Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 1925–1930

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Antinociceptive Activity of Pyrrolidinylnaphthalenes

Simona Collina,[a,*] Ornella Azzolina,[a] Dina Vercesi,[a] Massimo Sbacchi,[b] Mark Albert Scheideler,[b] Annalisa Barbieri,[c] Enrica Lanza[c] and Victor Ghislandi[a]

[a]*Dipartimento di Chimica Farmaceutica, Università di Pavia, viale Taramelli, 12, I-27100, Pavia, Italy*
[b]*Department of Biology, SmithKline Beecham, via Zambeletti, I-20021 Baranzate di Bollate, Milan, Italy*
[c]*Dipartimento di Farmacologia sperimentale ed applicata, Università di Pavia, viale Taramelli, 14, I-27100, Pavia, Italy*

Received 27 January 2000; accepted 14 April 2000

Abstract—In this paper the synthesis of the racemates (2*R*,3*S*/2*S*,3*R*)-1,2-dimethyl-3-[2-(6-substituted naphthyl)]-3-hydroxypyrrolidine **1b–d** [(2*R*,3*S*/2*S*,3*R*)-**1b–d**] are reported. Compounds **1b–d** were prepared by reaction of the racemic 1,2-dimethyl-3-pyrrolidone **2** with the lithiation product obtained from 2-bromo-6-substituted naphthalene **3b–d**. Pharmacological properties of (2*R*,3*S*/2*S*,3*R*)-**1a–d** are also described. Analgesic activity was investigated by the hot plate test and binding affinities towards μ, δ and κ opioid receptors were evaluated. A preliminary evaluation of the in vivo side-effects was also accomplished using the rota-rod test. Interesting antinociceptive activity was shown by all compounds and in particular by **1d**, which is the most active compound, since it is six-fold more potent than morphine and has lower side effects on the locomotory activity.

Introduction

Our research during recent years has addressed the study of compounds with antinociceptive activity that interact with the opioid receptor system. The analgesia process of many opioid compounds is accompanied by multiple undesired side effects often due to the activation of multiple subtypes of receptors. Therefore, it is not always possible to establish clear relationships between the activation of specific opioid receptors and consequent numerous pharmacological effects. The development of ligands highly selective for each receptor type is an important goal since these ligands could be very useful for investigating the biological effects produced by the involvement of different receptors and could also be potential therapeutic agents.

Many peptide ligands with opioid activities have been investigated and the role of the topochemical features has already been emphasized.[1–3] Nonpeptide ligands have also been studied.[4] In this field we have examined the nonpeptide ligands dialkylaminoalkylnaphthalenes[5,6] and cycloaminoalkylnaphthalenes.[7] These compounds are structurally related to the heterosteroid 17-methyl-17-azaequilenine (Fig. 1) that was already investigated in opioid analgesia studies and was found to possess the most analgesic properties among phenolic heterosteroids.[8,9]

Now we report our study on the structure–activity relationships of 1,2-dimethyl-3-[2-(6-hydroxynaphthyl)]-3-hydroxypyrrolidine (2*R*,3*S*/2*S*,3*R*)-**1a**,[7] and its structural analogues 1,2-dimethyl-3-[2-(6-substituted naphthyl)]-3-hydroxypyrrolidines **1b–d** (Fig. 1).

In this paper the synthesis of compounds **1b–d** and the evaluation of the pharmacological properties of compounds **1a–d** by in vivo and in vitro methods are described. Antinociceptive activity was evaluated by the hot plate test (HPT). Binding affinities of the compounds to μ, δ and κ opioid receptors were investigated in vitro, by opioid receptor binding assays, and in vivo by means of HPT. Rota-rod test (RRT) was also performed to investigate the effects of the compounds on motor coordination.

Results and Discussion

The synthesis procedure of 1,2-dimethyl-3-[2-(6-substitutednaphthyl)]-3-hydroxypyrrolidines **1b–d** is reported in Scheme 1. The pyrrolidinols (2*R*,3*S*/2*S*,3*R*)-**1b–d**·HCl were prepared by reaction of the pyrrolidone (*R*,*S*)-**2** with the lithiation products obtained by treating the appropriate 2-bromo-6-substituted-naphthalenes **3b–d** with *tert*-BuLi and by successive treatment with 10% HCl. Using *tert*-BuLi instead of *n*-BuLi and less drastic cooling of the reaction mixuture, **1b–d**·HCl were obtained in higher yields than **1a**·HCl.[7]

