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Guanidinium and Aminoimidazolinium Derivatives of *N*-(4-Piperidyl)propanamides as Potential Ligands for μ Opioid and I_2-imidazoline Receptors: Synthesis and Pharmacological Screening

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Abstract—Derivatives of *N*-(1-phenethyl-4-piperidyl)propanamides incorporating guanidinium and 2-aminoimidazolinium groups have been prepared by a synthetic approach involving first introduction of a spacer between the piperidine and the functional group by reductive amination of piperidinone. The formation of each of these functional groups was carried out using *N*-*N*′-di(*tert*-butoxycarbonyl)thiourea and 2-methylthioimidazolinium iodide, respectively. These structures have been designed to incorporate two pharmacologic goals into one entity. Radioligand binding assays have been used to study their affinity for opioid (μ, δ and κ) and I_2-imidazoline receptors. Two of them, **10** and **16,** showed high affinity for μ opioid receptors and functionally they had moderate analgesic properties in the hot plate and writhing tests. The in vitro studies on guinea pig ileum (GPI) indicated that both compounds are μ opioid agonists. In what concerns I_2-imidazoline receptor activity, these derivatives showed low affinity around 6 to 7 times less than idazoxan.

Introduction

'Dual acting drugs', which are drugs containing two pharmacophoric groups combined in a single molecule is one of the strategies used in drug design.[1] These dual acting drugs can exert their action on two binding sites of a single receptor or on two different receptors.

Very recent examples include, among others, a new class of prodrug of an HIV protease inhibitor conjugated with a reverse transcriptase inhibitor,[2] dual inhibitors of cyclooxygenase-2 and 5-lipoxygenase as novel anti-arthritic agents,[3] a dual inhibitor of thymidilate synthase and dihydrofolate reductase as antitumor agent[4] and a novel compound for treating bronchial asthma which is a dual antagonist for leukotriene D4 and thromboxane A2 receptors.[5]

In this paper, we wish to report a new class of molecular entities combining the pharmacophoric features of μ opioid binding ligands with those of imidazoline receptor ligands (I_2-imidazoline preferring binding sites). Recent studies[6,7] have shown that imidazoline receptor agonists combined with some opioid agonists produce antinociceptive synergy and it has been recently demonstrated[8] that some I_2-imidazoline ligands can attenuate tolerance to opioid induced antinociception. The functional interaction between I_2-imidazoline and opioid receptors suggests a new effective therapeutic strategy to manage pain by administration of I_2-opioid ligands combinations. The rationale behind this combination in the same molecule would be to obtain compounds with enhanced opioid analgesic properties and with somewhat reduced side effects. On the basis of these considerations and within the framework of dual acting drugs, the structures here proposed combine in the same molecule a potent opioid moiety with I_2-imidazoline receptor features. The opioid moiety would provide analgesic properties and the imidazoline ligand moiety would enhance the activity and confer attenuation

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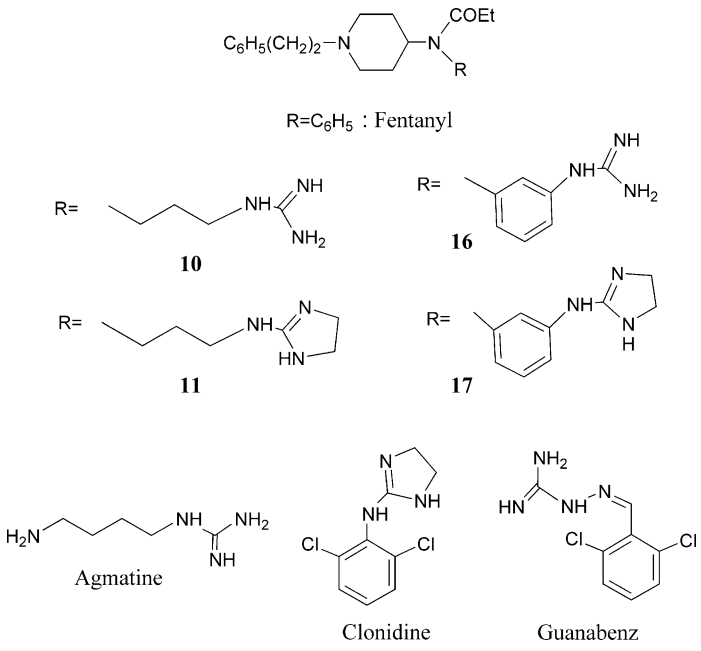


Figure 1. Chemical structure of fentanyl, compounds tested biologically included in this work and some known imidazoline ligands.

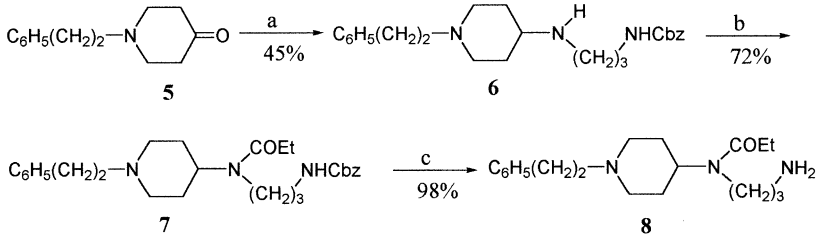
to the opioid induced tolerance, which is one of the major side effects of opioid therapy.

The general formulae of the compounds described is depicted in Figure 1 and corresponds to the combination of an *N*-[1-(2-phenethyl)-4-piperidyl]propanamide, present in fentanyl like analgesics[9] with a guanidinium or 2-aminoimidazolinium groups which are found in imidazoline ligands (see Fig. 1).[10] A synthetic methodology has been developed for these novel structures and their binding to both kinds of receptors studied. In the most promising compounds, in vitro and in vivo antinociceptive assays have also been carried out.

