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# Structure–activity studies on the nociceptin/orphanin FQ receptor antagonist 1-benzyl-*N*-{3-[spiroisobenzofuran-1(3*H*),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide

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ABSTRACT

Twelve derivatives of the nociceptin/orphanin FQ (N/OFQ) receptor (NOP) antagonist 1-benzyl-*N*-{3-[spiroisobenzofuran-1(3*H*),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide (Comp 24) were synthesised and tested in binding experiments performed on $CHO_{hNOP}$ cell membranes. Among them, a novel interesting NOP receptor antagonist (compound **35**) was identified by blending chemical moieties taken from different NOP receptor ligands. In vitro in various assays, Compound **35** consistently behaved as a pure, highly potent ($pA_2$ in the range 8.0–9.9), competitive and NOP selective antagonist. However compound 35 was found inactive when challenged against N/OFQ in vivo in the mouse tail withdrawal assay. Thus the usefulness of the novel NOP ligand compound 35 is limited to in vitro investigations.



## 1. Introduction

Nociceptin/orphanin FQ (N/OFQ)[1,2] modulates different biological functions via activation of the N/OFQ peptide receptor (NOP).[3] Few non-peptide molecules have been reported to selectively interact with the NOP receptor: these include the NOP agonist Ro 64-6198,[4] and the antagonists J-113397[5] and SB-612111[6] (see Chart 1). However, the chemical synthesis of such molecules, which are characterized by two chiral centres, is extremely complex, time consuming and of low yield, making the availability of these tools to the scientific community very limited.

Recently, a novel non-peptide NOP antagonist, 1-benzyl-*N*-{3-[spiroisobenzofuran-1(3*H*),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide, has been reported by Banyu researchers (as Comp **24**).[7] Comp **24** binds with high affinity ($pIC_{50}$ 9.57) to the human recombinant NOP receptor showing an impressive selectivity (>3000-fold) over classical opioid receptors. Comp **24** behaves as a pure NOP antagonist in the [$^{35}$S]GTPγS assay with very high potency ($pIC_{50}$ 9.82). Moreover, in vivo in mice Comp **24** at 10 mg/kg sc completely reverses the locomotor inhibitory effect elicited by a NOP receptor agonist.[7] Recently this molecule was synthesized and characterized in our laboratories confirming its excellent in vitro pharmacological profile in terms of high antagonist potency and selectivity of action over classical opioid receptors.[8] Moreover, in the mouse tail withdrawal assay, Comp **24** at 10 mg/kg ip did not modify per se tail withdrawal latencies but prevented the pronociceptive and antinociceptive effects of 1 nmol N/OFQ given supraspinally and spinally, respectively.[8] Collectively, these studies demonstrate that Comp **24** behaves as a pure, highly potent, selective and competitive NOP antagonist.

Interestingly, Comp 24 displays some chemical characteristics typical of N/OFQ related peptides (see Chart 1), these include: (i) a spacer of 12 atoms between the two phenyl rings which matches

*Abbreviations:* EtOAc, ethyl acetate; Bn-Cl, benzyl chloride; $Boc_2O$, di-*tert*-butyldicarbonate; BSA, bovine serum albumin; *n*-BuLi, normal butyllithyum; DCC, *N*,*N*′-dicicloexyl-carbodiimide; DCM, methylen chloride; DMF, *N*,*N*′-dimetilformammide; DPN, diprenorphine; $Et_3N$, triethylamine; $Et_2O$, diethylether; EtPt, light petroleum boiling fraction 40–60°; HOBt, 1-hydroxy-benzotriazole; *i*-PrOH, isopropanol; MeOH, methanol; rt, room temperature; NSB, non-specific binding; TBAF, tetrabutylamonium fluoride; TBDMS-Cl, *tert*-butyldimethylsilylchloride; TFA, trifluoro acetic acid; THF, tetrahydrofurane; TLC, thin layer chromatography; WSC, 1-ethyl-(3-dimethyl-amino-propyl)-carbodiimmide hydrochloride.

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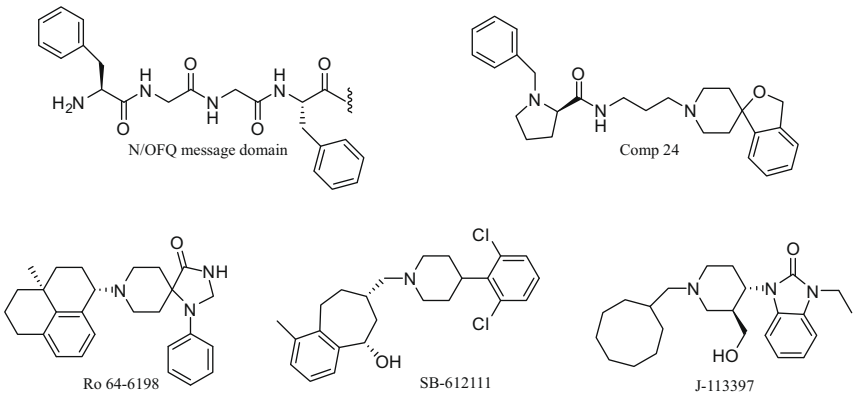
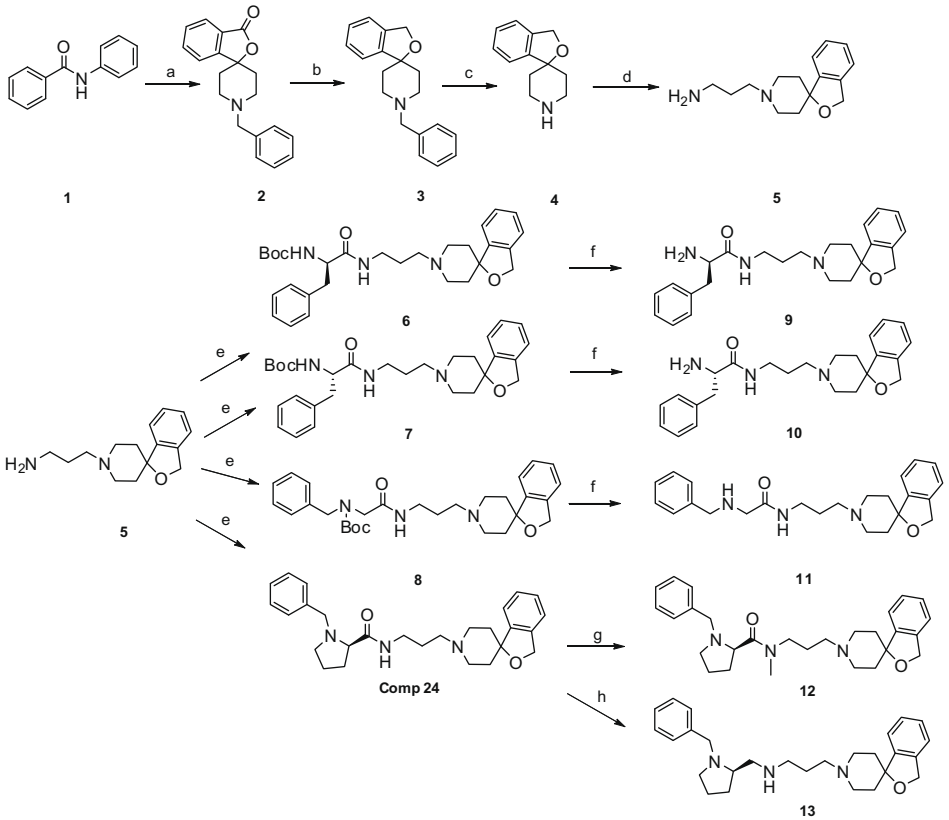