*Corresponding author. Tel.: +39-382-507376; fax: +39-382-422975; e-mail: collina@chifar.unipv.it

0968-0896/00/$ - see front matter
PII: S0968-0896(00)00117-6

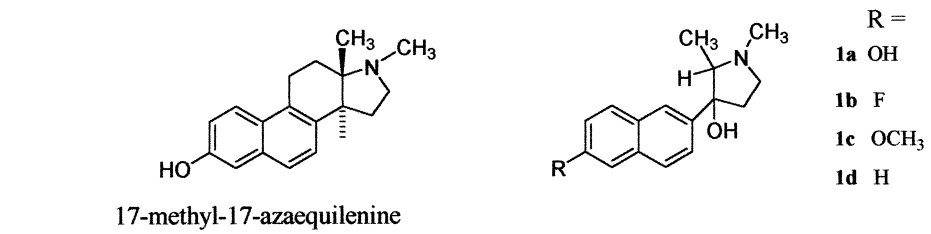
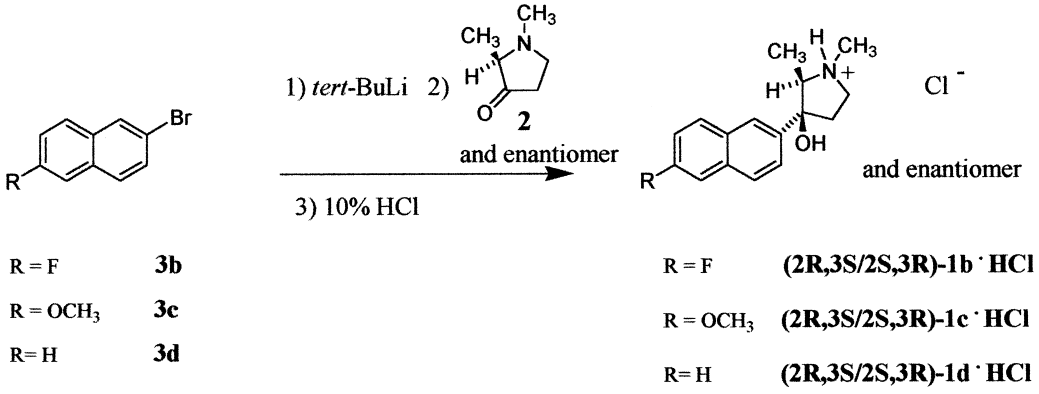


Figure 1. 17-Methyl-17-azaequilenine and pyrrolidinylnaphthalenes.



Scheme 1. Synthesis route of **1b–d**.

The configuration of the new chiral center C3 was predicted on the basis of our previous experience with the synthesis of the analogous 1,2-dimethyl-3-[2-(6-hydroxynaphthyl)]-3-hydroxypyrrolidine (2*R*,3*S*/2*S*,3*R*)-**1a**·HCl.[7] As already shown, the reaction occurs via a nucleophylous attack of the naphthalenic anion at the prochiral center of the pyrrolidone **2** on the less hindered side, consequentely 100% pure (2*R*,3*S*/2*S*,3*R*)-**1b–d**·HCl diastereomers were obtained as shown from ^{1}H NMR analysis of the crude products. The structures of the compounds were confirmed by means of elemental analysis (Table 1) and spectroscopic measurements (IR, ^{1}H NMR, ^{13}C NMR).

In our recent work[7] we found that the 1,2-dimethyl-3-[2-(6-hydroxynaphthyl)]-3-hydroxypyrrolidine **1a** displayed in vivo an analgesic activity comparable to that of morphine, exerting antinociceptive effects in HPT performed on mice. Therefore, the analogous compounds that we synthesized for this study were tested in the HPT and the results are shown in Table 2.

It is interesting to note that all compounds showed strong analgesic properties with potency relative to morphine ranging from 0.78 to 6.12. The analgesic activity is particularly noticeable for **1d** which does not bear any substituent on the naphthalene nucleus. Thus the activity in vivo seems to be related to the structural features of this moiety since substantial differences were recorded in the response to painful stimulus, according to the following sequence: OH < F < OCH_3 < H, as shown in Figure 2a.