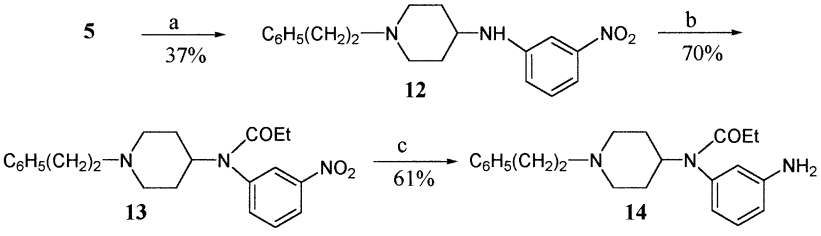
Results and Discussion

Chemistry

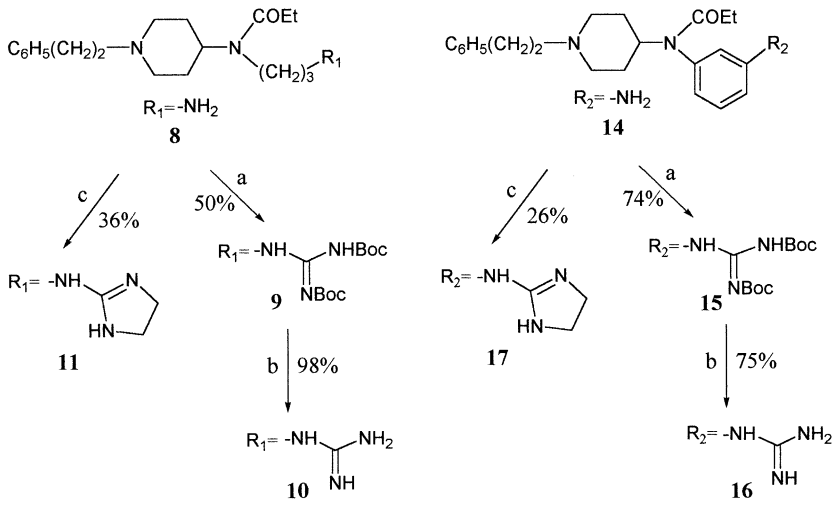
The syntheses of **10**, **11**, **16**, and **17** were achieved following the sequences described in Schemes 1 and 3 for the aliphatic series and in Schemes 2 and 3 for the aromatic series. The reaction sequences began with reductive amination of 1-phenethyl-4-piperidone (**5**). In the usual method of preparation of fentanyl derivatives,[11] this reductive amination proceeds in two steps with first, formation of the corresponding imine followed by $LiAlH_4$ reduction. The formation of aminopiperidines **6** and **12** from the piperidone **5** was carried out in an one



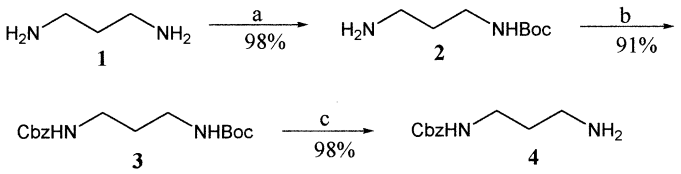
Scheme 1. (a) 4/$NaBH_3CN$/MeOH/3 Å sieves; (b) $(EtCO)_2O$; (c) H_2/Pd/C/AcOH/35–40 psi.



Scheme 2. (a) 3-Nitroaniline/$NaBH_3CN$/MeOH/3 Å sieves; (b) $(EtCO)_2O$; (c) H_2/Pd/C/AcOH/35–40 psi.



Scheme 3. (a) $(BocNH)_2CS/TEA/HgCl_2/CH_2Cl_2/0\,°C$; (b) TFA/CH_2Cl_2; (c) 2-methylthioimidazolinium iodide/MeOH.



Scheme 4. (a) $(Boc)_2O/CHCl_3/0\,°C$; (b) CbzCl/THF/NaOH(3.3 N); (c) TFA/CH_2Cl_2.

pot reaction with propanediamine **4** and 3-nitroaniline respectively, using $NaBH_3CN$ as reductive agent. The monoprotected propanediamine **4**, required for the reductive amination in the propyl series, was obtained (Scheme 4) through monoprotection of diaminopropane **1** with *tert*-butoxycarbonyl group, followed by substitution of the free amine with benzyloxycarbonyl group and finally the *tert*-butoxycarbonyl protecting group was removed. Acylation of aminopiperidines **6** and **12** with propionic anhydride led to the corresponding propanamides **7** and **13**. Catalytic hydrogenation of **7** allowed the deprotection of amine to provide **8** and catalytic hydrogenation of **13** resulted in reduction of the nitro group affording **14**. The guanidinylation of amines **8** and **14** with *N*-*N*′-di(*tert*-butoxycarbonyl)thiourea, a guanidinylation agent which was prepared as previously reported by Iwanowicz et al.,[12] in the presence of mercuric chloride and triethylamine provided **9** and **15** respectively (Scheme 3). Treatment of the protected guanidinium derivatives **9** and **15** with trifluoroacetic acid afforded the corresponding guanidinium derivatives **10** and **16** as trifluoroacetate salts. Formation of the aminoimidazoline from the amines **8** and **14** was attempted with *N*,*N*′-di(*tert*-butoxycarbonyl)imidazolin-2-thione but did not lead to the desired product. However, aminoimidazoline derivatives **11** and **17** were obtained according to the Aspinall et al.[13] procedure by treatment of the amines **8** and **14** with 2-(methylthioimidazolinium) iodide (Scheme 3). This reagent was prepared by reacting methyl iodide with *N*,*N*′-ethylenethiourea in refluxing methanol.

The NMR data of the propanamides **7**–**11**, corresponding to the aliphatic series and which are reported in the Experimental, show (i) in the ^1H NMR: duplicated signals for the methyl and methylenic protons of the amide group, for piperidine H-4 and for the methylenic protons of the propyl chain, (ii) in the ^{13}C NMR: two signals for the carbonyl and for piperidine C-4. These data indicate that the rotation around the N–CO bond is slow enough to observe *cis–trans* isomerism in NMR studies. For the propanamides **13**–**17**, corresponding to the aromatic series, *cis–trans* conformers were not observed by NMR studies at room temperature. However, in the ^1H NMR spectra, the methylenic protons of the amide group form an ABX_3 pattern indicating restricted rotation around $N–C_{phenyl}$ bond due probably of the presence of the meta-substituted group.

The biological activities of compounds **16** and **17** have been studied on their oxalate salt. For compound **11**, the iodide salt was used. Concerning product **10**, binding assays have been realized on its trifluoracetate salt and in vivo and in vitro studies have been carried on its chloride salt.

Pharmacology

Binding assays. Compounds **10, 11, 16** and **17** were evaluated for their binding affinities at the μ, δ and κ opioid receptors and at I_2-imidazoline receptors following reported procedures[14,15] with minor modifications. Fentanyl and idazoxan were also tested for comparative purposes. Mouse brain membranes (P_2 fraction) were used with [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U-69593 ligands for the μ, δ and κ receptors, respectively, and [^{3}H]2-BFI for I_2-imidazoline receptor. Data of radioligand binding assays are reported in Table 1.

Table 1. Affinity data [K_i (nM)] for μ, δ, κ and I_2-imidazoline receptor[a]

Receptors	[^{3}H]DAMGO (2 nM) μ	[^{3}H]DPDPE (4 nM) δ	[^{3}H]U-69593 (2 nM) κ	[^{3}H]2-BFI (1 nM) I_2
Idazoxan	n.d.	n.d.	n.d.	307±183
Fentanyl	6±1.5	663±184	298±40	5462±1343
10	37±12	1744±185	355±134	2022±949
11	1751±1135	24955±17612	5043±483	2327±811
16	7.8±2.5	181±42	841±282	1890±499
17	7119±4089	12078±9686	11551±4381	9630±6731

[a]Values are expressed as mean±standard error of the mean of three experiments.

Concerning the opioid receptors, compounds **10** and **16** show high affinity for the μ receptor, the latter having a K_i similar to that of fentanyl, a well-established potent μ opioid used as the reference compound.

The affinity values obtained for the I_2-imidazoline receptor are less relevant than for the μ opioid receptor, with the compounds showing low or no affinity. Nevertheless, the best μ opioid ligands **16** and **10** show some affinity for the I_2-imidazoline receptor although their potencies are 6 times lower than idazoxan.