**Chart 1.** Chemical formulae of nociceptin/orphanin FQ receptor ligands.

the Phe-Gly-Gly-Phe sequence of the N/OFQ message domain; (ii) an amide bond which is quite uncommon in non-peptide NOP ligands; (iii) a *N*-benzyl amino acid, a chemical moiety also present in the N-terminal of the NOP receptor peptide antagonists [Nphe[1]]N/OFQ(1-13)-$NH_2$[9] and UFP-101.[10] Based on these considerations, in the present study, the importance of the *N*-benzyl D-Pro of Comp 24 was assessed by replacement with L- or D-Phe, and Nphe. In addition, the amide bond of Comp 24 was substituted with other amide bond isosters. Moreover, the spiroisobenzofurane nucleus of Comp 24 was replaced with chemical moieties derived from other non-peptide NOP ligands that is, Ro 64-6198,[4] SB-612111[6] and J-113397[5] (Chart 1). The novel molecules were evaluated for their ability to bind the human recombinant NOP receptor expressed in CHO cell ($CHO_{hNOP}$) membranes. The molecule with the highest affinity, that is, compound **35** has been further characterized in vitro at the recombinant human NOP in [$^{35}$S]GTPγS binding and calcium mobilization assays and at native NOP receptors expressed in isolated animal (mouse, rat, guinea-pig) tissues. Finally compound **35** was assayed in vivo against the effects elicited by N/OFQ in the mouse tail withdrawal assay.

## 2. Chemistry

Compound **24** was synthesized following procedures reported by Goto et al.[7] The key intermediate **5** (Scheme 1) was obtained starting from commercially available benzanilide **1** that reacts with *n*-butyllithium at −78 °C and subsequentially with the *N*-benzyl-




**Scheme 1.** Reagents and conditions: (a) *n*-BuLi 2.5 M, −78 °C, *N*-benzyl-piperidine-4-one, 0 °C, THF; (b) $(CH_3)_2S^*BH_3$, THF, 0 °C to reflux; (c) $H_2$, C/Pd 10%, EtOH, overnight; (d) *N*-Boc-3-bromo-propyl amine DMF, 60 °C, 3 h, then DCM TFA 1 h; (e) L-Phe, D-Phe, Nphe or *N*-Bn-D-Pro, DMF, HOBt. WSC, 0 °C to rt, 24 h; (f) TFA, 0 °C, 1 h; (g) $(CH_3)_2S^*BH_3$, THF, 0 °C to reflux O.N.; (h) $CH_3I$, NaH, DMF, 0 °C to rt, O.N.

piperidone to give the spiroisobenzofuran-2-one **2**. Compound **2** was reduced with borane dimethyl sulfide complex in THF to give the corresponding isobenzofurane **3**. The deprotection of the piperidine nitrogen using $H_2$ in presence of palladium on charcoal 10% afforded the desired spiroisobenzofurane piperidine **4** in good yield.

Alkylation with *N*-Boc-3-bromo-propyl amine followed by TFA treatment gave the free amine **5.** The substitution in compound 24 of the D-Pro amino acid with the D-Phe, L-Phe and Nphe was achieved using compound **5** and the corresponding protected aminoacid for the coupling reaction, removing the Boc protected group from the amino function with TFA at 0 °C. This allowed us to obtain compounds **9**, **10** and **11** (Scheme 1).

The *N*-methyl alkylation or the reduction of the amide bond of compound **24** produced the final compounds **12** and **13**.

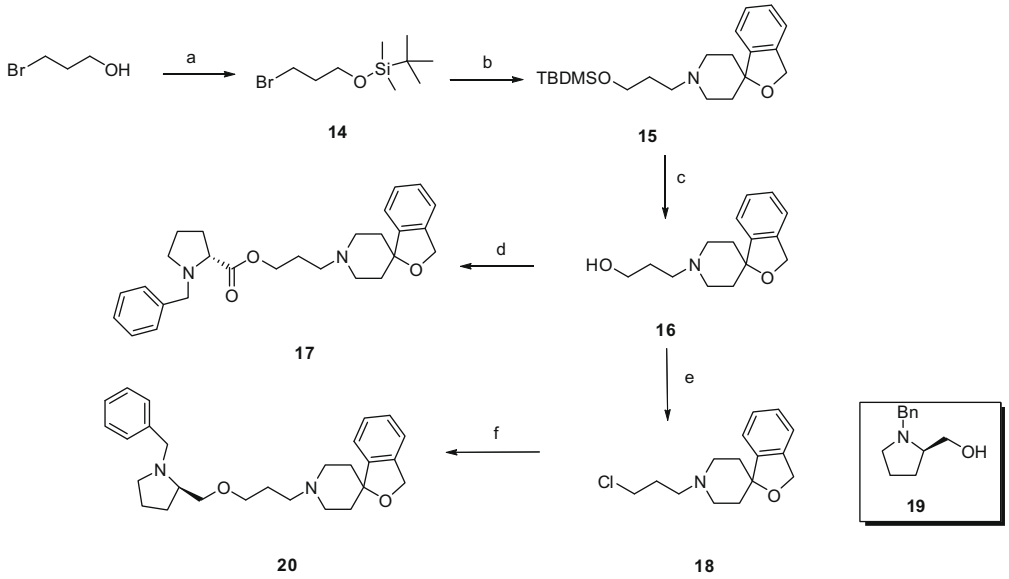
The substitution of the amide bond in Comp 24 with ester or ether bond was achieved following Scheme 2. 3-Bromo-1-propanol, was protected as TBDMS derivative (**14)** and reacted with **4** in DMF at 60 °C in the presence of potassium carbonate to obtain compound **15**. Treatment of **15** with TBAF in THF allows us to obtain the corresponding alcohol **16** which was condensed with the *N*-Bn-D-Pro using the Steglich esterification[11] to obtain the desired ester **17**

All the attempt to prepare the tosylate of **16** using tosyl chloride and $Et_3N$, generated the corresponding chloride **18**, which is employed in the esterification reaction[12] with Bn-D-prolinol **19** using tetrabutyl ammonium iodide as an exchanging agent to obtain the ether **20.**