The antinociceptive activity of the **1a–d** compounds was found to be completely reversed by the non specific opioid antagonist naloxone (NLX) administered at 10 mg/kg, confirming that they are opioid analgesics. In order to identify which opioid receptor type was mainly involved in the antinociceptive activity, a preliminary investigation on selective opioid antagonists was performed by the HPT (a model that produces low variability). As shown in Table 3 and in Figure 2b, the activities of all compounds were influenced to a various extent by naloxone 0.5 mg/kg (NLX, a μ specific antagonist when used at low dose), naltrindole 1 mg/kg (NTN, a δ specific antagonist) and nor-binaltorphimine 5 mg/kg (nor-BNI, a κ specific antagonist). In particular it is evident that the antinociceptive activity of **1a**, **1c** and **1d** is highly antagonized by NTN and consequently we can suppose that the activation of mainly δ receptors is involved in the analgesic properties of these compounds. However,

Table 1. Analytical data

Compound	Formula	Calcd.%			Found%		
		C	H	N	C	H	N
1b·HCl	$C_{16}H_{19}NOFCl$	64.97	6.47	4.74	65.02	6.54	4.48
1c·HCl	$C_{17}H_{22}NO_2Cl$	66.33	7.20	4.55	66.50	7.15	4.28
1d·HCl	$C_{16}H_{20}NOCl$	69.18	7.26	5.04	69.24	7.45	4.91
3b	$C_{10}H_6BrF$	53.37	2.69	—	53.06	2.58	—

Table 2. Analgesic activity in the hot plate test

Compound	AD_{50} mg/kg	Conf. limits	AD_{50} μmol/kg	Relative potency (vs morphine)
Morphine HCl·$3H_2O$	5.38	3.89–7.43	14.3	1.00
(2*R*, 3*S*/2*S*, 3*R*)-**1a**·HCl	5.41	1.65–3.41	18.4	0.78
(2*R*, 3*S*/2*S*, 3*R*)-**1b**·HCl	4.12	2.56–6.62	13.9	1.03
(2*R*, 3*S*/2*S*, 3*R*)-**1c**·HCl	2.11	1.11–4.03	6.8	2.09
(2*R*, 3*S*/2*S*, 3*R*)-**1d**·HCl	0.65	0.21–1.99	2.3	6.12

Table 3. Efficacy of NLX, NTN, nor-BNI as selective antagonists of μ, δ and κ opioid receptors

Compound	% of inhibition[a]		
	NLX (0.5 mg/kg)	NTN	nor-BNI
(2*R*, 3*S*/2*S*, 3*R*)-**1a**.HCl	46.0 (±7.1)	100**	91.3 (±4.8)**
(2*R*, 3*S*/2*S*, 3*R*)-**1b**.HCl	73.8 (±5.8)	51.8 (±4.6)	54.5 (±5.7)
(2*R*, 3*S*/2*S*, 3*R*)-**1c**.HCl	86.1 (±3.3)*	100**	16.2 (±5.11)
(2*R*, 3*S*/2*S*, 3*R*)-**1d**.HCl	22.1 (±5.3)	100**	28.9 (±5.4)

[a]In brackets the standard error is reported. *$P < 0.05$; **$P < 0.01$.

there is evidence from the results of the in vitro binding assays that all compounds exhibit very poor affinity for opioid receptors (K_i values of the μmolar order).

This knowledge suggests that compounds **1a–d** produce antinociceptive effects by an indirect activation of the opioid system. This hypothesis could justify the results of HPT in the presence of receptor subtype selective antagonists (NLX, NTN and nor-BNI) if an inhibition of specific enzymes, responsible for degradation of endogenous opioid peptides (endorphins, dynorphins and enkephalins) is invoked.

A wide pharmacological screening in vitro is in progress on the most potent compound **1d**, to investigate the mode of action of our compounds and to establish whether and which enzymatic or receptor system is involved in the biological activities. Antinociceptive properties of our compounds may result either by an inhibition at enzymatic level of endogenous opioid peptides degradation

