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Long-Acting Fentanyl Analogues: Synthesis and Pharmacology of *N*-(1-Phenylpyrazolyl)-*N*-(1-phenylalkyl-4-piperidyl)propanamides

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Abstract—The synthesis of new fentanyl analogues in which the benzene ring of the propioanilido group has been replaced by phenylpyrazole is described. Antinociceptive activity was evaluated using the writhing and hot plate tests in mice. Two compounds, **3b** and **3d**, showed interesting analgesic properties, being more potent than morphine and less than fentanyl but with longer duration of action. These compounds inhibited the electrically evoked muscle contraction of guinea pig ileum and mouse vas deferens but not that of rabbit vas deferens and the effects could be reversed by antagonists (naloxone and/or CTOP), thus indicating that the compounds acted as μ agonists. Finally, the binding data confirmed that the compounds had high affinity and selectivity for the μ receptor.

Introduction

Pain is the most common reason that patients seek advice from pharmacists and other health professionals and represents important medical and economic costs for the community. Current analgesic therapy can be divided in three large groups: NSAIDS (non-steroidal antiinflammatory agents), opioids, and the so-called analgesic adjuvants which include antidepressants and local anesthetics. Despite their proven efficacy in alleviating symptoms and providing pain relief, all have considerable side effects including gastrointestinal and renal damage for the first, and respiratory depression, emesis, and tolerance and/or addiction for opioids.[1] This together with the fact that many pain sufferers are not satisfied with their pain care, makes the search for new analgesics, that can more effectively treat pain either chronic, neuropathic or from any other origin an important challenge in medicinal chemistry. Among the new analgesic drugs on the market, gabapentin used for neuropathic pain and tramadol, with a dual mode of action (opioid and noradrenaline uptake inhibitor) are worth mentioning.[2]

In what opioids are concerned,[3] and despite the considerable research effort of the past two decades, the only new opioid analgesics either on the market, or in clinical development are mainly alternative dosage forms of the classic opioids, including controlled-released morphine suppositories and supensions, and transdermal fentanyl, a patch that allows 3-day dosing and avoids the first-pass effect of the liver.[4]

Since the discovery of fentanyl by Janssen in 1962,[5] many anilidopiperidines have been synthesized and evaluated for SAR studies[6] and for providing insight into the key structural features required for high affinity binding to the μ receptor.[7] However, only three fentanyl-like compounds are commercially available (alfentanil, remifentanil and sufentanil) (Chart 1) and due to their high potency and short duration of action they are mainly used for induction of general anesthesia.

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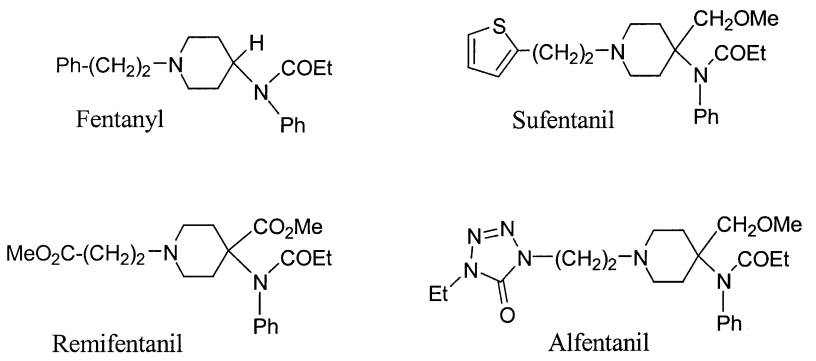
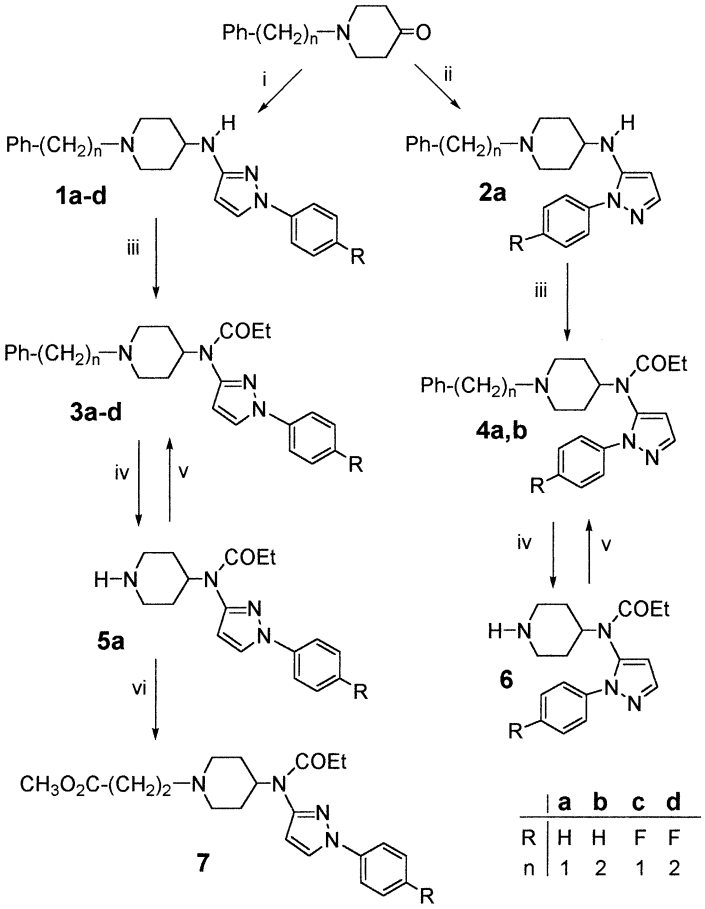


Chart 1.

Therefore, fentanyl itself still represents the reference drug in this kind of compounds and the increasing use of its transdermal formulation for the treatment of chronic and cancer pain suggests that the search of new analogues with longer duration of action may represent an interesting approach for novel analgesics.

Within this context, we decided to modify the fentanyl molecule, and taking into account our experience in azole chemistry[8] we decided to substitute the benzene of the propioanilido group by a phenylpyrazole. It should be commented that despite the many different structural variations that have been performed on the fentanyl structure, worth mentioning the recent description of the potent 4-methylfentanyl derivative,[9] the substitution of the propioanilido benzene by five-membered rings has not, in general, been explored. It has actually been replaced by pyridine, six-membered heterocycles[10] and fused heteroaryl derivatives[11] but with no substantial improvement of the analgesic profile over fentanyl itself. Therefore, in this paper we wish to report the synthesis and pharmacological studies of new fentanyl analogues bearing a phenylpyrazole moiety, some of which have shown interesting analgesic properties with potency higher than morphine, somewhat lower than fentanyl but with considerably longer duration of action.



Scheme 1. (i) 3-aminophenylpyrazole, $NaBH_4$, $PhCH_3$, *p*-TsOH or $NaBH_3CN$, MeOH; (ii) 5-aminophenylpyrazole, $NaBH_4$, $PhCH_3$, *p*-TsOH or $NaBH_3CN$, MeOH; (iii) $(Et_2OC)_2O$; (iv) 1-ACE-Cl, $ClCH_2CH_2CI$; (v) $PhCH_2CH_2Br$, DMA; (vi) $CH_3O_2CCH{=}CH_2$, CH_3CN.