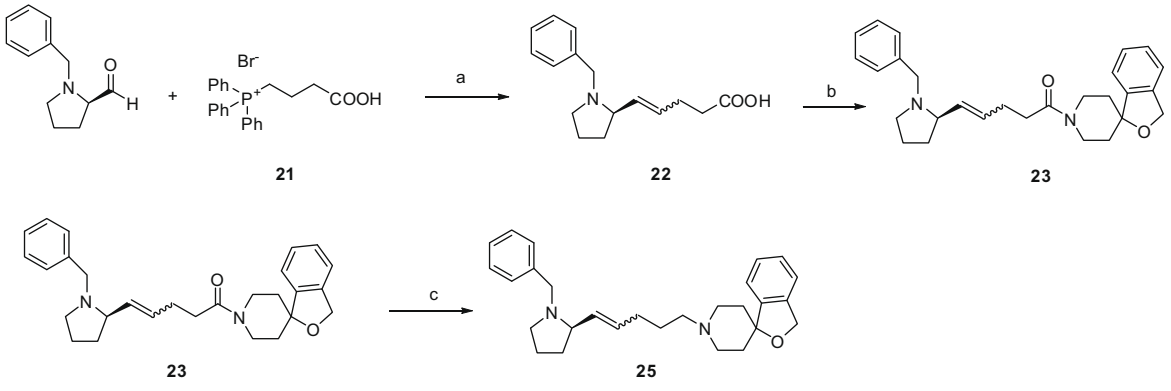
The replacement of the amide bond present in Comp 24 with a olefin moiety was the most difficult procedure, in particular, all the attempts to prepare the desired olefin via Wittig[13] or Horner–Wadsworth–Emmons[14] reaction on the *N*-Bn-Prolinale[15] using the (3-carboxy-propyl)-triphenylphosphonium bromide **21**, failed. In contrast, using the dimethylsulfynil anion[16,17] to generate the corresponding ylide allowed us to obtain the corresponding olefin **22** in moderate yield.

Compound **22** was reacted with **4** in the presence of WSC/HOBt and the amide bond of **23** reduced using borane dimethylsulfide complex to obtain the desired olefin derivative **25** as a inseparable mixture of *E/Z* isomers (Scheme 3).

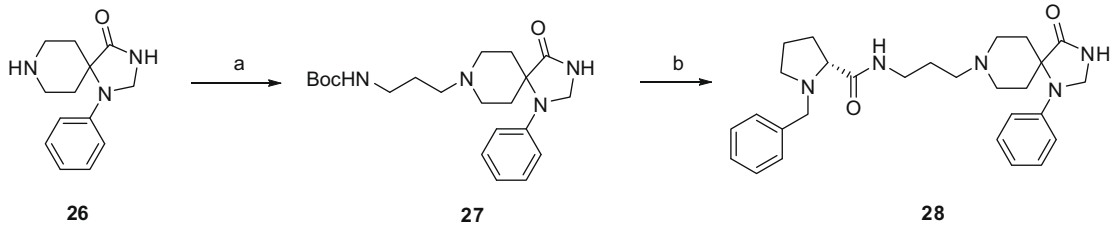
The synthesis of compound **28** (Scheme 4) started from the commercially available 1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one **26** that was alkylated using the conditions previously reported for **5** with *N*-Boc-3-bromo-propyl amine to obtain in a good yield the derivative **27**. Deprotection of *tert*-butoxycarbonil moiety and the coupling with *N*-Bn-D-Pro allowed us to obtain the chimeric compound **28** as depicted in Scheme 4.



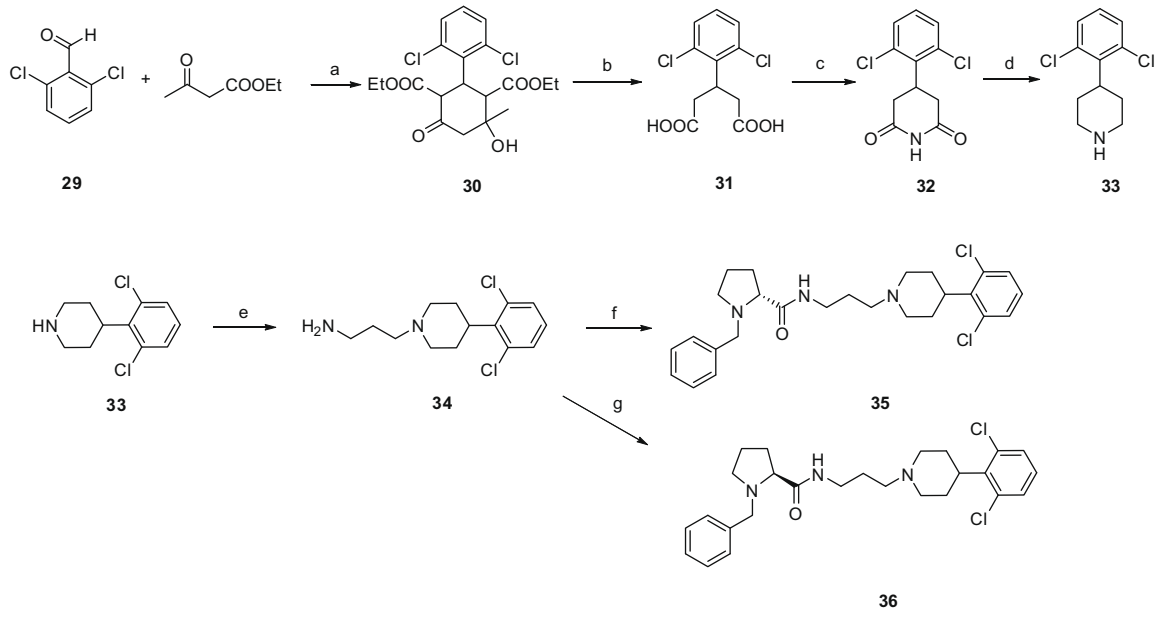

**Scheme 2.** Reagents and conditions: TNDMS-Cl, imidazole, THF, rt, 24 h; (b) (**4**), $K_2CO_3$, DMF, 60 °C, O.N.; (c) TBAF, THF, 24 h; (d) *N*-Bn-D-Pro, HOBt, WSC, DMF, rt, O.N.; (e) tosyl chloride, $Et_3N$, $CH_2Cl_2$, 0 °C to rt, O.N.; (f) (**15**) TBAI, NaH, DMF, 0 °C to rt, O.N.




**Scheme 3.** Reagents and conditions: (a) NaH, DMSO, 77 °C 40 min, than *N*-Bn-D-Pro at 0 °C to rt, O.N.; (b) (**4**) DMF, HOBt, WSC, rt, O.N.; (c) THF, $(CH_3)_2S^* BH_3$, 0 °C to reflux O.N.



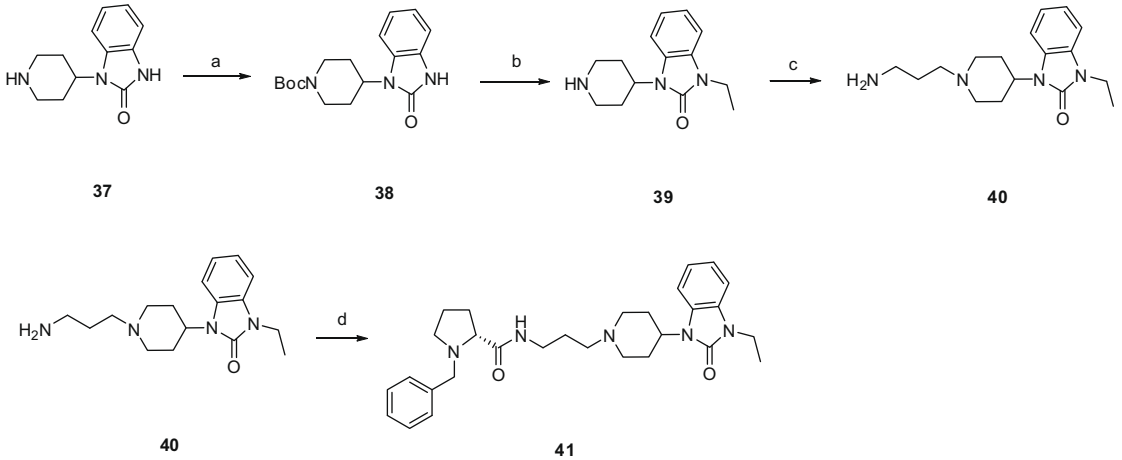
**Scheme 4.** Reagents and conditions: (a) DMF, BocNH-$(CH_2)_3$-Br, $K_2CO_3$, 60 °C, 1 h; (b) (i) TFA, 0 °C, 1 h; (ii) *N*-Bn-D-Pro, DMF, HOBt, WSC, 0 °C to rt, O.N.