Functional activity. The low affinity shown in the binding assays for the I_2-imidazoline receptor together with the fact that functional assays for this kind of receptor are not very specific, prompted us to performe in vitro and in vivo tests of the compounds only on the μ opioid receptors. Binding data show that two of the new compounds (**10** and **16**) selectively bind to μ opioid receptors, however these data give no information about intrinsic activity and the functional study is necessary in order to determine whether they are agonists or antagonists.

To obtain this information, the analgesic activity of these two compounds was tested using the writhing test and the hot plate test in mice. The writhing test was used because it is widely employed at the first stages of the evaluation of antinociceptive drugs[16] and it was used as a first approach to determine their antinociceptive potency. Then they were also studied using the hot plate test,[17] to corroborate the analgesia by this more sensitive test. Reversal of the antinociceptive effect with naloxone, a selective opioid antagonist, was used to verify that the analgesia might be attributed to interaction with opioid receptors.

When the antinociceptive activity of the new compounds **10** and **16** was determined carrying out the writhing test, they elicited a dose-dependent inhibition of the number of writhes induced by the intraperitoneal administration of acetic acid. In the writhing test, the percentage of inhibition of writhes induced by compound **10** were: 2 mg/kg−19.5±2.3% (n=12); 10 mg/kg−20.5±1.6% (n=12) and 25 mg/kg−47.4±2.2% (n=12). The increase of the dosage induced behavioral impairments and results were disregarded. Naloxone was able to antagonize the antinociceptive effect of the largest tested dose of compound **10**, after the ip administration of 1 mg/kg of naloxone, the percentage of inhibition of the writhes induced by 25 mg/kg of compound **10,** was only of 5±2.7% (n=10). Non toxic doses (≤ 25 mg/kg) were not able to induce analgesia (25 mg/kg−% MEP 12.3±3.8%, n=12) when the hot plate test was done.

Compound **16** also inhibited the number of writhes (2 mg/kg−20±4.4% (n=6), 5 mg/kg−55.3±1.3% (n=12), 10 mg/kg−84.1±0.9% (n=13) in mice. In hot plate test the% MPE induced by compound **16** was: 10 mg/kg−15.8±8% (n=10) and 20 mg/kg−50.7±10% (n=11), naloxone (1 mg/kg) antagonized the analgesia induced by **16** (% MPE after naloxone 13.2±11.8 (n=10). Larger doses (40 mg/kg) of this compound were tested but must be disregarded because of their toxicity.

Intraperitoneal administration of morphine induced a dose-dependent antinociceptive effect in both writhing test and hot plate test. In the writhing test, the percentage of inhibition of writhes induced by morphine were: 1.3 mg/kg−19.5±0.5% (n=10); 2.5 mg/kg−46.6±1.4% (n=12); 3.5 mg/kg−80.4±0.5% (n=11); 5 mg/kg−96±0.3% (n=10) and 10 mg/kg−100% (n=10). In hot plate test the percentage MEP induced by morphine were: 2.5 mg/kg−29.8±4.8% (n=12), 5 mg/kg−41±8.9% (n=12), 7.5 mg/kg−64.1±7.1% (n=12) and 10 mg/kg−86.3±7.5% (n=11).

To assess the participation of the opioid system on the effect of these compounds their functional activity on different subtypes of receptors myenteric plexus—longitudinal muscle strips of the guinea pig ileum[18–20]—was analyzed. In guinea pig ileum, **10** and **16**, as well as the reference compound, morphine, induced a dose-dependent inhibition of the electrically-induced contractions (Fig. 2). Table 2 shows the EC_{50} values. The in vitro effect of all the tested drugs was completely reversed by the in vitro administration of low concentration of naloxone (5×10^{-8} M) a μ opioid antagonist. The inhibition of the contractile response in this tissue may be mediated by activation of μ or κ receptors[18,19,21–23] but considering

Table 2. Inhibitory effect (EC_{50}) of morphine, **10** and **16** on the electrically-induced contractions in guinea pig ileum

Compound	EC_{50} (M)	Confidence intervals 95%
Morphine	2.1×10^{-7}	8.7×10^{-8} to 5.4×10^{-7}
10	6.61×10^{-6}	5×10^{-6} to 8.9×10^{-6}
16	1.9×10^{-6}	4.5×10^{-7} to 7.9×10^{-6}

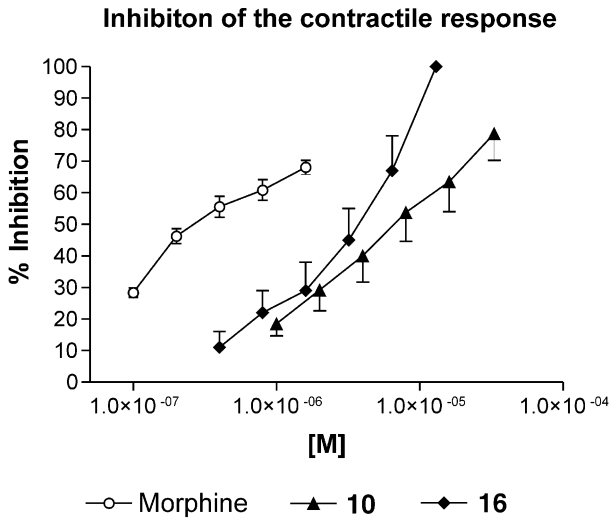


Figure 2. Lines show the mean percentage ±SEM of inhibition of the electrically induced contractile responses induced by cumulative addition of morphine (open circles) or of **10** (triangles) or **16** (squares).

that their effect was antagonized by low concentrations of naloxone, it could be suggested that the effect on this tissue could be mediated by the μ opioid receptor. It is interesting to note that there is a very good correlation between the antinociceptive potency of tested drugs (**10** and **16** and morphine) and their effectiveness on guinea pig ileum which is an interesting result because it is characteristic of opioid analgesic drugs.[24]

In summary, although compounds **10** and **16** were not well tolerated after their in vivo administration and the behavioral impairment restrained their analgesic utility, data obtained from the study of the antinociception and of the naloxone antagonism permits the suggestion that the activation of opioid receptors plays a role in their effect. Moreover, when isolated guinea pig ileum, a tissue containing mainly μ and κ receptors, was used, it was confirmed that **10** and **16** are μ opioid agonists.

Conclusions

In this paper, we have described compounds with dual action on two types of receptors, μ opioid and I_2-imidazoline receptors. A synthetic methodology has been developed for incorporating in the same molecule the two pharmacophoric groups required, *N*-phenylpiperidine and guanidinium and aminoimidazolinium groups. Concerning the affinity for the two kinds of receptors, radioligand binding assays indicate that two compounds (**10** and **16**) are potent μ opioid ligands, one of them (**16**) with potency similar to that of fentanyl, and they also show moderate analgesic properties in vivo. The results for the other receptor, the I_2-imidazoline one, are less significant and the compounds (**10** and **16**) show only low affinity. This kind of hybrid structures descrived here for the first time, can be regarded as a lead for structural modifications mainly aimed at improving their binding to the I_2-imidazoline receptors, in order to get analgesic compounds with reduced-tolerance side effects.