Results

Chemistry

Compounds **1**–**7** described in this study were prepared as outlined in Scheme 1.[12] The benzyl derivatives of the propanamides **3a**, **3c** and **4a** were prepared starting from 1-benzyl-4-piperidone. The phenethyl derivatives were obtained either from 1-phenethyl-4-piperidone, as was the case for the propanamide **3d**, or from 1-benzyl-4-piperidone, which was how the propanamide **4b** was prepared through the deprotected piperidine **6**. In the case of the phenethyl derivative of the propanamide **3b**, we describe both procedures: from 1-benzyl-4-piperidone **3b** was obtained through **5a** with an overall yield of $\sim$ 10% and from 1-phenethyl-4-piperidone the overall yield improved (24%). The first step of the reaction sequences consists on a reductive amination of the piperidones. This reaction was carried out in two steps: formation of the corresponding imines by addition of the corresponding aminopyrazole followed by reduction with sodium borohydride (procedure A) or in a one pot reaction using sodium cyanoborohydride as reductant[13] (procedure B). Next, reflux of the resulting amines in propionic anhydride afforded the desired amides **3a**–**d** and **4a**. The debenzylation could not be achieved by catalytic hydrogenation; however, treatment with 1-chloroethylchloroformate[14] followed by methanolysis removed the benzyl group in good yield. Alkylation of the *N*-deprotected derivatives **5a** and **6** with 1-phenyl-2-bromoethane gave the respective phenethyl derivatives **3b** and **4b**. Finally, the methoxycarbonylderivative **7**, which can be considered an analogue of remifentanil, has been synthesized in good yield by alkylation of the *N*-deprotected derivative **5a** with methyl acrylate by Michael reaction. This compound was prepared in order to study the variation of the pharmacokinetic profile of this series, since it is well known that modulation of the duration of action can be achieved through alkyl ester substitution.[15]

The synthesized compounds of this study have been characterized by electrospray mass spectrometry and by NMR spectroscopy: the signals have been attributed by a combined used of ^{1}H, ^{13}C, HMQC and literature references.[16] The analgesic evaluation of compounds **3a**–**d**, **4a**, **4b** and **7** has been carried on their oxalates.

Pharmacology

Target compounds **3a**–**d**, **4a**, **4b** and **7** were tested in order to determine their antinociceptive capacity. The writhing test in mice was used because it is widely employed at the first stages of the evaluation of antinociceptive drugs.[17] This test allows an easy and quick discarding of the non-analgesic derivatives. Compounds showing antinociceptive activity, were also studied using the hot plate test, to corroborate the analgesia by this more sensitive test. Naloxone is a selective opioid antagonist that is generally used to verify any opioid involvement in the effect of drugs. Data obtained from the study of the antinociception and of the naloxone antagonism permits to suggest that the activation of opioid receptors plays a role in the effect of a drug but it is also interesting to determine the receptor subtype.

The opioid activity profile of the new compounds was functionally determined by in vitro bioassays using guinea pig ileum for μ and κ receptors, mouse vas deferens for δ receptors and rabbit vas deferens for κ receptors.[18–21]

Finally, compounds **3b** and **3d** showing μ opioid profile were evaluated for their binding affinities at the μ, δ and κ binding sites following reported procedures[22] with minor modifications. The inactive isomeric structure of **3b**, **4b**, and fentanyl 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. Since the binding data indicated that the compounds had an extremely high affinity for the μ receptor which did not correlate linearly with the analgesic potencies and inhibitory effects observed, further binding tests were performed in a different system, in human recombinant CHO-K1 cells following the methods of Wang[23] and Maguire.[24]

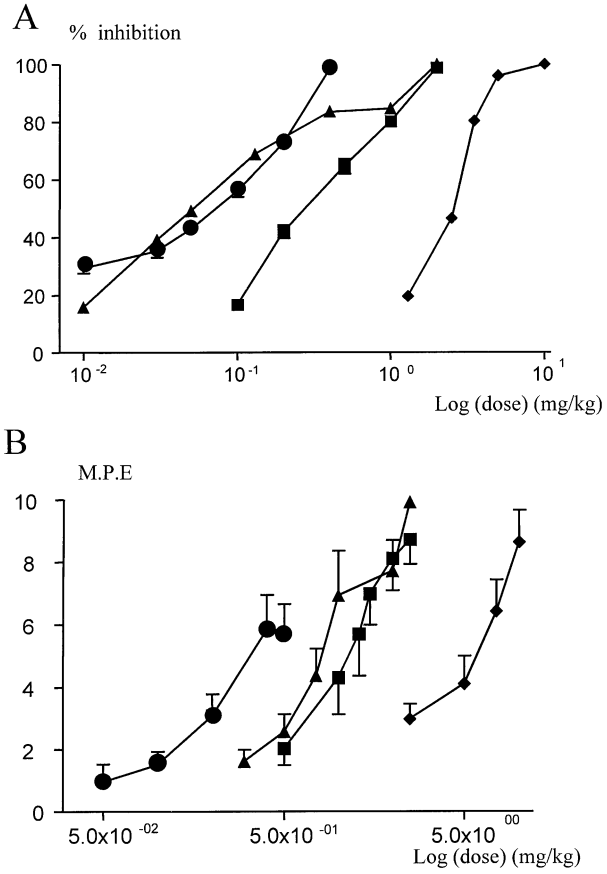


Figure 1. Lines show: **A** the % of inhibition ± SEM of the number of contractions induced by acetic acid administration, number of writhes in saline solution treated animals (control group): 19 ± 0.9 (n = 12) and **B** the % of Maximum Possible Effect (MPE) ± SEM in the hot plate test, induced by the administration of **3b** (squares), **3d** (triangles), morphine (diamonds) and fentanyl (circles) in mice, 30 mm before the test.

Discussion

First of all, the antinociceptive activity of the new compounds **3a–d**, **4a–b** and **7** was determined carrying out the writhing test. Using this test, the phenethyl derivatives **3b** and **3d** were able to induce a dose-dependent antinociceptive effect, while the corresponding benzyl derivatives **3a** and **3c** and the methoxycarbonyl derivative **7** did not demonstrate any significant analgesic activity. Compounds **4a** and **4b** corresponding to the positional isomers of **3a** and **3b** turned out to be inactive. Then, a more selective test, the hot plate, was used and the antinociception induced by morphine and by fentanyl was also analysed in order to evaluate the effect of the new compounds. Compounds **3b** and **3d** were more potent than morphine but less potent than fentanyl in both the writhing test and the hot plate test (Fig. 1). Naloxone was able to completely antagonize the analgesia induced by the new compounds as well as that induced by morphine or by fentanyl. From these findings, it could be suggested that the antinociception induced by both **3b** and **3d** is mediated through the activation of opioid receptors.

The duration of the analgesic effect of the new derivatives was compared with that of fentanyl. In order to perform the study, the analgesic effectiveness of several doses of the compounds was tested 5 min after their administration and equipotent (85–95% of inhibition of the nociceptive response) doses of each compound were selected. We found that whereas the fentanyl antinociception ceased 120 min after the ip administration, the effect of **3b** and **3d** persisted for at least 360 min (Fig. 2). It is worth mentioning that no substantial difference in the duration of action was observed between the parent compound **3b** and **3d**, in which on of the probable metabolic degradation routes is blocked.

To study the functional activity of the opioid agonists, isolated tissues such as guinea-pig ileum,[18] mouse vas deferens[19] and rabbit vas deferens[21] were used. In guinea pig ileum, **3b** and **3d**, as well as control opioids, morphine and fentanyl, induced a dose-dependent inhibition of the electrically induced contractions (Fig. 3).

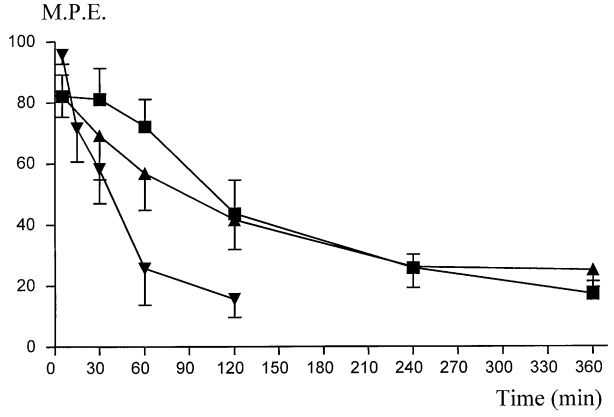


Figure 2. Lines show the duration ± SEM of the analgesic effect of **3b** (2 mg/kg) (squares), **3d** (1 mg/kg) (triangles) and fentanyl (0.4 mg/kg) (inverted triangles) in the hot plate test in mice.