**Scheme 5.** Reagents and conditions: (a) EtOH, Pip, rt, O.N.; (b) NaOH 35%, EtOH, reflux, O.N.; (c) $NH_4OH$, 200 °C, 6 h; (d) $(CH_3)_2S^* BH_3$, THF, 0 °C to reflux O.N.; (e) DMF $K_2CO_3$, BocNH-$(CH_2)_2$-Br, 60 °C, O.N., then TFA; (f) DMF, HOBt, WSC, *N*-Bn-D-Pro, 0 °C to rt, O.N.; (g) DMF, HOBt, WSC, *N*-Bn-L-Pro, 0 °C to rt O.N.

The synthesis of the 4-(2,6-dichlorophenyl)-piperidine **33** starts from the reaction of 2,6-dichloro-benzaldehyde **29** with ethyl acetoacetate to give the diester **30**. Hydrolysis under basic conditions of **30** allowed us to obtain the corresponding diacid **31** that was treated with ammonia at 200 °C for 6 h to gave the immide **32**. Reduction using borane dimethyl sulfide complex of **32** gave the desired 4-(2,6-dichloro-phenyl)-piperidine **33** in moderate yield. The final compounds **35** and **36** were prepared using **33** following procedures reported in Scheme 2d (Scheme 5).

The compound **41** was prepared starting from the *tert*-butyloxycarbonyl protection of the commercially available 1-piperidin-4-yl-1,3-dihydro-benzoimidazol-2-one **37**. N3 ethylation of **38** with ethyl



**Scheme 6.** Reagents and conditions: (a) $CH_2Cl_2$, $(Boc)_2O$, DMAP; (b) (i) DMF, EtBr, NaH, 0 °C to rt, 5 h; (ii) TFA, 1 h,rt; (c) (i) DMF, $K_2CO_3$, BocNH-$(CH_2)_3$-Br, 60 °C, 1 h; (ii) TFA, 1 h; (d) DMF, HOBt, *N*-Bn-D-Pro, 0 °C to rt, O.N.

bromide in presence of sodium hydride followed by TFA treatment gave compound **39**. The reaction of **39** with *N*-Boc-3-bromo-propyl amine followed by deprotection and acylation with *N*-Bn-D-Pro allowed us to obtain the final compound **41** (Scheme 6).

Compound **35** was selected as the most interesting molecule of this series. It was obtained with an overall yield of 21%. This value is similar to the overall yield of Comp **24** (25% in our laboratories[8], 31% in Banju laboratories[7]). On the contrary the overall yield of other NOP antagonists such as J-113397 and SB-612111 is much lower (approx. 1–2% in our laboratories). Moreover, the synthesis of the 2,6-dichlorophenyl-piperidine moiety of SB-612111 used for generating compound **35** has been performed avoiding the tedious, money and time consuming chromatographic purifications. In addition, the complete synthesis of compound **35** has been obtained in six steps while those of Comp **24** and of SB-612111 require 8 and 12 steps, respectively.

## 3. Results and discussion

The ability of the reference ligand, Comp **24**, and the novel molecules to bind to the NOP receptor was evaluated using $CHO_{hNOP}$ cell membranes (Table 1). Comp **24** produced a concentration dependent inhibition of [$^3$H]N/OFQ binding with a p$K_i$ value of 9.62. This result perfectly matches with that previously reported by Goto et al.[7] (p$IC_{50}$ 9.57). The substitution of the *N*-benzyl D-Pro with D or L-Phe (compounds **9** and **10**) or Nphe (compound **11**) produced a profound (>100-fold) loss of NOP affinity suggesting a pivotal role of the *N*-benzyl D-Pro moiety for Comp **24** bioactivity. This result is not surprising since the simple inversion of Pro chirality was reported to generate an analogue 200-fold less potent than Comp **24**.[7]

Modifications of the amide bond obtained by N-methylation (compound **12**) or by replacement with a methyleneamino (compound **13**), an ester (compound **17**), a methyleneoxy (compound **20**), or an alkene bond (compound **25**), produced a drastic reduction in NOP receptor binding or, in the case of compound **25**, a complete loss of affinity. These results indicated that the amide bond represents a chemical feature crucial for the bioactivity of Comp 24. It is worthy of note, that an amide bond is a relatively uncommon chemical feature in non-peptide NOP ligands. However, this chemical bond is also present in the NOP receptor antagonist JTC-801 and its chemical modification (i.e., N-methylation and retro-inverso bond) was reported to be detrimental for binding affinity.[18] Thus, the amide bond might play a similar important role in both Comp 24 and JTC-801. In particular, according to the non-peptide NOP ligand pharmacophoric model proposed by Zaveri,[19] the amide bond may be part of the B-moiety that links and contributes to the maintainance of the correct spatial arrangement of the A-moiety (important for ligand affinity and selectivity) and the C-moiety (important for ligand efficacy).

Next, we considered replacement of the benzoisofurane group in position 4 of the Comp 24 piperidine scaffold with chemical moieties taken from the some position of other high affinity NOP receptor ligands. The substitution with 1-phenylimidazolidin-4-one, the chemical group of the NOP receptor agonist Ro 64-6198 (compound **28**), or with 1-ethylbenzoimidazol-2-one, the chemical group of the NOP receptor antagonist J-113397 (compound **41**), in position 4 generated inactive molecules. In contrast, the insertion of the piperidine scaffold of the 2,6-dichlorophenyl moiety (compound **35**), the pharmacophore of the NOP receptor antagonist SB-612111, in position 4 generated a molecule with high affinity for the NOP receptor (p$K_i$ 9.14). In particular compound **35** is only threefold less potent than Comp 24. The results obtained with this limited series of chimeric compounds suggest that it is possible to combine pharmacophoric moieties taken from different NOP receptor ligands to generate novel biologically active molecules. In particular, the chemical groups benzoisofurane and 2,6-dichlorophenyl seem to contribute to NOP receptor binding in a very similar manner. This is corroborated by the finding that the substitution of D-Pro with L-Pro in the chimeric molecule compound **36** produced a 100-fold reduction in receptor affinity. This result is in agreement with compounds **22** and **23** of the Goto series.[7] Thus, D chirality of the Pro residue represents a crucial requirement for high affinity NOP receptor recognition for both Comp 24 and compound **35**.