Experimental

Chemistry

The melting points were determined with a Reichert Jung Thermovar apparatus and are uncorrected. Electrospray mass spectra were recorded on a MSD-Serie 1100 Hewlett Packard apparatus. Elemental analysis were performed on a Heraeus CHNO Rapid Analyser. Flash column chromatographies were run on silica gel 60 (230–400 mesh ASTM, Merck) or on medium pressure 40i prepacked KP-Sil cartridge (Biotage). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 spectrometer and on Varian 300 and 400 unity spectrometers. Dry MeOH was obtained by distillation over $Mg(OH)_2$. Anhydrous CH_2Cl_2 was obtained by distillation over $CaCl_2$. THF and toluene were distilled over sodium-benzophenone. TEA and propylamine were purified by distillation over KOH.

***N*-(*tert*-Butoxycarbonyl)-1,3-propanediamine (2).** To 1,3-propanediamine (550 mL) in $CHCl_3$ (1 L) at 0 °C was added $(Boc)_2O$ (24.0 g, 110 mmol) dissolved in $CHCl_3$ (150 mL) dropwise during 0.5 h. The reaction mixture was allowed to warm to ambient temperature and stirred for 2 h. After filtrating the mixture, the filtrate was concentrated in vacuo and the oily residue was dissolved in EtOAc (500 mL). The solution was washed with brine and dried over $MgSO_4$. Evaporation of the solvent in vacuo gave **2** (18.8 g) as a white solid. Yield 98%, mp 54–56 °C. ^{1}H NMR ($CDCl_3$) δ 5.14 (br, 1H), 3.23 (br, 2H), 3.16 (m, 2H), 2.75 (t, 2H, J = 6.7 Hz), 1.61 (tt, 2H, J = 6.7 and 6.7 Hz), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 156.2, 79.0, 38.9, 38.1, 32.3, 28.3; MS (ES^+) m/z 175 ($[MH]^+$, 100).

***N*-(*tert*-Butoxycarbonyl)-*N*′-(benzyloxycarbonyl)-1,3-propanediamine (3).** To propanediamine **2** (18.7 g, 107 mmol) in THF (600 mL) at 0 °C was added benzyl chloroformate (23 mL, 161 mmol) in NaOH (3.2 N). The reaction mixture was allowed to warm to ambient temperature and stirred for 2 h. The solution was acidified with HCl (6 N), extracted with EtOAc. The organic layer was dried over $MgSO_4$. The solvent was removed in vacuo to give the crude product which was flash chromatographed on silica gel (CH_2Cl_2/EtOAc, 9:1) to provide **3** (30.2 g) as a white solid. Yield 91%, mp 45–46 °C. ^{1}H NMR ($CDCl_3$) δ 7.26–7.33 (m, 5H), 5.42 (br, 1H), 5.06 (s, 2H), 4.95 (br, 1H), 3.20 (m, 2H), 3.14 (m, 2H), 1.58 (tt, 2H, J = 6.7 and 6.7 Hz), 1.41 (s, 18H); ^{13}C NMR ($CDCl_3$) δ 156.7, 156.4, 136.5, 128.4, 128.0, 127.4, 126.8, 79.2, 66.5, 37.6, 37.0, 30.4, 28.3; MS (ES^+) m/z 331 ($[MNa]^+$, 100).

***N*-(Benzyloxycarbonyl)-1,3-propanediamine (4).** A solution of **3** (30.0 g, 97 mmol) in CH_2Cl_2 (100 mL) and TFA (100 mL) was stirred for 4 h at room temperature. The solvents were removed in vacuo. The residue was dissolved in CH_2Cl_2 and extracted with NaOH (2.5 N). The organic layer was dried over $MgSO_4$. The solvent was removed in vacuo to give **4** (20.0 g). Yield 98%, mp 83–84 °C. ^{1}H NMR ($CDCl_3$) δ 7.28–7.35 (m, 5H), 5.28 (br, 1H), 5.08 (s, 2H), 3.28 (m, 2H), 2.77 (t, 2H, J = 6.6 Hz), 1.63 (tt, 2H, J = 6.6 and 6.6 Hz); ^{13}C NMR

($CDCl_3$) δ 156.6, 136.6, 128.5, 128.1, 66.6, 39.6, 39.1, 32.6; MS (ES^+) *m*/*z* 209 ($[MH]^+$, 100).

1-(2-Phenethyl)-4-(*N*-benzyloxycarbonyl-*N'*-diaminopropyl)piperidine (6). To **4** (2.2 g, 10.5 mmol) in anhydrous MeOH (25 mL) was added a solution of concd HCl_g in anhydrous MeOH until pH 6. To this solution were successively added 1-phenethyl-4-piperidone (1.8 g, 8.75 mmol), $NaBH_3CN$ (820 mg, 13.1 mmol) and 3 Å molecular sieves. The solution was then acidified to pH 6 with a solution of concd HCl_g in MeOH and stirred for 24 h at ambient temperature. The reaction mixture was filtered over a Celite bed which was then washed with MeOH. Concentration of the combined filtrates in vacuo gave a brown precipitate which was purified by medium pressure chromatography on silica gel ($CH_2Cl_2/NH_{3(g)}$ in MeOH, 9:1) to provide **6** (1.2 g) as a yellowish solid. Yield 45%, mp 131–133 °C. ^1H NMR ($CDCl_3$) δ 7.13–7.17 (m, 3H), 7.22–7.25 (m, 2H), 7.25–7.29 (m, 5H), 5.87 (br, 1H), 5.04 (s, 2H), 4.17 (br, 1H), 3.27 (t, 2H, *J* = 6.5 Hz), 3.0 (d, 2H, *J* = 12.0 Hz), 2.83 (t, 2H, *J* = 6.6 Hz), 2.75 (m, 3H), 2.56 (m, 2H), 2.06 (t, 2H, *J* = 12.0 Hz), 2.02 (d, 2H, *J* = 12.0 Hz), 1.91 (tt, 2H, *J* = 6.7 and 6.7 Hz), 1.70 (q, 2H, *J* = 12.0 Hz); ^{13}C NMR ($CDCl_3$) δ 158.1, 139.8, 136.6, 128.7, 128.5, 128.4, 128.1, 128.0, 126.2, 67.7, 60.9, 56.1, 52.7, 43.8, 39.5, 34.4, 30.5, 28.8; MS (ES^+) *m*/*z* 396 ($[MH]^+$, 100).