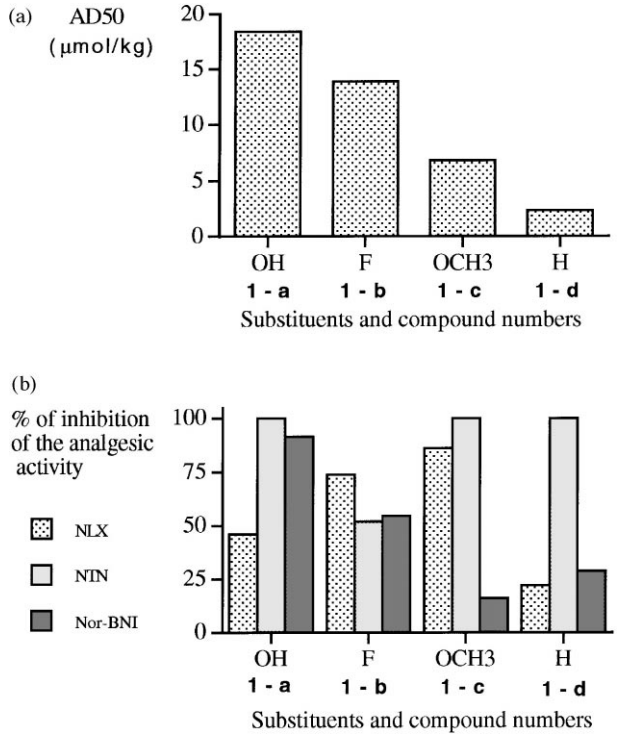


Figure 2. (a) Influence of the substituent on the antinociceptive activity of **1a–d**; (b) Inhibition of the antinociceptive activity of **1a–d** from selective opioid antagonists.

either by an interaction with a receptor having a stimulatory activity on an opioid neuron, such as Cholecystokinin and neuropeptide FF analogues.[10,11]

Furthermore, in order to assess the possible non-specific muscle-relaxant or sedative effects of the compounds **1a–d** (which could generate false positives in the HPT) the integrity of motor coordination of the mice was evaluated with the rota-rod test (RRT) to distinguish analgesia from drug-induced motor changes. The monitoring was effected after 30 s and was repeated after 120 s. After 30 s all compounds were found to influence the locomotory activity to a lesser extent than morphine. In fact, the percentage of mice that remained on the bar ranges from 75% (compound **1c**) to 100% (compounds **1a–b–d**), whereas only 65% of the mice treated with morphine remained on the bar. When motor coordination was tested for a longer period of time (120 s) the sedative effects of compound **1d** were found again to be inferior to those of morphine (75% instead of 50% of the mice remained on the bar). Experimental data are reported in Table 4.

In conclusion, the pyrrolidinylnaphthalenes investigated in this work possess very interesting pharmacological properties. It has been shown that both the analgesic activity (HPT) as well as the side effects on locomotory activity (RRT) are related to the features of the naphthalene moiety. Nevertheless, on the basis of actual knowledge, any correlation can not be done between the structural features of the compounds and their intrinsic activity. Differences in biological effects could depend also on different metabolic stability or pharmacokinetic properties.

Anyway, owing to its pharmacological profile (2.3 μmol/kg the AD_{50} in the HPT and low side effects evident from the RRT), **1d** is the most interesting compound, thus it will be considered for further investigation in order to elucidate the mode of action of these novel compounds. Furthermore, since the stereoselectivity can play an important role on the drug–receptor interaction, our purpose in the near future will be the study of the single stereoisomers.

Experimental

Chemistry

General methods. Melting points were determined with a Büchi apparatus and are uncorrected. Elemental analyses

Table 4. Locomotor activity in the rota-rod test: percentage of mice which remain on the bar after 30 or 120 s

Compound	30 s	120 s
Morphine·HCl·3H_2O	65[a]	50[a]
(2*R*, 3*S*/2*S*, 3*R*)-**1a**·HCl	100	25[b]
(2*R*, 3*S*/2*S*, 3*R*)-**1b**·HCl	100	12.5[b]
(2*R*, 3*S*/2*S*, 3*R*)-**1c**·HCl	75	37.5[b]
(2*R*, 3*S*/2*S*, 3*R*)-**1d**·HCl	100	75

[a]$P < 0.05$.
[b]$P < 0.01$.

were carried out with a Perkin–Elmer 240 C,H,N analyzer and were within ±0.4% of the theoretical values. IR spectra were obtained on a Perkin–Elmer 682 spectrophotometer and ^{1}H NMR and ^{13}C NMR spectra [TMS as internal standard (δ = 0.00)] were obtained using a Bruker AMX 400 (^{1}H 400 MHz, ^{13}C 100.617 MHz) apparatus. Differential scanning calorimetry was carried out with a Mettler TA 4000 apparatus equipped with DSC 25 cell. Detection of compounds in TLC was done with UV light or iodine vapor. ICN silica gel 60 (70–230 mesh) was used for flash chromatography. Anhydrous sodium sulfate was always used to dry organic solutions. Evaporation was performed in vacuum with a rotatory evaporator. All reagents and solvents were purchased from commercial suppliers and employed without further purification.