Table 1 shows the EC_{50} values. The in vitro effect of all the tested drugs was completely reversed by the in vitro administration of naloxone (10^{-6} M). Since these tissues mainly present μ and κ opioid receptors,[25] it could be suggested that μ and/or κ opioid receptors could be involved in the effect of new compounds, although effects on the δ opioid receptor may not be discarded.

To clarify these possibilities, we used mouse vas deferens preparations that mainly contain δ receptors (although they have also μ and κ receptors), and rabbit vas deferens, having κ receptors only. The effect of **3b** and **3d** in these tissues was compared with that of two selective agonists: [D-Pen^2,D-Pen^5]-enkephalin (DEPEN) (δ)[26] and U-50,488H (κ).[27] Naltrindole [28] was used as a δ selective antagonist and norbinaltorfimine[29] as a κ antagonist. In comparison with the δ agonist DEPEN, **3b** and **3d** were slightly less potent in inhibiting the electrically induced contractions in the mouse vas deferens (Table 1). However, the inhibition of the contractile response induced by DEPEN (6×10^{-8} M) was 71.6±3.4% and in naltrindole (10^{-9} M) treated tissues was 41.0±3.5%, whilst the inhibition induced by **3b** (2×10^{-7} M,% of inhibition: 70.5±8.1) and **3d** (2×10^{-7} M,% of inhibition: 70.2±9.4) was not significantly modified by the δ antagonist (10^{-9} to 10^{-8} M). From this result it could be suggested that the effect of **3b** and

Table 1. Inhibitory effect (EC_{50}) of morphine, fentanyl, **3b** and **3d** on the electrically-induced contractions in guinea pig ileum and mouse vas deferens

Compounds	EC_{50} (M)	Confidence intervals (95%)
Guinea pig ileum		
Morphine	5.2×10^{-7}	2.1×10^{-7}–1.3×10^{-6}
Fentanyl	5.2×10^{-9}	4.1×10^{-9}–6.5×10^{-9}
3b	1.3×10^{-7}	8.2×10^{-8}–2.0×10^{-7}
3d	9.4×10^{-8}	5.4×10^{-8}–1.6×10^{-7}
Mouse vas deferens		
D-Pen	4.0×10^{-9}	2.5×10^{-9}–6.6×10^{-9}
3b	6.0×10^{-8}	3.9×10^{-8}–9.2×10^{-8}
3d	4.7×10^{-8}	3.0×10^{-8}–7.5×10^{-8}

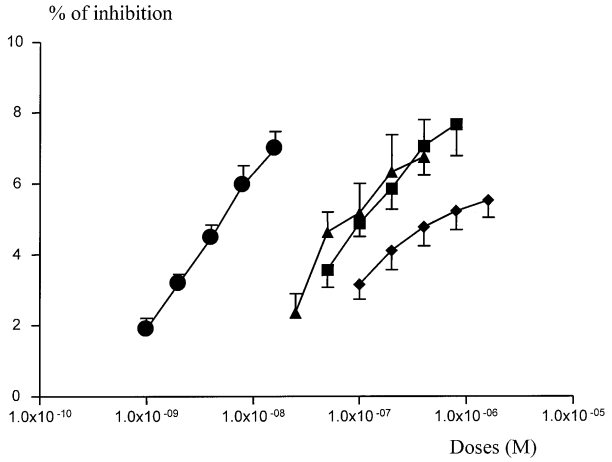


Figure 3. Lines show the % of inhibition ± SEM of the electrically-induced contractions in the MP-LM strips of guinea pig ileum incubated with **3b** (squares), **3d** (triangles), morphine (diamonds) and fentanyl (circles).

Table 2. Affinity data [K_i (nM)] of Fentanyl, **3b**, **3d** and **4b** for μ, δ and κ opioid receptors[a]

Receptors:	[^{3}H]DAMGO (2 nM) μ	[^{3}H]DPDPE (4 nM) δ	[^{3}H]U-69593 (2 nM) κ
Fentanyl	5.9±1.4	568±159	298±40
3b	0.032±0.01	89±38	828±431
3d	0.0025±0.0006	86±23	1383±227
4b	427±124	7321±1656	4554±558

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

3d on the mouse vas deferens was not mediated by δ opioid receptors.

When the μ selective antagonist CTOP (10^{-6} M)[30,31] was used to assess that the inhibitory effect was mediated by μ receptors a complete reversion of the effect of **3b** and **3d** was observed in guinea pig ileum and mouse vas deferens. In rabbit vas deferens, as expected, U-50,488H induced a dose dependent inhibition of the electrically induced contractions and the selective κ antagonist norbinaltorfimine completely antagonized this effect. On the contrary, neither **3b** nor **3d** significantly modified the contractile response of the rabbit vas deferens. This result discards any functional activity of the new fentanyl derivatives on κ receptors. The possibility of these new compounds to selectively antagonize the effect of U-50,488H on κ receptors was also tested in rabbit vas deferens: neither **3b** (up to 5×10^{-7} M) nor **3d** (up to 2.5×10^{-7} M) were able to recover the contractile activity of the tissue after treatment with the κ agonist.

These in vitro results indicated that the new compounds are μ opioid selective agonists. Their affinity for the μ opioid receptor was corroborated by radioligand binding assays.[22] The binding data in mouse brain membranes for the μ, δ and κ receptors of **3b** and **3d** together with those of **4b** and fentanyl are provided in Table 2. These data are in agreement with results obtained on isolated tissues confirming that **3b** and **3d** are μ agonists, and corroborate the lack of affinity for the δ and κ receptors. On the other hand, compound **4b**, positional isomer of **3b**, which had shown no antinociceptive activity in vivo, had no affinity for the μ receptor suggesting that the position of the 1-phenylpyrazole is crucial for activity.

In terms of the potency of the binding affinity, the very low values of K_i for the μ receptors of **3b** and **3d** are difficult to explain. These values, which are comparable to those of some of the more potent isomers of ohmefentanyl, eg the oxalate salt of the 2*R*,3*R*,4*S* isomer has a $K_i = 0.013\pm0.002$ nM,[32] do not correlate well with the data obtained in functional assays and analgesic tests. There is, however, a good correlationship between the functional data obtained in isolated tissues and in the antinociceptive tests. The considerable difference between the binding data and the functional assays can be accounted for by the fact that the binding study provides information about affinity and selectivity upon different receptors whereas functional assays show the intrinsic activity. For example, low activity in the GPI assay despite strong binding to the κ receptor has been observed for a number of dynorphine analogues and recently this has been reported to reflect an intrinsic property of the ligand.[33]

In any case, the absolute K_i values should always be considered with certain care since for example, in the case of ohmefentanyl, a potent opioid already mentioned, in which eight optically active isomers are possible, there was controversy in the binding data of the more potent isomers reported by two different groups.[32,34]

Nevertheless, a different μ-binding study was performed in human recombinant CHO-K1 cells. The K_i values in this case were for **3b** 1.21±0.11 nM and for **3d** 1.04±0.05 nM. These values correlate well with all other pharmacological assays and confirm that **3b** and **3d** have high affinity and selectivity for the μ receptor.