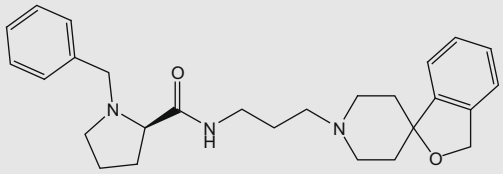
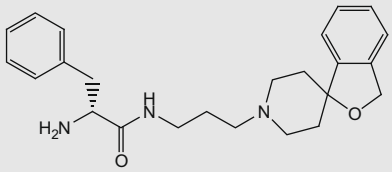
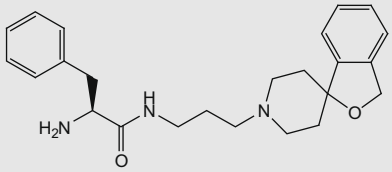
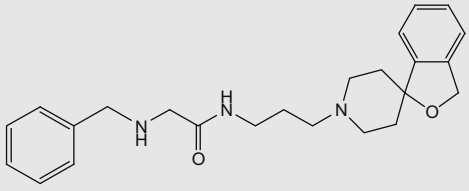
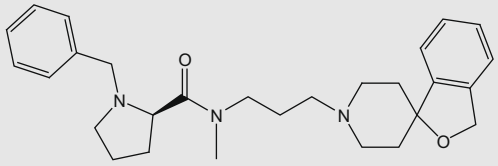
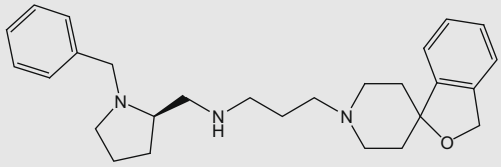
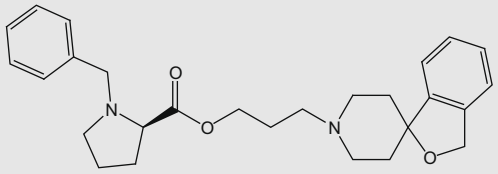
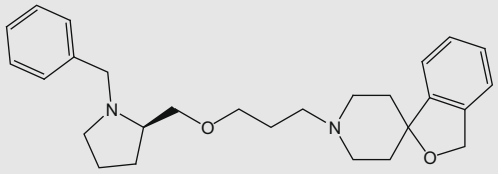
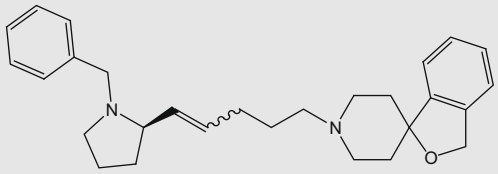
Based on its high affinity for the NOP receptor, compound **35** was selected for further pharmacological characterization in vitro and in vivo. As shown in Figure 1 (top left panel) in $CHO_{hNOP}$ cell membranes N/OFQ concentration dependently stimulated [$^{35}$S]GTPγS binding with p$EC_{50}$ and $E_{max}$ values of 8.91 and 10.93 ± 0.18 (stimulation factor), respectively. Over the concentration range of 1–100 nM, compound **35** was inactive per se but produced a rightward shift of the concentration response curve to N/OFQ in a parallel manner and without modifying the agonist maximal effect. Schild analysis of these data (Fig. 1 top right panel) is compatible with a competitive type of antagonism and a p$A_2$ value of 9.91 was derived. This potency value is close to that previously reported for Comp **24** by Goto et al. (p$IC_{50}$ 9.82)[7] and by us (p$A_2$ 9.98).[8] These results suggest that the benzoisofurane and 2,6-dichlorophenyl moieties of Comp **24** and compound **35** play a similar role not only in receptor binding but also in determining pharmacological activity that is, pure and competitive antagonism. This may be derived from the common ability of the spiro junction (Comp **24**) and of the 2,6-dichloro substitution (compound **35**) to favour an orthogonal spatial disposition between their piperidine and phenyl nuclei. Therefore, this conformational feature may likely be crucial for both NOP receptor binding and antagonist activity.

The pharmacological actions of compound **35** were further assessed at the hNOP receptor coupled to calcium signalling via the chimeric protein Gαqi5; this assay has been previously validated with a large panel of NOP receptor full and partial agonists and antagonists.[20] In CHO cells stably expressing the hNOP receptor and the Gαqi5 protein, N/OFQ produced a concentration dependent stimulation of intracellular calcium with a p$EC_{50}$ of 9.24 and $E_{max}$ of 198 ± 12% over the basal values. Compound **35** was inactive per se up to 10 μM while inhibiting the stimulatory effect of 10 nM N/OFQ in a concentration dependent manner. A p$K_b$ value of 8.47 was derived from these experiments (Table 2). This potency value is approx threefold lower than that obtained with Comp **24** (p$K_b$ 9.03).[8]

The competitive antagonist behaviour of compound **35** was confirmed at the native NOP receptor expressed in the mouse vas deferens. In this preparation, N/OFQ inhibited electrically evoked twitches in a concentration dependent manner with p$EC_{50}$ and $E_{max}$ values of 7.49 and −80 ± 2%, respectively (Fig. 1, right top panel). Compound **35** was inactive per se but caused a concentration dependent (10–1000 nM) and a parallel rightward shift of the concentration response curve to N/OFQ without modifying the agonist maximal effects. The relative Schild plot, depicted in Figure 1 right bottom panel, demonstrated a competitive type of antagonism with a p$A_2$ value of 8.00. This value of potency is close to that previously reported for Comp **24** that is, 8.24.[8] Similar results were obtained in other N/OFQ sensitive preparations such as the rat vas deferens and guinea pig ileum where compound **35** at 100 nM was inactive per se while antagonizing N/OFQ inhibitory actions with p$K_b$ values of 8.06 and 8.84, respectively (Table 2).

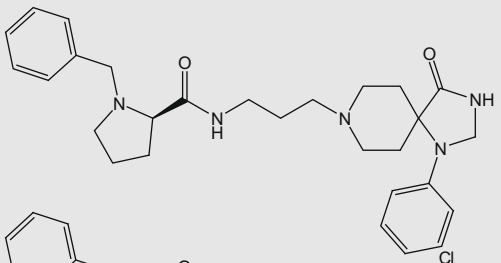
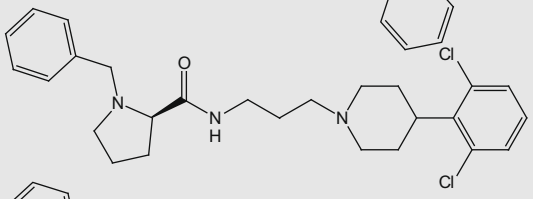
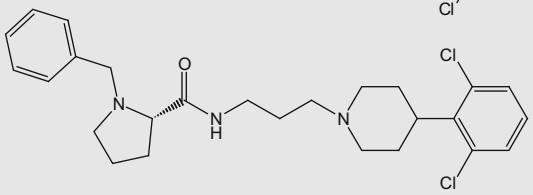
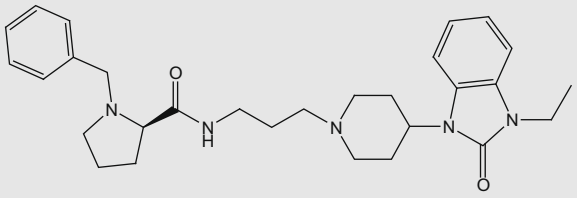
Finally the selectivity of action of compound **35** over classical opioid receptors was assessed in animal tissues expressing native receptors and at recombinant human proteins. Compound **35** at 1 μM was inactive per se and did not modify the inhibitory effects