2-Bromo-6-fluoronaphthalene 3b. The synthesis of **3b** was essentially accomplished according to Newman et al.[12] The reaction of 2-bromo-6-naphthol with ammonium sulfite and 30% ammonium hydroxide under pressure (27 h at 150 °C) produced the crude brown-yellow 2-bromo-6-naphthylamine (mp 118–120 °C). This compound was purified by flash chromatography (mobile phase CH_2Cl_2 70/hexane 30) and the recovered colorless solid (mp 120–122 °C) was converted to the corresponding diazonium hexafluorophosphate (mp 107–110 °C) by treatment at 0 °C with 3.5% HCl, 50% $NaNO_2$ and 60% hexafluorophosphoric acid. The thermal decomposition of the diazonium salt produced the crude **3b** which was purified by sublimation and crystallization (90% aqueous MeOH); mp 63–65 °C; TLC analysis [stationary phase: Merck RP-18, F_{254}; mobile phase: 85% (v/v) aqueous MeOH]: R_f 0.31. IR (nujol) main absorptions (cm^{-1}): 3070, 1625, 1598, 1572, 1505, 1260, 1250, 1200, 1140, 1118, 1062, 960, 905, 890, 878, 868, 815. ^{1}H NMR (in CD_3OD) δ: 7.30 (dt, 1H, C*H* 7, *J* = 2.5–9.0–9.0; 7.47 (dd, 1H, C*H* 5, *J* = 2.5–9.5); 7.53 (ddd, 1H, C*H* 3, *J* = 9.0–2.0–1.0); 7.75 (d, 1H, C*H* 4, *J* = 9.0); 7.83 (dd, 1H, C*H* 8, *J* = 5.5–9.0); 8.02 (d, 1H, C*H* 1, *J* = 2.0). Anal. $C_{10}H_6BrF$ (C,H).

General procedure for the synthesis of (2*R*,3*S*/2*S*,3*R*)-1,2-dimethyl-3-[2-(6-substituted-naphthyl)]-3-hydroxypyrrolidines hydrochlorides [(2*R*,3*S*/2*S*,3*R*)-1b–d·HCl]. The synthesis of **1b–d** was performed essentially according to the procedure that we have already reported for the preparation of **1a**, suitably modified, as described.[7] A 1.7 M solution of *tert*-BuLi (21 mL, 35.7 mmol) in pentane was added under nitrogen at −40 °C to a solution of **3b**, **3c** and **3d** respectively (17.8 mmol) in anhydrous ether (60 mL). After stirring at −40 °C for 1 h the temperature was allowed to warm to −5 °C and then freshly prepared (R,S)-1,2-dimethyl-3-pyrrolidone [(*R*,*S*)-**2**][13] [1.7 g, 15 mmol, bp_{18} 47–48 °C, purity = 99.9% (GC)] in anhydrous ether (20 mL) was added dropwise while maintaining the temperature at −5 °C. The mixture was stirred for a further 3 h. A 10% solution of HCl (approximately 25 mL) was added to bring the pH to 2; after 15 min a fine white solid precipitated. The organic phase was then separated from the aqueous phase containing the white solid in the form of a very fine suspension. The crude product was recovered by filtration and recrystallised. Differential scanning calorimetry (DSC) evidenced only an endothermic process corresponding to the melting point of the substances; no thermal phenomena attributable to the evaporation of the crystallization solvent were present.

(2*R*,3*S*/2*S*,3*R*)-1b·HCl. 44.1% yield; mp 202–204 °C (isopropyl alcohol); TLC analysis [stationary phase:-Merck silica gel 60 F_{254}; mobile phase: hexane 87/isopropyl alcohol 13/methyl alcohol/$(C_2H_5)_2NH$ 2]:R_f 0.34. IR (nujol) main absorptions (cm^{-1}): 3250, 2675, 1610, 1510, 1407, 1190, 1103, 969, 949, 897, 862, 802; ^{1}H NMR (in CD_3OD) δ: 1.15 (d, 3H, C*H*$_3$CH, *J* = 6.5); 2.28 (ddd, 1H, HC*H*CH$_2$N, J_{gem} = 13.7, J_{vic} = 3.4–8.6); 2.75 (ddd, 1H, *H*CHCH$_2$N, J_{gem} = 13.7, J_{vic} = 8.6–11.6); 2.95 (s, 3H, C*H*$_3$N); 3.42 (t, 1H, HC*H*N, J_{gem} = 11.26, J_{vic} = 4); 3.70 (q, 1H, CH$_3$C*H*, *J* = 6.4); 3.90 (dt, 1H, *H*CHN, J_{gem} = 11.26, J_{vic} = 8.4–8.4); 7.28 (dt, 1H, aromatic, C*H* 7, *J* = 2.5–9.0–9.0); 7.49 (d, 1H, aromatic, C*H* 5, *J* = 9.8); 7.62 (d, 1H, aromatic, C*H* 3, *J* = 8.7); 7.85 (d, 1H, aromatic, C*H* 4, *J* = 8.7); 7.90 (dd, 1H, aromatic, C*H* 8, *J* = 5.6); 8.03 (s, 1H, aromatic, C*H* 1); ^{13}C NMR (in CD_3OD) δ: 164.32 (*C*4, aromatic); 132.31, 132.19, 129.32, 126.10, 125.46, 118.18, 117.84, 111.96 and 111.68 (9C, aromatic, *C*8, *C*10, *C*9, *C*1, *C*2, *C*6, *C*7, *C*3 and *C*5); 82.27 (*C*3); 73.66 (*C*2); 55.05 (*C*5); 39.93 and 39.70 (N*C*H$_3$ and *C*4); 8.70 (C2*C*H$_3$). Anal. $C_{16}H_{18}FNO$·HCl (C,H,N).