Conclusions

New piperidylpropanamides, structurally related to fentanyl and incorporating a 1-phenylpyrazole rest have been synthesized. Two derivatives **3b** and **3d** are able to induce a dose dependent antinociceptive effect that may be blocked by the opioid antagonist naloxone, and their in vitro pharmacological profile suggests that they are potent and selective μ opioid agonists. Furthermore, their in vitro and in vivo potency is greater than that of morphine and, the duration of their analgesic effect is longer than for fentanyl. Considering that the use of fentanyl in chronic pain is mainly limited by the short duration of its antinociceptive activity, it could be suggested that 1-phenylpyrazole may represent an interesting substitution for the benzene ring in fentanyl and deserves further investigation. Studies on the tolerance induced by these analgesics are currently underway.

Experimental

Chemistry

The melting points were determined with a Reichert Jung Thermovar apparatus and are uncorrected. Mass spectra were recorded using Fast Atom Bombardment in NBA matrix or using electrospray. Flash column chromatographies were run on silica gel 60 (230–400 mesh) or on medium pressure flash system with prepacked silica gel cartridge. ^{1}H and ^{13}C NMR spectra were recorded on a 200, 300 and 400 unity spectrometers. Dry ethanol was obtained by distillation over $Mg(OH)_2$. Tetrahydrofuran and toluene were distilled over sodium-benzophenone. The oxalate salts were precipitated from a solution of the corresponding free bases in EtOAc to which was added oxalic acid in a light excess. 1-Phenyl-3-amino-Δ^2-pyrazoline, 1-(4-fluorophenyl)-3-amino-Δ^2-pyrazoline and 1-phenyl-5-aminopyrazole were synthesized as reported in the

literature.[35] 1-Phenyl- and 1-(4-fluorophenyl)-3-aminopyrazoles were prepared by oxidation of the corresponding pyrazoline as follows:

1-Phenyl-3-aminopyrazole. 1-Phenyl-3-amino-Δ^2-pyrazoline (3.7g, 23 mmol) was dissolved in dioxane (100 mL). DDQ (5.77 g, 25 mmol) was added to the solution. The reaction mixture was stirred at room temperature for 0.5 h. Then the solution was filtrated. The filtrate was acidified with 1 N HCl and CH_2Cl_2 was added (150 mL). The organic layer was separated, extracted with 1 N HCl. The combined aqueous layers were then made alkaline with 10% NaOH and extracted with CH_2Cl_2. The organic layers were dried over $MgSO_4$ and the solvent was evaporated to yield 2.9 g (80%) of a white solid: mp 89–90 °C [lit.[35a] (H_2O) mp 101 °C].

***N*-(1-Phenylpyrazol-3-yl)-*N*-(1-benzyl-4-piperidyl)-amine (1a). Procedure A**. To 1-benzyl-4-piperidone (0.7 mL, 3.78 mmol) in toluene (100 mL) was added 1-phenyl-3-aminopyrazole (600 mg, 3.77 mmol) and few crystals of *p*-TsOH. The reaction mixture was stirred and heated to reflux for 18 h eliminating the H_2O formed with a Dean-Stark apparatus. The solvent was then evaporated and the reddish oily residue was dissolved in MeOH (20 mL). Sodium tetrahydroborate (150 mg, 4.1 mmol) was added portionwise and the solution was stirred for 3 h at room temperature. The solvent was then evaporated, the residue was dissolved in CH_2Cl_2, washed with H_2O, dried over Na_2SO_4. The solvent was evaporated and the crude product was purified on silica gel [EtOAc/TEA(200/1)] to yield 662 mg (53%) of a yellowish solid: mp 52–54 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.61 (1H, d, $J=2.5$ Hz, H-5(Pz)), 7.48 (2H, d, $J=8$ Hz, *o*-H(C_6H_5Pz), 7.30 (2H, t, $J=8$ Hz, *m*-H(C_6H_5Pz)), 7.25 (5H, m, $C_6H_5CH_2$), 7.07 (1H, t, $J=8$ Hz, *p*-H(C_6H_5Pz)), 5.70 (1H, d, $J=2.5$ Hz, H-4(Pz)), 3.45 (2H, s, $C_6H_5CH_2$), 3.29 (1H, m, H-4(Pip)), 2.77 (2H, d, $J=13$ Hz, H-2(Pip)), 2.10 (2H, t, $J=9.5$ Hz, H-2(Pip)), 2.01 (2H, d, $J=13$ Hz, H-3(Pip)), 1.47 (2H, m, H-3(Pip)); ^{13}C NMR ($CDCl_3$) δ (ppm) 157.0 (C-3(Pz)), 139.9 (*ipso*-(C_6H_5Pz)), 138.1 (*ipso*- ($C_6H_5CH_2$)), 128.8 (*m*-($C_6H_5CH_2$)), 127.9 (C-5(Pz)), 126.9 (*o*-($C_6H_5CH_2$)), 126.7 (*m*-(C_6H_5Pz)), 124.3 (*p*-(C_6H_5Pz); *p*-($C_6H_5CH_2$)), 117.1 (*o*-(C_6H_5Pz)), 94.5 (C-4(Pz)), 63.7 ($C_6H_5CH_2$), 52.9 (C-2(Pip)), 51.9 (C-4(Pip), 33.4 (C-3(Pip)). MS (electrospray) [M + 1]333.

***N*-(1-Phenylpyrazol-3-yl)-*N*-(1-benzyl-4-piperidyl)-propanamide (3a).** Amine **1a** (620 mg, 1.87 mmol) was heated to 135 °C in propionic anhydride for 4 h. Then the reaction mixture was poured on ice–H_2O, made alkaline with concd NH_4OH and extracted with CH_2Cl_2. The combined organic layers were washed with H_2O, dried over $MgSO_4$, and the solvent was evaporated. The crude product was chromatographed on silica gel (EtOAc) to yield 380 mg (52%) of a yellowish oil which solidified on standing: mp 110–112 °C; ^{1}HNMR ($CDCl_3$) δ (ppm) 7.92 (1H, d, $J=2.4$ Hz, H-4(Pz)), 7.66 (2H, d, $J=7.5$ Hz, *o*-H(C_6H_5Pz)), 7.47 (2H, t, $J=7.5$ Hz, *m*-H(C_6H_5Pz)), 7.30 (1H, t, $J=7.5$ Hz, *p*-H(C_6H_5Pz)), 7.26 (5H, m, $C_6H_5CH_2$), 6.23 (1H, d, $J=2.4$ Hz, H-5(Pz)), 4.60 (1H, m, H-4(Pip), 3.46 (2H, s, $C_6H_5CH_2$), 2.90 (2H, d, $J=11$ Hz, H-2(Pip)), 2.13 (2H, q, $J=7.5$ Hz, CH_2CH_3) 2.10 (2H, m, H-2(Pip)), 1.83 (2H, d, $J=11$ Hz, H-3(Pip)),1.56 (2H, m, H-3(Pip)), 1.07 (3H, t, $J=7.5$ Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm) 174.0 (CO), 148.7 (C-3(Pz)), 139.6 (*ipso*-(C_6H_5Pz)), 138.1 (*ipso*-($C_6H_5CH_2$)), 129.4 (*m*-($C_6H_5CH_2$)), 129.0 (*m*-(C_6H_5Pz)), 127.7 (C-5(Pz)), 127.6 (*o*-($C_6H_5CH_2$)), 126.8 (*p*-($C_6H_5CH_2$)), 126.7 (*p*-(C_6H_5Pz); 118.9 (*o*-(C_6H_5Pz)), 107.3 (C-4(Pz)), 62.9 ($C_6H_5CH_2$), 52.9 (C-2(Pip)), 51.9 (C-4(Pip), 30.3(C-3(Pip)), 28.0 (CH_2CH_3), 9.4 (CH_2CH_3). Anal. ($C_{24}H_{28}N_4O$): calcd C 74.20, H 7.26, N 14.42; found C 73.58, H 7.06, N 14.22.