***N*-[(*N'*-Benzyloxycarbonyl)aminopropyl]-*N*-[1-(2-phenethyl)-4-piperidyl]propanamide (7).** A solution of **6** (6.1 g, 15.4 mmol) in propionic anhydride (50 mL) was stirred and heated to reflux for 3 h. After cooling, the solution was poured on ice, basified with NH_4OH (30%) and extracted with EtOAc. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. To the residue dissolved in EtOAc was added Et_2O, the precipitate was filtered. The filtrate was concentrated to give the crude product which was purified by flash chromatography on silica gel (EtOAc/MeOH, 9:1) to provide **7** (5.0 g) as an orange oil. Yield 72%. ^1H NMR ($CDCl_3$) δ 7.32–7.25 (m, 10H), 7.23–7.17 (m, 4H), 7.12–7.10 (m, 6H), 5.0 (s, 4H), 4.32 (t, 1H, *J* = 12.0 Hz), 3.47 (t, 1H, *J* = 12.0 Hz), 3.25 (m, 4H), 3.06 (m, 4H), 2.97 (d, 4H, *J* = 12.0 Hz), 2.69 (m, 4H), 2.51 (m, 4H), 2.27 (q, 2H, *J* = 7.4 Hz), 2.20 (q, 2H, *J* = 7.4 Hz), 1.98 (t, 4H, *J* = 12.0 Hz), 1.77 (d, 4H, *J* = 12.0 Hz), 1.72–1.60 (m, 8H), 1.06 (t, 3H, *J* = 7.3 Hz), 1.03 (t, 3H, *J* = 7.3 Hz); ^{13}C NMR ($CDCl_3$) δ 173.8, 156.4, 139.8, 136.6, 128.4, 128.2, 127.8, 127.7, 127.6, 125.8, 66.1, 60.0, 55.4, 53.0, 38.5, 38.2, 33.5, 30.6, 29.5, 26.6, 9.5; MS (ES^+) *m*/*z* 452 ($[MH]^+$, 100), 474 ($[MNa]^+$); MS (ES^-) *m*/*z* 486 ($[MCl]^-$).

***N*-(Aminopropyl)-*N*-[1-(2-phenethyl)-4-piperidyl]propanamide (8).** A mixture of **7** (340 mg, 0.76 mmol), 10% Pd/C (15 mg) and glacial AcOH (1 mL) in MeOH (30 mL) was shaken under H_2 (35–40 psi) for 6 h. After filtration and evaporation of the solvent, the residue was dissolved in NaOH (2.5 N) and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated in vacuo to give **8** (235 mg) as a lighly yellowish oil. Yield 98%. ^1H NMR ($CDCl_3$) δ 7.23–7.18 (m, 4H), 7.12–7.10 (m, 6H), 5.78 (s, 4H, 2H), 4.45 (t, *J* = 12.0 Hz, 1H), 3.50 (t, *J* = 12.0 Hz, 1H), 3.26 (t, 2H, *J* = 6.7 Hz), 3.18 (t, 2H, *J* = 6.7 Hz), 3.0 (d, 4H, *J* = 12.0 Hz), 2.72 (m, 4H), 2.66 (m, 4H), 2.52 (m, 4H), 2.36 (q, 2H, *J* = 7.4 Hz), 2.34 (q, 2H, *J* = 7.3 Hz), 2.0 (t, 4H, *J* = 12.0 Hz), 1.82 (d, 4H, *J* = 12.0 Hz), 1.67 (q, 4H, *J* = 12.0 Hz), 1.62 (m, 4H), 1.61 (t, 2H, *J* = 7.3 Hz), 1.63 (t, 2H, *J* = 7.3 Hz); ^{13}C NMR ($CDCl_3$) δ 173.7, 173.5, 140.0, 128.5, 128.3, 126.0, 60.1, 55.4, 53.1, 39.6, 39.1, 33.6, 32.7, 30.7, 26.7, 9.5; MS (ES^+) *m*/*z* 318 ($[MH]^+$, 100); MS (ES^-) *m*/*z* 352 ($[MCl]^-$, 100).

***N*-[(2,3-di-*tert*-Butoxycarbonyl)guanidinopropyl]-*N*-[1-(2-phenethyl)-4-piperidyl]propanamide (9).** To a solution of **8** (2.0 g, 6.3 mmol), thiourea (1.7 g, 6.3 mmol) and 2 mL of TEA (18.9 mmol) in 20 mL of anhydrous CH_2Cl_2 under N_2 and at 0 C was added 2.2 g (8.2 mmol) of $HgCl_2$. The reaction mixture was stirred at 0°C for 1 h. The temperature was allowed to warm to ambient temperature and the mixture was stirred for additional 24 h. The mixture was diluted with EtOAc, filtered over Celite. The filtrate was washed with H_2O, washed with brine, and dried over $MgSO_4$. The solvent was evaporated in vacuo. The residue was purified by medium pressure chromatography on silica gel (EtOAc) to provide **9** (1.9 g) as a yellowish oil. Yield 51%. ^1NMR ($CDCl_3$) δ 12.01 (s, 2H), 11.50 (s, 2H), 7.26–7.21 (m, 4H), 7.17–7.14 (m, 6H), 4.42 (t, 1H, *J* = 12.0 Hz), 3.53 (t, 1H, *J* = 12.0 Hz), 3.40 (t, 2H, *J* = 7.0 Hz), 3.38 (t, 2H, *J* = 7.0 Hz), 3.24 (m, 4H), 3.08 (d, 4H, *J* = 12.0 Hz), 2.73 (m, 4H), 2.56 (m, 2H), 2.34 (q, 1H, |J| = 7.3 Hz), 2.31 (q, 1H, *J* = 7.3 Hz), 2.05 (t, 4H, *J* = 12.0 Hz), 1.73–1.84 (m, 8H), 1.67 (m, 4H), 1.46 (s, 36H), 1.13 (t, 6H, *J* = 7.3 Hz); ^{13}C NMR ($CDCl_3$) δ 173.7, 173.5, 163.3, 156.1, 155.9, 140.0, 128.5, 128.3, 126.0, 83.2, 82.7, 60.2, 55.3, 53.1, 39.2, 38.7, 33.7, 30.7, 29.6, 28.2, 27.9, 26.8, 9.5; MS (ES^-) *m*/*z* 352 ($[MCl]^-$, 100).

***N*-[1-(2-Phenethyl)-4-piperidyl]-*N*-(guanidinopropyl)propanamide (10).** A solution of **9** (1.6 g, 2.89 mmol) in a mixture 1:1 of CH_2Cl_2/TFA was stirred at room temperature for 4 h. After evaporation of the solvents in vacuo, the residue was dried in vacuo to give **10** (1.6 g) as a trifluoroacetic salt. Yield 98%, mp 158–160 °C. ^1H NMR (D_2O) δ 7.31–7.26 (m, 4H), 7.23–7.19 (m, 6H), 4.12 (t, 1H, *J* = 12.0 Hz), 4.0 (t, 1H, *J* = 12.0 Hz), 3.59 (m, 4H), 3.25 (m, 4H), 3.23 (m, 4H), 3.08 (m, 4H), 3.06 (m, 2H), 2.98 (m, 4H), 2.36 (q, 2H, |J| = 7.4 Hz), 2.27 (q, 2H, *J* = 7.4 Hz), 2.06 (q, 2H, *J* = 12.0 Hz), 1.96 (d, 4H, *J* = 1.02 Hz), 1.67 (tt, 2H, *J* = 7.4 and 7.4 Hz), 1.64 (tt, 2H, *J* = 7.4 and 7.4 Hz), 0.95 (t, 3H, *J* = 7.4 Hz), 0.94 (t, 3H, *J* = 7.4 Hz); ^{13}C NMR (D_2O) δ 177.8, 177.5, 163.0 (d, J_{CCF} = 35.2 Hz), 156.9, 136.3, 129.2, 128.9, 127.5, 116.4 (d, J_{CF} = 291.6 Hz), 58.0, 52.5, 52.1, 51.3, 43.0, 39.0, 30.0, 28.1, 27.4, 26.5, 9.1. The chloride salt of **10** was formed by treating an aqueous solution of the trifluoroacetic salt with Amberlite IRA 400 resine: MS (ES^+) *m*/*z* 180 $[(M+2H)/2]^{2+}$, 360 ($[MH]^+$, 100%). Anal. ($C_{20}H_{33}N_5O.2HCl{\cdot}1.5H_2O$) (found) C: 52.26(52.10); H: 8.35(8.15); N: 15.24(14.96).