(2*R*,3*S*/2*S*,3*R*)-1c·HCl. 70.7% yield; mp 204–206 °C (isopropyl alcohol 90/H_2O 10); TLC analysis [stationary phase: Merck silica gel 60 F_{254}; mobile phase: hexane 87/isopropyl alcohol 13/methyl alcohol 3/$(C_2H_5)_2NH$ 2]:R_f 0.28. IR (nujol) main absorptions (cm^{-1}): 3220, 2662, 1630, 1605, 1200, 1028, 962, 905, 850, 812; ^{1}H NMR (in CD_3OD) δ: 1.15 (d, 3H, C*H*$_3$CH, *J* = 6.5); 2.28 (ddd, 1H, HC*H* CH$_2$N, J_{gem} = 13.5, J_{vic} = 3.5–8.5); 2.74 (ddd, 1H, *H*CH CH$_2$N, J_{gem} = 13.5, J_{vic} = 8.5–11.5); 2.95 (s, 3H, C*H*$_3$N); 3.42 (dt, 1H, HC*H*N, J_{gem} = 11.3, J_{vic} = 3.5); 3.68 (q, 1H, CH$_3$C*H*, *J* = 6.5); 3.85 (s, 3H, OC*H*$_3$); 3.895 (dt, 1 H, *H*CHN, J_{gem} = 11.3, J_{vic} = 8.5, 8.5); 7.10 (dd, 1H, aromatic, C*H* 5, *J* = 3.0–9.0); 7.19 (dt, 1H, aromatic, C*H* 7, *J* = 2.5); 7.53 (dd, 1H, aromatic, C*H* 3, *J* = 2.0–9.0); 7.73 (d, 1H, aromatic, C*H* 4, *J* = 9.0); 7.77 (d, 1H, aromatic, C*H* 8, *J* = 8.7); 7.91 (s, 1 H, aromatic, CH 1). ^{13}C NMR (in CD_3OD) δ: 151.49 (*C*4, aromatic); 128.46, 127.42, 122.42, 121.83, 120.22, 117.22, 116.22, 112.06 and 98.32 (9 C, aromatic, *C*8, *C*10, *C*1, *C*9, *C*2, *C*6, *C*7, *C*3 and *C*5); 73.75 (*C*3); 65.10 (*C*2); 47.46 (*C*5); 46.36 (O*C*H$_3$); 41.27–39.99 (C2*C*H$_3$); 31.06 and 31.29 (N*C*H$_3$ and *C*4). Anal. $C_{17}H_{21}NO_2$·HCl (C,H,N).

(2*R*,3*S*/2*S*,3*R*)-1d·HCl. 77.7% yield; mp 204–206 °C (isopropyl alcohol 95/H_2O 5); TLC analysis [stationary phase:Merck silica gel 60 F_{254}; mobile phase: hexane 87/isopropyl alcohol 13/methyl alcohol 3/$(C_2H_5)_2NH$ 2] : R_f 0.45. IR (nujol) main absorptions (cm^{-1}): 3300, 2680, 1602, 1505, 1112, 1075, 960, 900, 860, 819, 750; ^{1}H NMR (in CD_3OD) δ: 1.15 (d, 3H, C*H*$_3$CH, *J* = 6.50); 2.285 (ddd, 1H, HC*H*CH$_2$N, J_{gem} = 14.0, J_{vic} = 3.5–8.7); 2.78 (ddd, 1H, *H*CHCH$_2$N, J_{gem} = 14.0, J_{vic} = 8.3–10.5); 2.97 (s, 3H, C*H*$_3$N); 3.45 (dt, 1H, HC*H*N, J_{gem} = 11.0, J_{vic} = 3.4); 3.75 (q, 1H, CH$_3$C*H*, *J* = 6.3); 3.91 (dt, 1H, *H*CHN, J_{gem} = 11.0, J_{vic} = 8.0); 7.45 (m, 2H, aromatic); 7.61 (dd, 1H, aromatic, C*H* 4, *J* = 8.5); 7.83–8.01 (s + m,