**Table 1**
Binding affinities at $CHO_{hNOP}$ cell membranes of Comp 24 and related compounds

| Compound | Structure | p$K_i$ |
|---|---|---|
| Comp **24** |  | 9.62 ± 0.07 |
| **9** |  | 7.23 ± 0.19 |
| **10** |  | 7.23 ± 0.05 |
| **11** |  | 7.22 ± 0.08 |
| **12** |  | 5.94 ± 0.28 |
| **13** |  | 7.31 ± 0.06 |
| **17** |  | 6.97 ± 0.02 |
| **20** |  | 6.80 ± 0.80 |
| **25** |  | <5 |

(*continued on next page*)

**Table 1** (*continued*)

| Compound | Structure | p$K_i$ |
|---|---|---|
| **28** |  | <5 |
| **35** |  | 9.14 ± 0.05 |
| **36** |  | 7.08 ± 0.02 |
| **41** |  | <5 |

Data are means ± sem of four separate experiments performed in duplicate.

elicited by the DOP selective agonist DPDPE in the mouse vas deferens (control $pEC_{50}$ 8.38 ($CL_{95\%}$ 8.20–8.56), $E_{max}$ −98 ± 1%; 1 μM compound **35** $pEC_{50}$ 8.44 ($CL_{95\%}$ 8.26–8.62), $E_{max}$ −98 ± 1%) or those produced by the MOP agonist dermorphin in the guinea pig ileum (control $pEC_{50}$ 8.52 ($CL_{95\%}$ 8.35–8.69), $E_{max}$ −90 ± 3%; 1 μM compound **35** $pEC_{50}$ 8.44 ($CL_{95\%}$ 8.19–8.69), $E_{max}$ −85 ± 5%). Results obtained with compound **35** and the universal opioid receptor antagonist naloxone in selectivity studies performed with receptor binding and calcium mobilization assays are summarized in Tables 3 and 4, respectively. Compound **35** up to 10 μM did not displace [$^3$H]DPN from DOP sites and showed very low affinity (less than 300-fold compared to NOP) at MOP and KOP sites (Table 3). In contrast, naloxone did not bind to the NOP receptor up to 10 μM, while it displaced [$^3$H]DPN at classical opioid receptors showing very high affinity for MOP and lower affinities for KOP and DOP (Table 3). Similar results were found in functional studies measuring calcium mobilization in CHO cells stably expressing the hNOP receptor or classical opioid receptors and the Gαqi5 protein (Table 4). Dermorphin, DPDPE and dynorphin A were used in these experiments as agonists for MOP, DOP and KOP receptors, respectively; they produced a concentration dependent stimulation of intracellular calcium with the following $pEC_{50}$ values: 7.93 ($CL_{95\%}$ 7.67–8.19), 8.82 ($CL_{95\%}$ 8.43–9.21) and 8.47 ($CL_{95\%}$ 8.16–8.78). Naloxone inhibited the effects of these agonists with higher potency at MOP than KOP and DOP while being inactive against N/OFQ (Table 4). Thus, compound **35** was at least 300-fold less potent at classical opioid receptors than at the NOP receptor (Table 4).

These in vitro results demonstrated that compound **35** behaves as a pure, potent and competitive NOP receptor antagonist. Moreover selectivity studies indicated that the substitution of the benzoisofurane with the 2,6-dichlorophenyl moiety not only allows maintenance of high affinity and antagonist potency but also of high selectivity over classical opioid receptors.

Finally the pharmacological activity of compound **35** was assessed in vivo using the mouse tail withdrawal assay. In this test N/OFQ was reported to elicit opposite effects depending on the route of administration: the peptide induced pronociceptive actions when injected intracerebroventricularly (icv) while antinociceptive effects were reported after intrathecal (i.t.) injection (see for reviews[3,21] and for results obtained in our laboratories[22,23]). As shown in Figure 2 left panel, mice injected with saline displayed similar tail withdrawal latencies (≈6 s) over the time course of the experiment. N/OFQ 1 nmol given icv produced a clear but short lasting pronociceptive effect. Compound **35** at 10 mg/kg given intraperitoneally 30 min before icv injection, did not modify tail withdrawal latencies per se and did not affect the pronociceptive action of the peptide. When 1 nmol N/OFQ was given i.t., the peptide elicited a robust antinociceptive effect (Fig. 2 right panel). Again compound **35** at 10 mg/kg did not modify the effect of N/OFQ. Thus, in the mouse tail withdrawal assay compound **35** at 10 mg/kg was found inactive against the pronociceptive and antinociceptive effects of N/OFQ injected supraspinally and spinally, respectively. Under these same experimental conditions Comp 24 at 10 mg/kg significantly counteracted the actions of N/OFQ[8], while SB-612111 already at 1 mg/kg fully prevented the effects of the peptide.[24] Thus, despite a very similar in vitro pharmacological profile, the three NOP antagonists displayed very different in vivo potency that is, SB-612111 > Comp 24 ≫ compound **35**. Although the reasons for such variable in vivo potency are at present unknown, they might be due to