***N*-(1-Phenylpyrazol-3-yl)-*N*-(4-piperidyl)-propanamide (5a).** Propanamide **3a** (270 mg, 0.70 mmol) was dissolved in dry 1,2-dichloroethane (6 mL). At 0 °C was added 1-chloroethylchloroformate (127 mL, 1.16 mmol). The reaction mixture was kept stirring at this temperature for 15 min then was heated to reflux for 1 h. After evaporating the solvent, the residue was heated in refluxing dry MeOH (15 mL) for 1.5 h. The solution was then concentrated and the solid residue was dissolved in 0.5 N HCl (30 mL), this aqueous solution was washed with Et_2O. The aqueous layer was made alkaline with 10% NaOH, then it was extracted with Et_2O. The ethereal layers were dried over $MgSO_4$ and the solvent was evaporated yielding 200 mg (95%) of a white solid: mp 128 °C; ^{1}H NMR($CDCl_3$) δ (ppm) 7.98 (1H, d, $J=2.4$ Hz, H-5(Pz)), 7.69 (2H, d, $J=7.5$ Hz, *o*-H(C_6H_5Pz)), 7.45 (2H, t, $J=7.5$ Hz, *m*-H(C_6H_5Pz)), 7.30 (1H, t, $J=7.5$ Hz, *p*-H(C_6H_5Pz)), 6.25 (1H, d, $J=2.4$ Hz, H-4(Pz)), 4.66 (1H, m, H-4(Pip), 3.05 (2H, d, $J=11$ Hz, H-2(Pip)), 2.69 (2H, t, H-2(Pip)), 2.15 (2H, q, $J=7.5$ Hz, CH_2CH_3), 1.85 (2H, d, $J=11$ Hz, H-3(Pip)), 1.41 (2H, dd, H-3(Pip)), 1.10 (3H, t, $J=7.5$ Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm) 173.6 (CO), 148.6 (C-3(Pz)), 139.4 (*ipso*-(C_6H_5Pz)), 129.2 (*m*-(C_6H_5Pz)), 127.6 (C-5(Pz)), 126.6 (*p*-(C_6H_5Pz); 118.7 (*o*-(C_6H_5Pz)), 107.1 (C-4(Pz)), 51.9 (C-4(Pip), 45.9 (C-2(Pip)), 31.5 (C-3(Pip)), 27.8 (CH_2CH_3), 9.3 (CH_2CH_3). MS (electrospray) [M + 1] 299.2.

N-(1-phenylpyrazol-3-yl)-*N*-(1-phenethyl-4-piperidyl)-amine (**1b**) was prepared following the same procedure as for the benzyl derivatives starting with the 1-phenethylpiperidone.

***N*-(1-phenylpyrazol-3-yl)-*N*-(1-phenethyl-4-piperidyl)-amine (1b).** (Yield, 43% from procedure A): mp 107–110 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.76 (1H, d, H-5(Pz)), 7.63 (2H, d, *o*-H(C_6H_5Pz)), 7.40 (2H, t, *o*-H(C_6H_5Pz)), 7.32 (6H, m, $C_6H_5CH_2$, *p*-H(C_6H_5Pz)), 5.85 (1H, d, H-4(Pz)), 3.82 (1H, brm, NH), 3.48 (1H, m, H-4(Pip)), 3.03 (2H, d, H-2(Pip)), 2.83 (2H, m, $C_6H_5CH_2CH_2$), 2.62 (2H, m, $C_6H_5CH_2CH_2$), 2.24 (2H, t, H-2(Pip)), 2.21 (2H, t, H-3(Pip)), 1.62 (2H, m, H-3(Pip)); ^{13}C NMR ($CDCl_3$) δ 156.8 (C-3(Pz)), 140.0 (*ipso*-(C_6H_5Pz)), 139.8 (*ipso*-($C_6H_5CH_2$)), 128.8 (*m*-($C_6H_5CH_2$)), 128.2 (*o*-($C_6H_5CH_2$)), 127.9 (C-5(Pz)), 126.6 (*m*-(C_6H_5Pz)), 125.6 (*p*-($C_6H_5CH_2$)), 124.2 (*p*-(C_6H_5Pz)), 117.1 (*o*-C_6H_5Pz)), 94.4 (C-4(Pz)), 60.2 ($C_6H_5CH_2CH_2$), 52.0 (C-2(Pip)), 50.8 (C-4(Pip), 33.4 ($C_6H_5CH_2CH_2$), 32.4 (C-3(Pip)). MS (electrospray) [M + 1] 347.3.

***N*-(1-Phenylpyrazol-3-yl)-*N*-[1-(2-phenethyl)-4-piperidyl]-propanamide (3b).** From **1b**: same procedure than for the preparation of **3a**, (yield = 55%). From **5a**: to propanamide **5a** (200 mg, 0.67 mmol) in DMA (5 mL) were added 1-phenyl-2-bromoethane (92 mL, 0.67 mmol) and TEA (110 mL, 0.79 mmol). This solution was stirred at 70 °C for 18 h, then the solution was poured on H_2O. The white precipitate was extracted with CH_2Cl_2, the combined organic layers were washed with H_2O, dried over $MgSO_4$ and concentrated. The crude was purified by chromatography on silica gel [EtOAc/cyclohexane (1:1)] to yield 73 mg (36%) of a white solid: mp 75–78 °C; oxalate, mp 182 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.97 (1H, d, H-5(Pz)), 7.72 (2H, d, *o*-H(C_6H_5Pz)), 7.53 (2H, t, *m*-H(C_6H_5Pz)), 7.30 (6H, m, *p*-H(C_6H_5Pz), $C_6H_5CH_2$), 7.31 (1H, d, H-4(Pz)), 4.68 (1H, m, H-4(Pip)), 3.08 (2H, d,H-2(Pip)), 2.80 (2H, m, $C_6H_5CH_2CH_2$), 2.60 (2H, m, $C_6H_5CH_2CH_2$), 2.20 (2H, m, H-2(Pip)), 2.21 (2H, q, J = 7.5 Hz, CH_2CH_3), 1.91 (2H, d, H-3(Pip)), 1.65 (2H, m, H-3(Pip)), 1.13 (3H, t, J = 7.5 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm) 174.1 (CO), 148.6 (C-3(Pz)), 140.1 (C-*ipso*-(C_6H_5Pz)), 139.9 (*ipso*-($C_6H_5CH_2$)), 129.4 (*m*-(C_6H_5Pz)), 128.6 (*m*-($C_6H_5CH_2$)), 128.3 (*o*-($C_6H_5CH_2$)), 127.8 (C-5(Pz)), 126.8 (*p*-(C_6H_5Pz)), 125.9 (*p*-($C_6H_5CH_2$)), 119.0 (*o*-(C_6H_5Pz)), 107.4 (C-4(Pz)), 60.5 ($C_6H_5CH_2CH_2$), 53.0 (C-2(Pip)), 51.8 (C-4(Pip), 33.7($C_6H_5CH_2CH_2$), 30.3 (C-3(Pip)), 28.0 (CH_2CH_3), 9.5 (CH_2CH_3). MS (electrospray) [M + 1] 403.2. Anal. ($C_{25}H_{30}N_4O.C_2H_2O_4$): calcd C 65.84, H 6.55, N 11.37; found C 65.59, H 6.55, N 11.26.