4 H, aromatic); ^{13}C NMR (in CD_3OD) δ: 167.00 (*C*8, aromatic); 162.53, 162.24, 157.39, 157.10, 156.48, 155.41, 155.36, 153.62 and 151.91 (9C, aromatic, *C*9, *C*10, *C*4, *C*6, *C*1, *C*2, *C*5, *C*3 and *C*7); 110.01 (*C*3); 101.36 (*C*2); 82.71 (*C*5); 67.63 and 67.38 (NCH_3 and *C*4);36.34 (C2*C*H$_3$). Anal. $C_{16}H_{19}NO$·HCl (C,H,N).

Pharmacology

Pharmacological studies in vivo were performed on male adult Swiss mice weighting 30±5 g. To assess the antinociceptive effects the hot plate test (HPT) was utilized. Compounds were dissolved in saline solution and administered within 1 h from dissolution.

The HPT experiments of all compounds **1a**–**d** were run simultaneously.

Hot plate test (HPT)

The response to a thermal stimulus was evaluated using a copper plate heated to 55 °C. The reaction time was measured in seconds.

The response of the mouse included the sitting on its hind legs and licking.[14]

Once the basal animal reaction time was determined, groups of 10 mice were treated (via ip injection) with increasing doses of compound. Control animals received the same volume of saline solution. The reaction time to the pain stimulus was measured 20 min after ip injection. The reaction time of the control animals was 23±2 s.

AD_{50} values were determined using a computerized program.[15]

Experimental data are reported in Table 2.

Antagonist activity test

To assess an involvement of the opioid system (μ, κ and δ receptors) in the antinociceptive activity (determined by HPT), the following opioid antagonists were used: naloxone (NLX) at high doses (10 mg/kg) as a non selective opioid inhibitor, naloxone at low doses (0.5 mg/kg) as a μ-preferential antagonist,[16,17] nor-binaltorphimine (nor-BNI) 5 mg/kg as a κ preferential antagonist[18] and naltrindole (NTN) 1 mg/kg, as a δ selective antagonist.[19]

Antagonists were administered ip before agonists at the following times: NLX 30 min, nor-BNI 24 h, NTN 5 min. Experimental data are reported in Table 3 and in Figure 2b.

Rota-rod test

In order to assess the possible non-specific muscle-relaxant or sedative effects of the investigated compounds, the integrity of motor coordination of the mice was tested on the rota-rod apparatus by using a light modification of the method described by Vaught et al.[20] The apparatus consisted of a platform equipped with a rotating rod (3 cm diameter) with a non-slippery surface. The rod was placed at a 15 cm height from the base and was divided by six disks into five equal sections in order to simultaneously test up to five mice. The speed of rota-rod was fixed at 17 rpm. The animals were selected randomly and divided in groups of 10 mice for any experiment.

Before the beginning of the test the mice were trained to remain on the rod for the maximum time used (120 s) and those animals that did not remain on the bar during this time were rejected. Performance time was monitored before (control animals) and 15 min after treatment with the substances, tested at AD_{50} of HPT. The integrity of motor coordination was evaluated by counting the number of mice of each group that fell from the rod during the periods of 30 and 120 s.

In all sets of experiments a group of mice was treated with morphine as a standard antinociceptive agent in order to make a suitable comparison with the tested compounds.

Results are expressed as the percentage of mice that fell from the bar during the fixed period of time. Statistical analysis was performed by Student *t* test for grouped data. *P* Values less than 0.05 were considered significant. Experimental data are reported in Table 4.

Radioligand binding test

Opioid receptor binding experiments were carried out using membranes prepared by hypotonic lysis from CHO (μ and δ) or HEK-293 (κ) cells expressing cloned human opioid receptors.[21] [^{3}H]-U-69593, [^{3}H]-DADLE (D-Ala^2, D-Leu^5 enkephalin) and [^{3}H]-DAMGO (D-Ala^2, MePhe^4, Gly-ol^5 enkephalin) were used as the radioligands to label κ, δ and μ receptors respectively.