***N*-[1-(2-Phenethyl)-4-piperidyl]-*N*-[*N'*-(4,5-dihydro-1*H*-imidazol-2-yl)aminopropyl]propanamide (11).** A solution of **8** (248 mg, 0.78 mmol) and 2-(methylthio)imidazoli-

nium iodide (400 mg, 1.64 mmol) in anhydrous MeOH (3 mL) was stirred and heated to reflux for 48 h. A stream of N_2 was passed through the solution for 15 min then the solution was allowed to cool to room temperature. A precipitate was formed on adding diethyl ether to the solution. The solid was filtered, washed with diethyl ether and recrystallized from water at 5 °C. The crystals were dried in vacuo at 30 °C to give **11** (140 mg) as a iodide salt. Yield 36%, mp 143–145 °C. ^{1}H NMR (CD_3OD) δ 7.30–7.25 (m, 4H), 7.21–7.18 (m, 6H), 4.10 (t, 1H, J = 12.0 Hz), 4.0 (t, 1H, J = 12.0 Hz), 3.40 (m, 4H), 3.5 (s, 8H), 3.22 (m, 2H), 3.20 (m, 2H), 3.06 (m, 2H), 3.0 (m, 4H), 2.96 (m, 4H), 2.27 (q, 2H, J = 7.4 Hz), 2.36 (q, 2H, J = 7.4 Hz), 2.06 (q, 4H, J = 12.0 Hz), 1.88 (d, 4H, J = 12.0 Hz), 1.70 (tt, 2H, J = 7.4, 7.4 Hz), 1.64 (tt, 2H, J = 7.4 and 7.4 Hz), 1.16 (t, 3H, J = 7.4 Hz), 1.12 (t, 3H, J = 7.4 Hz); ^{13}C NMR (CD_3OD) δ 177.6, 177.4, 158.9, 136.3, 130.2, 130.0, 128.8, 58.5, 52.8, 52.2, 51.4, 44.1, 43.5, 40.0, 30.1, 28.3, 27.6, 26.2, 9.7; MS (ES^+) m/z 386 ($[MH]^+$). Anal. ($C_{22}H_{35}N_5O.2HI$) (found) C: 41.20 (41.11); H: 5.81 (5.37); N: 10.92 (10.49).

1-(2-Phenethyl)-4-(3-nitroanil-1-yl)piperidine (12). To 3-nitroaniline (1.5 g, 7.4 mmol) in anhydrous MeOH at pH 6 were successively added 1-phenethyl-4-piperidone (1.2 g, 8.8 mmol), $NaBH_3CN$ (700 mg, 11.1 mmol) and 3 Å molecular sieves. During 78 h of stirring of the reaction mixture at room temperature the solution was kept acidifying to pH 6 with a solution of concd HCl_g in MeOH. Then the reaction mixture was filtered over a Celite bed. Concentration of the filtrate in vacuo gave a crude which was purified by flash chromatography on silica gel (EtOAc/hexane, 9:1) to provide **12** (3.6 g) as a yellow solid. Yield 37%, mp 115–119 °C. ^{1}H NMR ($CDCl_3$) δ ^{1}H NMR (acetone-d_6) δ 7.02 (ddd, 1H, J = 8.0, 2.0 and 1.0 Hz), 7.13–7.18 (m, 1H), 7.20–7.29 (m, 3H), 7.30–7.33 (m, 1H), 7.34–7.38 (m, 1H), 7.41 (t, 1H, J = 2.0 Hz), 3.40 (t, 1H, J = 12.0 Hz), 2.96 (d, 2H, J = 12.0 Hz), 2.77 (m, 2H), 2.56 (m, 2H), 2.22 (t, 2H, J = 12.0 Hz), 2.01 (d, 2H, J = 12.0 Hz), 1.52 (q, 2H, J = 12.0 Hz); ^{13}C NMR (acetone-d_6) δ 150.4, 150.0, 141.7, 130.7, 129.5, 129.0, 126.6, 119.4, 110.8, 106.8, 61.1, 52.9, 50.5, 34.4, 32.7; MS (ES^+) m/z 326 ($[MH]^+$, 100).

***N*-[1-(2-Phenethyl)-4-piperidyl]-*N*-(3-nitrophenyl)propanamide (13).** A solution of **12** (538 mg, 1.65 mmol) in propionic anhydride (80 mL) was stirred and heated to reflux for 4 h. After allowing to cool to room temperature, the excess of propionic acid was decomposed by heating to reflux the solution with water (100 mL). The solution was then basified with NH_4OH (30%) and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. To the residue dissolved in EtOAc was added an excess of oxalic acid in diethylether, this mixture was stirred at room temperature for 1 h. The precipitate was filtered, washed with EtOAc then with Et_2O. The crude was first crystallized from MeOH/Et_2O then from H_2O at 5 °C to provide the oxalate salt of **13** (540 mg). Yield 70%, mp 142–143 °C. ^{1}H NMR ($CDCl_3$) δ 8.27 (ddd, 1H, J = 8.0, 2.0 and 1.0 Hz), 7.98 (t, 1H, J = 2.0 Hz), 7.63 (t, 1H, J = 8.0 Hz), 7.46 (ddd, 1H, J = 8.0, 2.0 and 1.0 Hz), 7.28–7.23 (m, 2H), 7.20–7.13 (m, 3H), 4.72 (tt, 1H, J = 12.0 and 4.0 Hz), 3.01 (dd, 2H, J = 12.0 and 4.0 Hz), 2.70 (m, 2H), 2.54 (m, 2H), 2.18 (dt, 2H, J = 12.0 and 2.0 Hz), 1.92 (m, 2H), 1.85 (dd, 2H, J = 12.0 and 2.0 Hz), 1.38 (dq, 2H, J = 12.0 and 4.0 Hz), 1.05 (t, 3H, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$) δ 172.8, 148.7, 140.2, 140.0, 136.8, 130.2, 128.6, 128.4, 126.1, 125.5, 123.4, 60.3, 52.9, 52.4, 33.8, 30.6, 28.9, 9.4; MS (ES^+) m/z 382 ($[MH]^+$, 100).