4-[(1-Oxopropyl)(1-phenylpyrazol-3-yl)amino]-*N*-piperidinepropanoic acid methyl ester (7). A mixture of propanamide **5a** (200 mg, 0.67 mmol) in dry acetonitrile and methylacrylate (120 mL, 1.32 mmol) was heated to 50 °C for 2.2 h. The solution was then concentrated and the residue was chromatographed on silica gel [EtOAc/MeOH (8.5:0.5)] to yield 243 mg (96%) of a white solid: mp 88–91 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.97 (1H, d, H-5(Pz)), 7.66 (2H, d,*o*-H(C_6H_5Pz)), 7.43 (2H, t, *m*-H(C_6H_5Pz)), 7.28 (2H, t, *p*-H(C_6H_5Pz)), 6.23 (1H, d, H-4(Pz)), 4.56 (1H, m, H-4(Pip)), 3.60 (3H, s, CH_3CO_2), 2.87 (2H, d, H-2(Pip)), 2.64 (2H, t, $CO_2CH_2CH_2$), 2.43 (2H, t, $CO_2CH_2CH_2$), 2.12 (4H, m, CH_2CH_3, H-2(Pip)), 1.82 (2H, d, H-3(Pip)), 1.52 (2H, m, H-3(Pip)), 1.05 (3H, t, CH_2CH_3); ^{13}CNMR ($CDCl_3$) δ (ppm) 172.4 (CO_2), 173.7 (CO), 148.3 (C-3(Pz)), 139.3 (C-*ipso*(C_6H_5Pz)), 129.1 (*m*-(C_6H_5Pz)), 127.6 (C-5(Pz)), 126.5 (*p*-(C_6H_5Pz)), 118.6 (*o*-(C_6H_5Pz)), 107.0 (C-4(Pz)), 53.0 ($CO_2CH_2CH_2$), 52.5 (C-2(Pip)), 51.5 (C-4(Pip), 31.8 ($CO_2CH_2CH_2$), 29.9 (C-3(Pip)), 27.7 (CH_2CH_3), 9.2 (CH_2CH_3). MS (electrospray) [M + 1] 385.3. Anal. ($C_{21}H_{28}N_4O_3$): calcd C 65.60, H 7.34, N 14.57; found C 65.28, H 7.05, N 14.40.

***N*-[1-(4-Fluorophenyl)pyrazol-3-yl]-*N*-(1-benzyl-4-piperidyl) - amine (1c). Procedure B.** 1-Benzyl-4-piperidone (0.88 mL, 4.7 mmol) and $NaBH_3CN$ (170 mg, 2.8 mmol) were mixed in MeOH (50 mL). A HCl–MeOH solution was added dropwise until pH 6. Then a solution of 1-(4-fluorophenyl)-3-aminopyrazole (1.23 g, 7 mmol) in MeOH (100 mL) was added. The reaction mixture was stirred at room temperature for 15 min maintaining the pH at 6 with HCl–MeOH addition. Molecular sieves were added, 10 min later the mixture was filtrated over a Celite bed. The filtrate was evaporated to dryness. The crude product was chromatographed on a flash 40i cartridge [CH_2Cl_2/MeOH (24:1)] to give 1.15 g (70%) of a yellowish solid: mp 58–62 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.55 (1H, d, J = 2.5 Hz, H-5(Pz)),7.46–6.94 (9H, *p*-F C_6H_4 and C_6H_5), 5.74 (1H, d, J = 2.5 Hz, H-4(Pz)), 3.81 (2H, s, $C_6H_5CH_2$), 3.72 (1H, m, H-4(Pip)), 3.10 (2H,m, H-2(Pip)), 2.50 (2H, m, H-2(Pip)), 2.22 (2H, m, H-3(Pip)), 1.85 (2H, m, H-3(Pip)); ^{13}C NMR ($CDCl_3$) δ (ppm) 160.2 (d, $J_{C\text{-}F}$ = 244 Hz, *p*-(*p*-F-C_6H_4)), 156.9 (C-3(Pz)), 136.7 (d, $J_{C\text{-}F}$ = 2.7 Hz, *ipso*-(*p*-FC_6H_4)), 133.6 (*ipso*-($C_6H_5CH_2$)), 130.3, 128.8, 128.6-(C_6H_5), 127.6 (C-5(Pz)), 119.3 (d, $J_{C\text{-}F}$ = 8 Hz, *0*-(*p*-F-C_6H_4)), 116.1 (d, $J_{C\text{-}F}$ = 23 Hz, *m*-(*p*-F-C_6H_4)), 95.2 (C-4(Pz)), 62.1 ($C_6H_5CH_2$), 51.6 (C-2(Pip)), 49.8 (C-4(Pip), 31.0 (C-3(Pip)).

***N*-[1-(4-Fluorophenyl)pyrazol-3-yl]-*N*-(1-benzyl-4-piperidyl) propanamide (3c) (oxalate salt).** Mp 192–193 °C, free base, mp 37–40 °C; ^{1}H NMR (CD_3OD) δ (ppm) 8.45 (1H, d, J = 2.3 Hz, H-5(Pz)), 7.94 (2H, m, (*p*-FC_6H_4), 7.62 (5H, brs, C_6H_5), 7.43 (2H, m, (*p*-FC_6H_4), 6.40 (1H, d, J = 2.3 Hz, H-4(Pz)), 4.94 (1H, m, H-4(Pip), 4.44 (2H, s, $C_6H_5CH_2$), 3.69 (2H, m, H-2(Pip)), 3.33 (2H, H-2(Pip)), 2.39 (2H, q, J = 7.4 Hz, CH_2CH_3), 2.06 (2H, m, H-3(Pip)), 2.01 (2H, m, H-3(Pip)), 1.20 (3H, t, J = 7.4 Hz,CH_2CH_3); ^{13}C NMR (CD_3OD) δ (ppm) 177.0 (C_2O_4), 166.7 (CO), 163 (d, $J_{C\text{-}F}$ = 245 Hz, *p-C*-(*p*-F-C_6H_4)), 149.5 (C-3(Pz)), 136.2 (d, $J_{C\text{-}F}$ = 3 Hz, *ipso*-(*p*-FC_6H_4)), 132.6, 131.4, 130.8, 130.5-(C_6H_5 and C-5(Pz)), 122.7 (d, $J_{C\text{-}F}$ = 8Hz, *0*-(*p*-F-C_6H_4)), 117.5 (d, $J_{C\text{-}F}$ = 24 Hz, *m*-(*p*-F-C_6H_4)), 108.8 (C-4(Pz)), 61.6 ($C_6H_5CH_2$), 53.0 (C-2(Pip)), 51.4 (C-4(Pip), 29.2 (C-3(Pip)), 28.8 (CH_2CH_3), 10.1 (CH_2CH_3). MS (electrospray) [M + 1] 407.3. Anal. ($C_{24}H_{27}N_4OF.C_2H_2O_4$): calcd C 62.90, H 5.84, N 11.29; found C 62.79, H 6.10, N 11.09.

***N*-[1-(4-Fluorophenyl)pyrazol-3-yl]-*N*-[1-(2-phenethyl)-4-piperidyl]-amine (1d).** (Yield, 44%): mp 100–104 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.76 (1H, d,H-5(Pz)), 7.63 (2H, d, *o*-H(C_6H_5Pz)), 7.40 (2H, t, *o*-H(C_6H_5Pz)), 7.32 (6H, m, $C_6H_5CH_2$, *p*-H(C_6H_5Pz)), 5.85 (1H, d, H-4(Pz)), 3.82 (1H, brm, NH), 3.48 (1H, m, H-4(Pip)), 3.03 (2H, d, H-2(Pip)), 2.83 (2H, m, $C_6H_5CH_2CH_2$), 2.62 (2H, m, $C_6H_5CH_2CH_2$), 2.24 (2H, t, H-2(Pip)), 2.21 (2H, t, H-3(Pip)), 1.62 (2H, m, H-3(Pip)); ^{13}C NMR ($CDCl_3$) δ (ppm) 160.1 (d, $J_{C\text{-}F}$ = 244 Hz, *p*-(*p*-F-C_6H_4)), 157.1 (C-3(Pz)), 136.7 (*ipso*-(*p*-FC_6H_4)), 128.9 (*ipso*-($C_6H_5CH_2$)), 128.7, 128.5, 126.2-(C_6H_5), 127.5 (C-5(Pz)), 119.2 (d, $J_{C\text{-}F}$ = 8 Hz, *0*-(*p*-F-C_6H_4)), 115.9 (d, $J_{C\text{-}F}$ = 23 Hz,*m*-(*p*-F-C_6H_4)), 94.9 (C-4(Pz)), 60.3 ($C_6H_5CH_2CH_2$), 52.3 (C-2(Pip)), 50.5(C-4(Pip), 33.3 ($C_6H_5CH_2CH_2$), 32.3 (C-3(Pip)). MS (electrospray) [M + 1] 365.2.