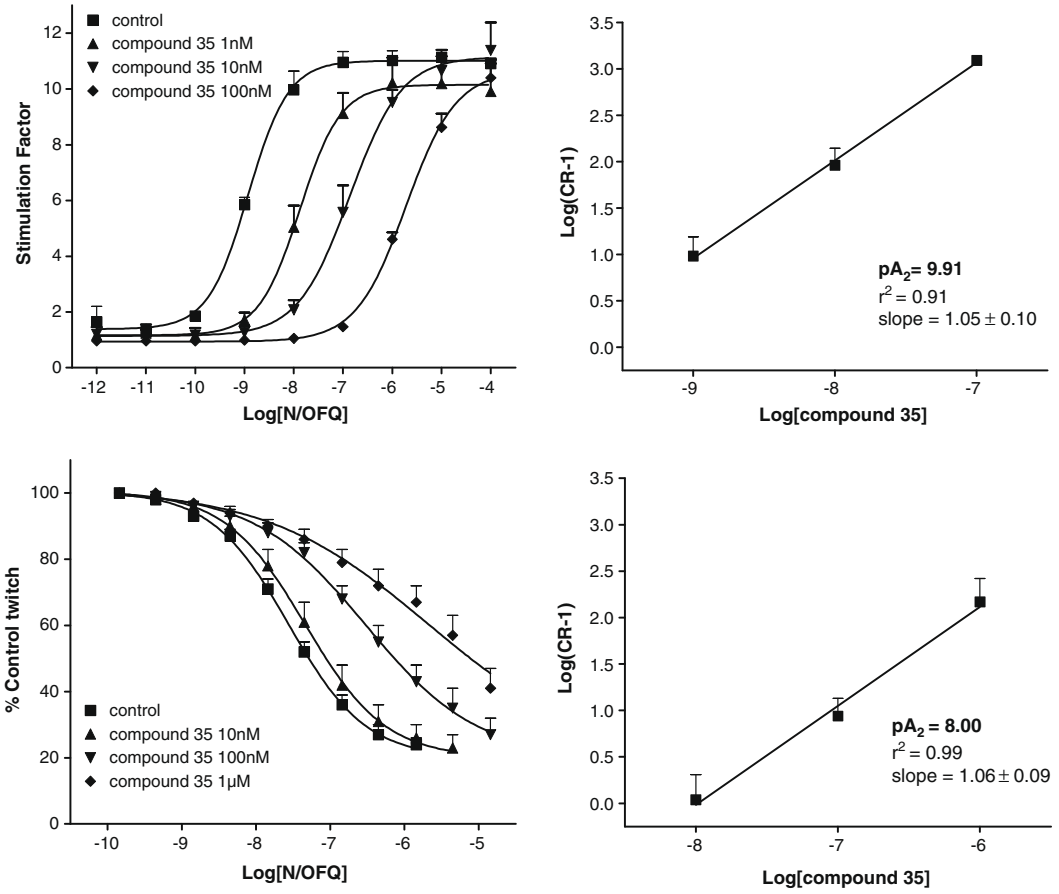



**Figure 1.** Concentration–response curve to N/OFQ in the absence and presence of increasing concentrations of compound **35** for [$^{35}$S]GTPγS binding to $CHO_{hNOP}$ cell membranes (top left panel) and in electrically stimulated mouse vas deferens (bottom left panel). The corresponding Schild plots are shown in the right panels. Data are means ± SEM of four separate experiments.

**Table 2**
Compound **35** affinity/antagonist potency in various pharmacological assays

| | $CHO_{hNOP}$ cell membranes | | $CHO_{hNOP/G\alpha qi5}$ cells | Electrically stimulated tissues | | |
|---|---|---|---|---|---|---|
| | Receptor binding | [$^{35}$S]GTPγS binding | $Ca^{2+}$ mobilization | Mouse vas deferens | Rat vas deferens | Guinea pig ileum |
| Compound **35** | 9.14 (9.04–9.24) | 9.91 (9.23–10.59) | 8.47 (8.31–8.63) | 8.00 (7.32–8.68) | 8.06 (7.58–8.54) | 8.84 (8.64–9.04) |

Data are expressed as means ($CL_{95\%}$) of at least four separate experiments.

**Table 3**
Affinities of compound **35** and naloxone at NOP and classical opioid receptors expressed in CHO cell membranes

| Receptor radioligand | NOP [$^3$H]N/OFQ | MOP [$^3$H]DPN | DOP [$^3$H]DPN | KOP [$^3$H]DPN |
|---|---|---|---|---|
| Naloxone | <6 | 9.25 (9.04–9.46) | 7.67 (7.59–7.75) | 8.35 (8.20–8.50) |
| Compound **35** | 9.14 (9.04–9.24) | 6.72 (6.47–6.97) | <6 | 6.50 (6.37–6.63) |

Data are expressed as mean ($CL_{95\%}$) of four separate experiments. Naloxone data at classical opioid receptors are obtained from Vergura et al.[33]

**Table 4**
Antagonist potencies of compound **35** and naloxone evaluated in calcium mobilization experiments performed in CHO cells expressing NOP or classical opioid receptors and the $G\alpha_{qi5}$ protein

| Receptor agonist | NOP N/OFQ 10 nM | MOP dermorphin 100 nM | DOP DPDPE 100 nM | KOP dynorphin A 100 nM |
|---|---|---|---|---|
| Naloxone | <6 | 9.09 (8.73–9.45) | 7.32 (6.80–7.84) | 7.14 (6.60–7.68) |
| Compound **35** | 8.47 (8.31–8.63) | 6.11 (5.92–6.30) | <6 | <6 |

Data are expressed as mean ($CL_{95\%}$) of four separate experiments performed in duplicate.

marked differences in pharmacokinetics between the three molecules. Therefore the blending of Comp 24 and SB-612111 chemical moieties to generate compound **35** did not affect pharmacodynamics (i.e., receptor affinity, antagonist potency and selectivity of action) while it seems to have a detrimental effect on pharmacokinetics.

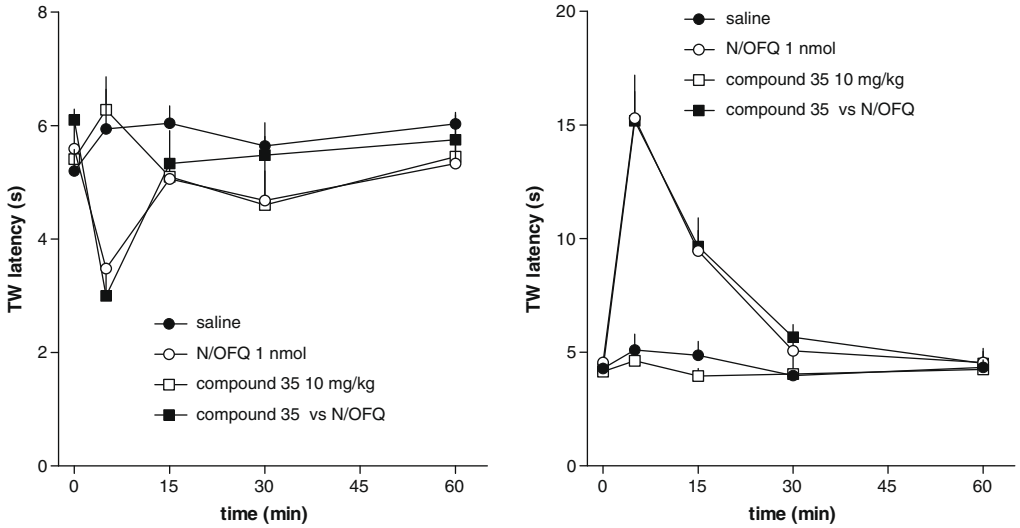



**Figure 2.** Mouse tail withdrawal assay. Effects of compound **35** (10 mg/kg ip, 30 min pre-treatment) on the pronociceptive or antinociceptive effects induced by 1 nmol N/OFQ injected icv (left panel) or i.t. (right panel). Data are mean ± SEM of four separate experiments.

## 4. Conclusions

The present study demonstrated that the *N*-benzyl D-Pro moiety as well as the amide bond of Comp 24 is essential for biological activity. In contrast the spirobenzoisofurane can be replaced with 2,6-dichlorophenyl moiety without significant loss of ligand potency, antagonist activity and selectivity of action. Thus by blending chemical moieties taken from known molecules (Comp 24 and SB-612111) we were able to identify compound **35** as a novel non-peptide selective NOP antagonist. While the in vitro pharmacological profile of compound **35** is similar to that of Comp 24 and SB-612111, the in vivo activities of these compounds are substantially different with the following order of antagonist potency SB-612111 > Comp 24 ≫ compound **35**. This limits the usefulness of compound **35** to in vitro investigations.

