estimate the convergence in the MC search. Graphical superimpositions were performed by the analysis module of the Maestro GUI.

4.5. Biological assays

The experiments were carried out on male adult Swiss mice (30 ± 5 g). Control and experimental groups consisted of 8–10 animals each. The salts of the investigated compounds were dissolved in saline solution and administered subcutaneously within 1 h of dissolution.

4.5.1. Hot plate test. The hot plate test was employed to assess the antinociceptive effects. Morphine·HCl was used as a standard antinociceptive agent. The response to a thermal stimulus was evaluated using a copper plate heated to 55 °C and proceeding according to a previously reported method.[12] The animal responded by sitting on its hind legs and licking. The experiment was conducted on mice treated with 2, 4, and 8 mg/kg of the compounds. The reaction time to the pain stimulus was measured 20 min after the injection. The cut-off was 24.7 ± 2.1 s. Experimental data are reported in Table 4.

4.5.2. Formalin test (FT). Nociception was induced by injecting 20 μL dilute formalin (1% in saline solution) under the skin of the dorsal surface of the hind paw of the mouse.[13] The licking of the treated hind paw was used as a measure of nociception. Each mouse was injected with formalin 10 min after being pretreated with analgesic compounds and then placed into a transparent plastic cage and observed. The licking response was monitored until 30 min, starting immediately after the injection of formalin. Experiments were performed on two separate groups of animals: the control group, receiving formalin only, and the treated one receiving formalin and each compound administered at 8 mg/kg. Antinociception was defined as a statistically significant reduction in the time spent licking, in comparison with the vehicle control group during the early (0–5 min) and late phase (20–25 min). Results obtained are expressed as licking time in seconds. Experimental data are reported in Figure 6.

4.5.3. Rota-rod test. The integrity of motor coordination was controlled with RRT on mice after treatment with the investigated substances.[14] Randomly selected mice, were examined 15 min after treatment with doses corresponding to 4 and 8 mg/kg. Results are expressed as the percentage of mice remaining on the rod during a 30 s period.

4.5.4. Statistical analysis. The results of HPT, FT, and RRT were expressed as means ± SEM and the means were compared using Student's t-test, * (p values <0.05) or ** ($p < 0.01$) being considered as statistically significant or highly significant, respectively.

All the statistical analyses were performed using the statistical software package SYSTAT.

References and notes

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