***N*-[1-(2-Phenethyl)-4-piperidyl]-*N*-(3-aminophenyl)propanamide (14).** A mixture of oxalate salt of **13** (1.3 g, 2.8 mmol) and 10% Pd/C (57 mg) in MeOH (30 mL) was shaken under H_2 (35–40 psi) for 0.5 h. After filtration and evaporation of the solvent, the residue was dissolved in CH_2Cl_2. The solution was washed with K_2CO_3 (0.5 N). The organic layer was dried over $MgSO_4$ and concentrated in vacuo to give **14** (600 mg) as a lighly yellowish oil. Yield 61%. ^{1}H NMR ($CDCl_3$) δ 7.23–7.06 (m, 6H), 7.05 (t, 1H, J = 8.0 Hz), 6.54 (d, 1H, J = 7.7 Hz); 6.46 (dd, 1H, J = 8.3 and 2.2 Hz), 4.56 (t, 1H, J = 12.0 Hz), 2.93 (d, 2H, J = 12.0 Hz), 2.68 (m, 2H), 2.50 (m, 2H), 2.06–1.91 (m, 4H), 1.75 (d, 2H, J = 12.0 Hz), 1.37 (q, 2H, J = 12.0 Hz), 0.93 (t, 3H, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$) δ 175.6, 164.6, 149.0, 139.3, 136.7, 130.8, 129.4, 129.2, 127.7, 116.9, 116.6, 113.7, 58.3, 52.8, 50.7, 31.0, 28.7, 28.1, 9.8. MS (ES^+) m/z 352 ($[MH]^+$, 100).

***N*-[*m*-(2,3-di-*tert*-Butoxycarbonyl)guanidinophenyl]-*N*-[1-(2-phenethyl)-4-piperidyl]propanamide (15).** To a solution **14** (500 mg, 1.42 mmol), *N*-*N*′-di(*tert*-butoxycarbonyl)thiourea (392 mg, 1.42 mmol) and TEA (0.6 mL, 4.26 mmol) in anhydrous MeOH (20 mL) under N_2 and at 0 °C was added $HgCl_2$ (390 mg, 1.82 mmol). The reaction mixture was stirred at 0 °C for 1 h. The temperature was allowed to warm to ambient temperature and the mixture was stirred for additional 20 h. The mixture was diluted with EtOAc, filtered over Celite. The filtrate was washed with H_2O, washed with brine, and dried over $MgSO_4$. The solvent was evaporated in vacuo. The residue was purified by flash chromatography on silica gel (EtOAc/hexane, 9:1) to provide **15** (625 mg) as an oil. Yield 74%. ^{1}H NMR (D_2O) δ 7.40–7.35 (t, 1H, J = 8.0 Hz), 7.25–7.13 (m, 6H), 7.10–7.04 (dd, 1H, J = 8.0 and 2.0 Hz), 7.0 (t, 1H, J = 2.0 Hz), 3.60 (t, 1H, J = 12.0 Hz), 3.13 (d, 2H, J = 12.0 Hz), 2.83 (m, 2H), 2.63 (m, 2H), 2.16 (t, 2H, J = 12.6 Hz), 1.83 (m, 4H), 1.58 (m, 2H), 1.53 (s, 9H), 1.51 (s, 9H), 0.91 (t, 3H, J = 7.4 Hz); ^{13}C NMR ($CDCl_3$) δ 173.6, 163.3, 153.4, 149.2, 140.3, 137.7, 129.1, 128.6, 128.4, 126.1, 109.7, 109.4, 107.4, 84.0, 79.6, 60.6, 55.7, 53.6, 33.9, 29.6, 28.1, 28.0, 22.6, 11.3; MS (ES^+) m/z 593 ($[MH]^+$, 100).

***N*-[1-(2-Phenethyl)-4-piperidyl]-*N*-(*m*-guanidinophenyl)-propanamide (16).** A solution of **15** (300 mg, 0.84 mmol) in a mixture 1:1 of CH_2Cl_2/TFA was stirred at room temperature for 4 h. After evaporation of the solvents in vacuo, the residue was dissolved in CH_2Cl_2 and washed with NaOH (2.5 N). The organic layer was dried over $MgSO_4$ and the solvent was evaporated. To the residue dissolved in EtOAc was added an excess of

oxalic acid dissolved in Et_2O. The white precipitate was filtered, washed with EtOAc then with Et_2O. The white solid was dried in vacuo at 30 °C to give **16** (303 mg) as an oxalate salt. Yield 75%, mp 110–112 °C. ^1H NMR (D_2O) δ 7.33 (t, 1H, $J=8$ Hz), 7.25–7.13 (m, 6H), 7.07 (dd, 1H, $J=8.0$ and 2.0 Hz), 7.0 (t, 1H, $J=2.0$ Hz), 3.8 (t, 1H, $J=12.0$ Hz), 3.57 (d, 2H, $J=12.0$ Hz), 3.23 (m, 2H), 2.95 (td, 2H, $J=12.0$ and 2.0 Hz), 2.90 (m, 2H), 2.06 (dq, 2H, $J=12.0$ and 2.0 Hz), 1.80 (dq, 2H, $J=12.0$ and 4.0 Hz), 1.29 (q, 1H), 1.24 (q, 1H), 0.69 (t, 3H, $J=7.3$ Hz); ^{13}C NMR (D_2O) δ 174.5, 167.7, 157.4, 143.0, 141.0, 136.28, 131.6, 129.2, 128.9, 127.5, 122.2, 119.0, 117.0, 58.3, 57.7, 51.7, 30.0, 26.0, 19.0, 10.3; MS (ES^+) m/z 394 (MH^+, 100). Anal. ($C_{23}H_{31}N_5O\cdot H_2C_2O_4$) (found) C: 62.10 (61.82); H: 6.88 (6.82); N: 14.48 (14.35).

***N*-[1-(2-Phenethyl)-4-piperidyl]-*N*-[*m*-[*N'*-(4,5-dihydro-1*H*-imidazol-2-yl)aminophenyl]propanamide (17).** A solution of **14** (300 mg, 0.85 mmol) and 2-(methylthio)imidazolinium iodide (410 mg, 1.70 mmol) in anhydrous MeOH (3 mL) was stirred and heated to reflux for 48 h. A stream of N_2 was passed through the solution for 15 min then the solution was allowed to cool to room temperature. A precipitate was formed on adding diethyl ether to the solution. The solid was filtered, washed with diethyl ether and recrystallized from water at 5 °C. To the crystals dissolved in EtOAc was added an excess of oxalic acid dissolved in Et_2O. The precipitate was filtered, washed with EtOAc then with Et_2O. The white solid was dried in vacuo at 30 °C to give **17** (102 mg) as an oxalate salt. Yield 26%, mp 130–132 °C. ^1H NMR (CD_3OD) δ 7.40 (t, 1H, $J=8.0$ Hz), 7.26–7.15 (m, 6H), 7.1 (dd, 1H, $J=8.0$ and 2.0 Hz), 6.9 (t, 1H, $J=2.0$ Hz), 3.9 (t, 1H, $J=12.0$ Hz), 3.62 (s, 4H), 3.55 (d, 2H, $J=12.0$ Hz), 3.20 (m, 2H), 3.0 (td, 2H, $J=12.0$ and 2.0 Hz), 2.92 (m, 2H), 2.0 (dq, 2H, $J=12.0$ and 2.0 Hz), 1.86 (dq, 2H, $J=12.0$ and 4.0 Hz), 1.32 (m, 2H), 0.94 (t, 3H, $J=7.3$ Hz); ^{13}C NMR (CD_3OD) δ 173.7, 167.0, 159.4, 143.0, 141.0, 136.0, 132.4, 128.8, 128.3, 127.7, 119.8, 116.5, 115.6, 57.8, 57.5, 52.8, 43.0, 31.0, 27.0, 20.7, 11.2; MS (ES^+) m/z 420 ($[MH]^+$, 100). Anal. ($C_{25}H_{33}N_5O\cdot H_2C_2O_4$) (found) C: 63.64 (63.41); H: 6.92 (6.67); N: 13.74 (13.61).