***N*-[1-(4-Fluorophenyl)pyrazol-3-yl]-*N*-[1-(2-phenethyl)-4-piperidyl)]propanamide (3d) (oxalate salt).** Mp 195–205 °C; ^{1}H NMR(CD_3OD) δ (ppm) 8.48 (1H, s, H-5(Pz)), 7.98 (2H, m, *m*-H(*p*-FC_6H_5)), 7.47 (7H, m, *o*-H(*p*-FC_6H_5), $C_6H_5CH_2$), 6.67 (1H, d, H-4(Pz)), 5.0 (1H,

m, H-4(Pip)), 3.88 (2H, d, H-2(Pip)), 3.43 (2H, m, $C_6H_5CH_2CH_2$), 3.39 (2H, m, H-2(Pip)), 3.17 (2H, m, $C_6H_5CH_2CH_2$), 2.39 (2H, q, CH_2CH_3), 2.14 (2H, d, H-3(Pip)), 2.06 (2H, m, H-3(Pip)), 1.24 (3H, t, CH_2CH_3); ^{13}C NMR (CD_3OD) δ (ppm) 176.7 (C_2O_4), 166.6 (CO), 162.9 (d, $J_{C\text{-}F}=245$ Hz, *p*-(*p*-F-C_6H_4)), 149.2 (C-3(Pz)), 137.7 (*ipso*-($C_6H_5CH_2$)), 137.5 (d, *ipso*-(*p*-FC_6H_4)), 129.9, 129.8, 128.2-(C_6H_5), 131.0 (C-5(Pz)), 122.4 (d, $J_{C\text{-}F}=9$ Hz, *0*-(*p*-F-C_6H_4)), 117.3 (d, $J_{C\text{-}F}=23$ Hz, *m*-(*p*-F-C_6H_4)), 108.6 (C-4(Pz)), 58.8 ($C_6H_5CH_2CH_2$), 53.1 (C-2(Pip)), 50.8 (C-4(Pip), 31.4 ($C_6H_5CH_2CH_2$), 29.0 (C-3(Pip)), 28.6 (CH_2CH_3), 9.8 (CH_2CH_3). MS (electrospray) [M + 1] 421.3. Anal. ($C_{25}H_{29}N_4OF.C_2H_2O_4$): calcd C 63.52, H 6.07, N 10.98; found C 63.03, H 6.04, N 10.52.

***N*-(1-Phenylpyrazol-5-yl)-*N*-(1-benzyl-4-piperidyl)-amine (2a).** (Yield, 84%): mp 94–96 °C; ^{1}H NMR($CDCl_3$) δ (ppm) 7.56–7.26 (10H, m, C_6H_5Pz, $C_6H_5CH_2$), 7.52 (1H, brs, H-3(Pz)), 5.52 (1H, H-4(Pz)), 3.63 (1H, d, NH), 3.52 (2H, s, $C_6H_5CH_2$), 3.13 (1H, m, H-4(Pip)), 2.78 (2H, m, H-2(Pip)), 2.08 (4H, m, H-2(Pip), H-3(Pip)), 1.47 (2H, m, H-3(Pip); ^{13}C NMR ($CDCl_3$) δ (ppm) 146.5 (C-5(Pz)), 140.4 (C-3(Pz)),138.0 (*ipso*-C_6H_5), 137.5 (*ipso*-C_6H_5), 124.1, 127.0, 127.3, 128.2, 129.0, 129.5 (*o, m, p*-C_6H_5), 87.7 (C-4(Pz), 62.9 ($C_6H_5CH_2$), 52.5 (C-4(Pip)), 51.9 (C-2(Pip)), 32.3 (C-3(Pip)). MS (electrospray) [M + 1] 333.3.

***N*-(1-Phenylpyrazol-5-yl)-*N*-(1-benzyl-4-piperidyl)-propanamide (4a).** (Yield 65%): mp 45–48 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.63 (1H, d, H-3(Pz)), 7.28 (10H, C_6H_5Pz, $C_6H_5CH_2$), 6.18 (1H, d, H-4(Pz)), 4.30 (1H, m, H-4(Pip)), 3.31 (2H, d, $C_6H_5CH_2$), 2.80 (1H, brd, H-2(Pip)), 2.60 (1H, brd, H-2(Pip)), 2.20–1.95 (5H, m, CH_2CH_3, 1H-3(Pip), 2H-2(Pip)), 1.80 (2H, m, H-3(Pip)), 1.49 (1H, m, H-3(Pip)), 0.99 (3H, t, CH_2CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm) 174.4 (CO), 139.9 (C-3(Pz)), 139.0 (C-5(Pz)), 138.0 (*ipso*($C_6H_5CH_2$)), 137.5 (*ipso*-(C_6H_5Pz)), 129.2 (*m*-(C_6H_5Pz)), 128.9 (*m*-($C_6H_5CH_2$)), 128.1 (*o*-($C_6H_5CH_2$)), 127.4 (*p*-(C_6H_5Pz)), 126.9 (*p*-($C_6H_5CH_2$)), 122.8 (*o*-(C_6H_5Pz)), 107.5 (C-4(Pz)), 62.7($C_6H_5CH_2$), 54.1 (C-4(Pip), 52.6 (C-2(Pip)), 30.1 (CH_2CH_3), 28.8 (C-3(Pip)), 28.4 (C-3(Pip), 9.2 (CH_2CH_3). MS (electrospray) [M + 1] 389.2. Anal. ($C_{24}H_{28}N_4O.C_2H_2O_4$): calcd C 65.26, H 6.32, N 11.71; found C 64.00, H 6.21, N 11.49.

***N*-(1-Phenylpyrazol-5-yl)-*N*-(4-piperidyl)-propanamide (6).** (Yield 66%): mp 198 °C (dec. 145–150 °C); ^{1}H NMR ($CDCl_3$) δ (ppm) 7.63 (1H, d, H-3(Pz)), 7.30 (5H, C_6H_5Pz), 6.21 (1H, d, H-4(Pz)), 4.38 (1H, m, H-4(Pip)), 3.72 (2H, d, $C_6H_5CH_2$), 3.05 (1H, brd, H-2(Pip)), 2.95 (1H, brd, H-2(Pip)), 2.80–2.40 (3H, m, H-(Pip)), 2.11 (1H, q, CH_2CH_3), 2.01 (1H, q, CH_2CH_3), 1.90–1.60 (2H, m, H-(Pip)), 1.44 (1H, qd, H-3(Pip)), 0.99 (3H, t, CH_2CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm) 174.3 (CO), 140.0 (C-3(Pz)), 138.8 (C-5(Pz)), 137.2 (*ipso*-(C_6H_5Pz)), 129.2 (*m*-(C_6H_5Pz), 127.6 (*p*-(C_6H_5Pz)), 122.8 (*o*-(C_6H_5Pz)), 107.3 (C-4(Pz)), 53.5 (C-2(Pip)), 50.9 (C-4(Pip)), 28.4 (CH_2CH_3), 9.14 (CH_2CH_3), 30.1 (CH_2CH_3), 28.8 (C-3(Pip)), 28.4 (C-3(Pip)). MS (electrospray) [M + 1] 299.2.