The assay was carried out in Tris buffer 50 mM pH 7.4 with final volume of 1 or 2 mL. The assay tubes were incubated at 25 °C for 60 min. The non specific receptor binding was evaluated in the presence of Naloxone, 10 μM. Bound ligand was separated from free ligand by filtration through Whatman GF/B filters using a Brandell Cell Harvester. The radioactivity on the filters was measured by liquid scintillation counting.

The data obtained from competition experiments were analyzed using non linear fitting analysis according to Benfenati and Guardabasso[22] and using the RS/1 software. K_i values were determined from IC_{50} using the Cheng and Prusoff equation and were > 1000 nM for all compounds.

Acknowledgements

This work was supported by a grant from MURST. The authors wish to thank Dr. Laura Linati for obtaining the NMR spectra.

References

1. Ronsisvalle, G.; Pappalardo, M. S.; Pasquinucci, L.; Vittorio, F.; Salvadori, S.; Spampinato, S.; Cavicchini, E.; Ferri, S. *Eur. J. Med. Chem.* **1990**, *25*, 29.
2. Yamazaki, T.; Ro, S.; Goodman, M.; Chung, N. N.; Schiller, P. W. *J. Med. Chem.* **1993**, *36*, 708.
3. Arttamangkul, S.; Murray, T. F.; De Lander, G. E.; Aldrich, J. V. *J. Med. Chem.* **1995**, *38*, 2410.
4. Ronsisvalle, G.; Pasquinucci, L.; Pappalardo, M. S.; Vittorio, F.; Fronza, G.; Romagnoli, C.; Pistacchio, E.; Spampinato, S.; Ferri, S. *J. Med. Chem.* **1993**, *36*, 1860.
5. Ghislandi, V.; Azzolina, O.; Collina, S.; Paroli, E.; Antonilli, L.; Giuseppetti, G.; Tadini, C. *Chirality* **1994**, *6*, 389.
6. Collina, S.; Azzolina, O.; Vercesi, D.; Benevelli, F.; Callegari, A.; Tadini, C.; Lanza, E.; Barbieri, A.; Ghislandi, V. *Biorg. Med. Chem.* **2000**, *8*, 769.
7. Ghislandi, V.; Collina, S.; Azzolina, O.; Barbieri, A.; Lanza, E.; Tadini, C. *Chirality* **1999**, *11*, 21.
8. Paroli, E.; Melchiorri, P. *Biochem. Pharmacology* **1961**, *6*, 18.
9. Paroli, E. *Atti Accademia Medica di Roma* **1989–90** *114*, 165.
10. Noble, F.; Smadja, C.; Roques, P. B. *J. Pharmacol. Exp. Ther.* **1994**, *271*, 1127–1134.
11. Gouardères, C.; Jhamandas, K.; Sutak, M.; Zajac, J. M. *British Journal of Pharmacology* **1996**, *117*, 493–501.
12. Newman, M. S.; Galt, R. H. B. *J. Org. Chem.* **1960**, *25*, 214.
13. Collina, S.; Gamba, A.; Ghislandi, V.; Azzolina, O. *Chirality* **1995**, *7*, 439.
14. Woolfe, G.; Mac Donald, A. D. *J. Pharmacol. Exp. Ther.* **1944**, *80*, 300.
15. Tallarida, R.J.; Murray, R.B. *Manual of Pharmacologic Calculation with Computer Programs*, Springer Verlag: Berlin, 1987.
16. Sawnok, J.; Pinsky, C.; La Bella, F. S. *Life Sci.* **1979**, *25*, 1621.
17. Von Voigtlander, P. F.; Lahti, R.; Ludens, J. H. *J. Pharmacol. Exp. Ther.* **1983**, *224*, 7.
18. Portoghese, P. S.; Lipkowski, A. W.; Takemori, A. E. *Life Sci.* **1987**, *40*, 1287.
19. Portoghese, P. S.; Sultana, M.; Nagase, H.; Takemori, A. E. *Eur. J. Pharmacol.* **1988**, *146*, 185.
20. Vaught, J.; Pelley, K.; Costa, L. G.; Sether, P.; Enna, S. J. *Neuropharmacology* **1985**, *24*, 211.
21. Scheideler, M. A.; Zukin, S. *J. Biol. Chem.* **1990**, *265*, 15176.
22. Benfenati, F.; Guardabasso, V. In *Principles and Methods in Receptor Binding*, Cattabeni, F.; Nicosia, S., Ed.; Plenum Press: New York, 1984; pp 41–63.