Pharmacology

Binding assays. Preparation of membranes. Neural membranes (P_2 fractions) were prepared from the whole brain of male Swiss Webster mice. Briefly, the tissue samples were homogenized in 5 mL of ice-cold Tris sucrose buffer (5 mM Tris–HCl, 250 mM sucrose, pH 7.4). The homogenates were centrifuged at 1100*g* for 10 min, and the supernatants were then recentrifuged at 40,000*g* for 10 min. The resulting pellet was incubated at 25 °C for 30 min to remove endogenous opioids. After that, the pellet was washed twice and resuspended in 50 mM Tris–HCl buffer (pH 7.5) to a final protein content of 0.87 ± 0.11 mg mL^{-1}.

Binding assay. Total binding was measured in 0.55 mL-aliquots (50 mM Tris–HCl, pH 7.5) of the neural membranes which were incubated with [^{3}H]DAMGO (2 nM), [^{3}H]U-69593 (2 nM), [^{3}H]DPDPE (4 nM) or [^{3}H]2-BFI (1 nM). Neural membranes were incubated for 60 min at 25 °C in [^{3}H]DAMGO and [^{3}H]U-69593 assays, 60 min at 37 °C in the [^{3}H]DPDPE assays or 45 min at 25 °C for [^{3}H]2-BFI, in the absence or presence of the competing compounds (10^{-12} M to 10^{-3} M, 10 concentrations). Total binding was determined and plotted as a function of the compound concentration.

Incubations were terminated by diluting the samples with 5 mL of ice-cold Tris incubation buffer (4 °C). Membrane bound was separated by vacuum filtration through Whatman GF/C glass fibre filters. Then, the filters were rinsed twice with 5 mL of incubation buffer and transferred to minivials containing 3 mL of Opti-Phase 'HiSafe' II cocktail and counted for radioactivity by liquid scintillation spectrometry.

Analysis of binding data. Analysis of competition experiments to obtain the inhibition constant (K_i) were performed by nonlinear regression using the EBDA-LIGAND program. All experiments were analysed assuming a one-site model of radioligand binding.

Drugs. [^{3}H]DAMGO (specific activity 50 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc., USA. [^{3}H]U69593 (specific activity 41.4 Ci/mmol) and [^{3}H]DPDPE (specific activity 45 Ci/mmol) were obtained from NEN Life Science Products Inc., USA. [^{3}H]2-BFI (specific activity 70 Ci/mmol) was purchased from Amersham International, UK. Idazoxan HCl was synthesized by Dr. F. Geijo at S.A. Lasa Laboratories, Barcelona, Spain. Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Functional activity. In vivo assays. CD1 male mice weighing 25–30 g were used. All the animals were supplied with food and water 'ad libitum' and were housed in a temperature-controlled room at 21 °C. Lighting was on a 12/12-h light/dark cycle. The mice were housed for at least 1 day in the test-room before experimentation.

To detect antinociceptive activity, several doses (2–50 mg/kg) of the tested compounds or saline solution were intraperitoneally (ip) administered to separated groups of mice ($n\geq10$), 30 min before the analgesic effect was tested. In order to evaluate the antinociception level, the effect of ip administration of morphine (1.3–10 mg/kg) was also tested. Each mouse was used only once and an observer who was unaware of the treatment performed the testing and data recording.

Writhing test. The mice were ip injected with a 2% acetic acid solution to produce the typical writhing reaction which is characterised by a wave of contraction of the abdominal musculature followed by extension of the hind limbs. After the acetic acid administration, mice were placed in individual transparent containers and, 5 min later, the number of writhes was counted during a 10 min period.

To test the antinociceptive effect writhing test was carried out 30 min after the administration of separated doses of: compound **10** (2, 10, 25 and 50 mg/kg), compound **16** (5 and 10 mg/kg) and morphine (1.3, 2.5, 3.5, 5 and 10 mg/kg).

Hot plate test. This test was carried out with a hot plate at 55 °C as a nociceptive stimulus. The control reaction latency of the animals was measured before the treatment. The time of licking of the front paw was taken as an index of nociception. The latency was measured before drug or saline administration (control) and 30 min after treatment. The cut-off time was 30 s and analgesia was quantified with the formula of the % of maximum possible effect (M.P.E.):

$$\text{M.P.E.} = (\text{latency aftertreatment} - \text{control latency})/(\text{cut-off time} - \text{control latency})$$

To test the antinociceptive effect hot plate test was carried out 30 min after the administration of separated doses of: compound **10** (2, 10, 25 and 50 mg/kg), compound **16** (5, 10 and 20 mg/kg) and morphine (2.5, 5, 7.5 and 10 mg/kg).

Naloxone antagonism. The opioid antagonist naloxone was used in order to assess the involvement of the opioid system. Separated groups of animals ($n \geq 10$) were ip treated with naloxone (1 mg/kg ip) and one of the new compounds (compound **10**—25 mg/kg, compound **16**—20 mg/kg), and 30 min later either the writhing test (**10**) or the hot plate test (**16**) were carried out. The inhibition of morphine (5 mg/kg) antinociception induced by naloxone was used as a control.

In vitro assays. 1 Isolated tissues. Male guinea-pigs weighing 300–450 g, were used for this study. Myenteric plexus-longitudinal muscle strips (MP-LM) were isolated from guinea-pig ileum as described by Ambache.[21] Tissues were suspended in a 10 mL organ bath containing Krebs solution (NaCl 118, KCl 4.75; $CaCl_2$ 2.54; KH_2PO_4 1.19; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11 mM). This solution was continuously gassed with 95% O_2 and 5% CO_2. Tissues were kept under 1 g of resting tension at 32 °C and were electrically stimulated through two platinum ring electrodes (rectangular pulses of 70 V, 0.1 ms duration and 0.3 Hz frequency). The isometric force was recorded on a Grass model 7A polygraph.

Cumulative concentration–response curves for reference compounds or for the new compounds were constructed in a step by step manner after the response to the previous concentration had reached a plateau. The interval between application of increasing concentrations was 5 min. The range effective concentrations tested were for:

1. Morphine: 10^{-7} to 1.6×10^{-6} M
2. Compound **10**: 10^{-6} to 3.2×10^{-5} M;
3. Compound **16**: 4×10^{-7} to 1.3×10^{-5} M

The effect of the drugs was evaluated 5 min after the addition of each dose, as percentage of inhibition, taking the amplitude of the last contraction before the first addition of agonist as 100%. The opioid agonists were added to the organ bath 15 min after the beginning of electrical stimulation. Each tissue was employed to construct only one concentration–response curve.

To corroborate that the inhibitory effect of the selective opioid agonists or of the new compounds was mediated through interaction with mu opioid receptors, one low dose naloxone (5×10^{-8} M) was added to the organ bath at the end of each experiment.

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