***N*-(1-Phenylpyrazol-5-yl)-*N*-[1-(2-phenethyl)-4-piperidyl) - propanamide (4b).** (Yield 64%): oxalate, mp 204 °C; ^{1}H NMR($CDCl_3$) δ (ppm) 7.69 (1H, d, H-3(Pz)), 7.30 (10H, C_6H_5Pz, $C_6H_5CH_2$), 6.26 (1H, H-4(Pz), 4.40 (1H, m, H-4(Pip)), 3.00 (2H, d, H-2(Pip)), 2.80 (2H, d, H-3(Pip)), 2.70 (2H, m, ($C_6H_5CH_2CH_2$), 2.48 (2H, m, ($C_6H_5CH_2CH_2$), 2.12 (2H, q, CH_2CH_3), 1.95 (2H, m, H-2(Pip)), 1.60 (2H, m, H-3(Pip)), 1.07 (3H, t, CH_2CH_3)); ^{13}C NMR ($CDCl_3$) δ (ppm) 174.3 (CO), 140.0 (*ipso*-($C_6H_5CH_2$)), 139.9 (C-3(Pz)), 138.9 (C-5(Pz)), 137.2 (*ipso*(C_6H_5Pz)), 129.1 (*m*-(C_6H_5Pz)), 128.4 (*m*-($C_6H_5CH_2$)), 128.2 (*o*-($C_6H_5CH_2$)), 127.4 (*p*-(C_6H_5Pz)), 125.8 (*p*-($C_6H_5CH_2$)), 122.6 (*o*-(C_6H_5Pz)), 107.4 (C-4(Pz)), 60.1 ($C_6H_5CH_2CH_2$), 53.8 (C-4(Pip), 52.6 (C-2(Pip)), 52.5 (C-2(Pip)), 33.6 ($C_6H_5CH_2CH_2$), 30.0 (CH_2CH_3), 28.7 (C-3(Pip)), 28.3 (C-3(Pip)), 9.15 (CH_2CH_3). MS (FAB) [M + 1] 403.2. Anal. ($C_{25}H_{30}N_4O.C_2H_2O_4$): calcd C 65.84, H 6.55, N 11.37; found C 65.02, H 6.51, N 10.97.

Pharmacological assays

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 23 °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 (0.1–20 mg/kg) of the tested compounds or saline solution were intraperitoneally (ip) administered to separated groups of mice ($n \geq 10$) 30 min before the analgesic effect was tested. In order to evaluate the antinociception level, the effect of ip morphine (2.5–10 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 characterized 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. The mean number of writhes in naïve animals was 19.8 ± 0.7 ($n = 12$). This value was not significantly modified after the ip administration of saline slution (19 ± 0.9) ($n = 12$). The effect of the studied compounds was expressed as percentage of modification of this control value.

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 Maximum Possible Effect (MPE):

MPE = (Latency after treatment − Control Latency)/(Cut-off time−Control latency). Control latency was evaluated in each individual experiment, the main value being 10.7 ± 0.3 ($n = 12$).

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 active compounds (0.1–2 mg/kg), and 30 min later either the writhing test or the hot plate test were carried out. The inhibition of morphine (5 mg/kg) antinociception induced by naloxone was used as a control.

Finally, the duration of the analgesic effect of the new compounds was compared with that of fentanyl. The analgesic effect of fentanyl (0.03–0.4 mg/kg), **3b** (0.4–2 mg/kg) and **3d** (0.3–1 mg/kg) was tested 5 min after their ip administration, from these dose–response curves equipotent (85–95% of inhibition of the nociceptive response) doses were selected and the analgesic effect of fentanyl (0.4 mg/kg) was tested 5, 15, 30, 60 and 120 min after its ip administration and the effect of **3b** (2 mg/kg) and of **3d** (1 mg/kg) were tested 5, 30, 60, 120, 240 and 360 min after its ip administration.

The compounds showing antinociceptive activity were tested for their functional activity on isolated tissues commonly used to study and characterize opioid effects (guinea pig ileum, mouse vas deferens and rabbit vas deferens).

In vitro assays

Isolated tissues. Male guinea-pigs weighing 300–450 g, male CD-1 mice weighing 25–30 g and male New Zealand rabbits weighing 3–3.5 kg were used for this study. Myenteric plexus-longitudinal muscle strips (MP-LM) were isolated from guinea-pig ileum as described by Ambache,[36] the rabbit vas deferens was prepared as described by Oka [37] and the mouse vas deferens as described by Hughes.[38] Tissues were suspended in a 20 mL organ bath containing Krebs solution (NaCl 118, KCl4.75; $CaCl_2$ 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 (guinea pig ileum) or 0.5 g (rabbit or mouse vas deferens) of resting tension at 32 °C and were electrically stimulated through two platinum ring electrodes. Guinea-pig MP-LM strips were stimulated with rectangular pulses of 70 V, 0.1 ms duration and 0.3 Hz frequency. Rabbit vas deferens were stimulated with rectangular pulses of 70 V, 0.1 ms duration and 0.1 Hz frequency, and mouse vas deferens with trains of 15 rectangular pulses of 70 V, 15 Hz and 2 ms duration each min. The isometric force was recorded on a Grass model 7A polygraph.

Cumulative concentration–response curves for selective μ, δ or κ opioid receptor agonists or the new antinociceptive compounds (**3b** and **3d**) 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. Curves were constructed for:

1. The μ agonist, morphine (10^{-7}–1.6×10^{-6} M) and for **3b** and **3d** (**3b**: 5×10^{-8}–8×10^{-7} mM; **3d**: 2.5×10^{-8} to 4×10^{-7} M) in guinea-pig MP-LM strips

2. The δ agonist, DEPEN ([D-Pen^2,D-Pen^5]enkephalin) (2×10^{-9}–1.6×10^{-8} M) and for **3b** and **3d** (**3b**: 5×10^{-8}–8×10^{-7} M; **3d**: 2.5×10^{-8}–4×10^{-7} M) in mouse vas deferens

3. The κ agonist, U-50,488H, (10^{-8} M–1.6×10^{-7} M) and for **3b** and **3d** (**3b**: 5×10^{-8}–8×10^{-7} M; **3d**: 2.5×10^{-8}–4×10^{-7} M) in rabbit vas deferens.

The effect of the drugs was evaluated 5 min after the addition of each dose, as % 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 their respective opioid receptors, one dose of selective antagonists was added to the organ bath at the end of each experiment. Naloxone (5×10^{-8} M), nor-binaltorphimine (10^{-8} M) and naltrindole (10^{-9} and 10^{-8} M) were used as μ, κ and δ antagonists, respectively. In mouse vas deferens the effect of active compounds, **3b** (2×10^{-7} M) and **3d** (4×10^{-7} M), was also antagonized by addition of the μ selective antagonist CTOP (10^{-6} M).[30,31]

Binding assays

Neural membranes from whole mice brain. 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 0.83 ± 0.14 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) or [^{3}H]DPDPE (4 nM) for 60 min at 25 °C, or 37 °C in the [^{3}H]DPDPE assays, in the absence or presence of the competing compounds (10^{-16}–10^{-4} M, 14 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.

Human recombinant CHO-KI cells. In vitro affinity of the compounds for μ opioid receptors sites was determined by their ability to displace the specific radioligand ([^{3}H]DAMGO) in human recombinant CHO.K1 cells according to the methods of Wang[23] and Maguire.[24] Average K_i (±SEM) values were calculated from at least three determinations of displacement curves, each consisting of 10 concentrations in triplicate. The inhibition constant K_i were calculated using the equation of Cheng and Prusoff[39] using the observed IC_{50} of the tested compound, the concentration of radioligand employed in the assay, and the historical values for K_d of the ligand.

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. Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

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