## 5. Experimental

### 5.1. Materials

Melting points (uncorrected) were measured with a Buchi-Tottoli apparatus, and $^{1}H$, $^{13}C$, and NMR spectra were recorded on a VARIAN400 MHz instrument unless otherwise noted. Chemical shifts are given in ppm ($\delta$) relative to TMS and coupling constants are in hertz. MS analyses were performed on a ESI-Micromass ZMD 2000. Infrared spectra were recorded on a Perkin–Elmer FT-IR Spectum 100 spectrometer. Flash chromatography was carried out on a silica gel (Merck, 230–400 Mesh). Elemental analyses were performed at the analytical laboratories of the Department of Chemistry, University of Ferrara.

### 5.2. Synthetic procedure

#### 5.2.1. 1′-Benzyl-spiro[isobenzofuran-1(3*H*),4′-piperidin]-3-one (2)

To a stirred solution of **1** (5 g, 25.38 mmol) in anhydrous THF at −78 °C and in argon atmosphere, *n*-BuLi (25.98 mL, 64.97 mmol) was added drop wise. The reaction was warmed at 0 °C for 1 h until a red-orange color appeared. At this time, *N*-benzyl-piperidone (10.68 mL, 57.61 mmol) was added and the reaction stirred at room temperature overnight. The reaction was checked by TLC (EtOAc/light petroleum, 3:2), quenched with HCl 3 N and the aqueous layer partitioned in chloroform and extracted three times with chloroform (30 mL each). The organic layer was dried and concentrated in vacuo to afford a yellow solid after crystallization with $Et_2O$ (6.54 g, 22.345 mmol, yield 88%). mp = 210–215 °C. MS (ESI): $[MH]^+ = 294$. $^{1}H$ NMR ($CDCl_3$): $\delta$ 7.872 (td, 1H, $J = 8$, 0.9 Hz); 7.660 (dt, 1H, $J = 7.4$, 1.2 Hz); 7.51 (dt, 1H, $J = 7.4$, 0.8 Hz); 7.42 (td, 1H, $J = 7.6$, 0.7 Hz); 7.38–7.31 (m, 4H); 7.29–7.25 (m, 1H); 3.62 (s, 2H); 2.92 (dd, 2H, $J = 9$, 2.2 Hz); 2.56 (dt, 2H, $J = 12$, 2.4 Hz); 2.23 (dt, 2H, $J = 13.2$, 4.4 Hz); 1.71 (dd, 2H, $J = 14.4$, 2.4 Hz). $^{13}C$ NMR ($CDCl_3$): 168.80; 151.37; 135.09; 131.33 (2C); 130.36; 130.21; 129.53 (2C); 126.14 (2C); 124.80; 121.79; 81.56; 61.31; 48.92 (2C); 33.02 (2C).

#### 5.2.2. 1′-Benzyl-spiro[isobenzofuran-1(3*H*),4′-piperidine] (3)

Under argon atmosphere 6.54 g (22.34 mmol) of compound **2** were suspended in anhydrous THF, the reaction mixture cooled at 0 °C and under vigorously stirring $(CH_3)_2S^{*}BH_3$ (4.30 mL, 44.68 mmol) was added drop wise. The reaction mixture was heated at reflux overnight. After this time the reaction was acidified (HCl 10%) until pH 2 and heated again at reflux for 4 h. The reaction was quenched with NaOH 2 N until pH 12, the THF was removed in vacuo and the aqueous layer extracted twice with EtOAc (30 mL each). The organic layer was dried, concentrated in vacuo and purified by flash chromatography (EtOAc/light petroleum, 1:1) to afford compound **3** in 55% yield. MS (ESI): $[MH]^+ = 280$. $^{1}H$ NMR ($CDCl_3$): $\delta$ 7.39–7.15 (m, 9H); 5.07 (s, 2H,); 3.60 (s, 2H); 2.85 (dd, 2H, $J = 10$, 2 Hz); 2.44 (dt, 2H, $J = 12$, 2.4 Hz); 2.01 (dt, 2H, $J = 13.2$, 4.4 Hz); 1.76 (dd, 2H, $J = 14.4$, 2.4 Hz). $^{13}C$ NMR ($CDCl_3$): 145.78; 138.98 (2C); 129.43 (2C); 128.28 (2C); 127.59; 127.38; 127.10; 121.10; 120.89; 84.81; 70.78; 63.54; 50.21 (2C); 36.64 (2C).

#### 5.2.3. Spiro[isobenzofuran-1(3*H*),4′-piperidine] (4)

A solution of **3** (3.43 g, 12.29 mmol) in 200 mL of ethanol was hydrogenated in the Parr apparatus (50 psi) in the presence of 10% palladium on charcoal (0.1 g) for 48 h. Filtration of the catalyst through a Celite pad and solvent evaporation gave (**4**) (2.20 g, 11.64 mmol, yield 70%) as a colorless oil. MS (ESI): $[MH]^+ = 190$. $^{1}H$ NMR ($CDCl_3$): $\delta$ 7.31–7.28 (m, 2H); 7.23–7.21 (m, 2H); 5.79 (br, 1H); 5.06 (s, 2H); 3.40–3.87 (m, 2H); 2.75–2.65 (m, 2H); 2.37–2.28 (m, 2H); 1.83–1.81 (m, 2H).

#### 5.2.4. 3-[Spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]] propylamine (5)

To a stirred solution of **4** (2 g, 10.58 mmol) in DMF a solution of *N*-Boc-3-bromo-propyl-amine (2.99 g, 12.59 mmol) in 10 mL of DMF was added followed by addition of $K_2CO_3$ (2.92 g, 21.16 mmol); the reaction was warmed at 60 °C for 1 h. The reaction was concentrated and the residue was diluted with EtOAc and washed twice with $NH_4Cl$ saturated solution (30 mL each) and brine (30 mL). The organic layer was dried and evaporated under reduced pressure. The crude material (3.45 g, 9.99 mmol) was treated with TFA at 0 °C for 1 h. NaOH 2 N was added until pH 12–14, the aqueous phase was extracted several times with EtOAc and the organic phases were combined, dried and evaporated under vacuum to give compound **5** (2.32 g, 9.417 mmol, 95% yield). MS (ESI): $[MH]^+ = 247$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.27–7.24 (m, 2H); 7.20–7.14 (m, 2H); 5.06 (s, 2H); 2.89 (dd, 2H, $J = 9.4$, 2 Hz); 2.81 (t, 2H, $J = 6.8$ Hz); 2.50 (t, 2H, $J = 7.2$ Hz); 2.38 (t, 2H, $J = 12.4$ Hz); 2.23 (br, 2H); 1.98 (dt, 2H, $J = 12.4$, 4.4 Hz); 1.79–1.70 (m, 4H).

#### 5.2.5. (D)-1-{3-[Spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]-propylcarbamoyl}-2-phenyl-ethyl)-carbamic acid-*tert*-butyl estere (6)

To a stirred solution of Boc-D-Phe-OH (539 mg, 2.032 mmol) in DMF at 0 °C, HOBt (373 mg, 2.44 mmol) and WSC (467 mg, 2.44 mmol) were added and the reaction stirred for 10 min. After this time compound **5** (500 mg, 2.032 mmol) dissolved in DMF (20 mL) was added and the reaction mixture stirred for 24 h. The DMF was removed in vacuo and the product partitioned between EtOAc and $NaHCO_3$ 5%. The organic phase was dried, concentrated in vacuum and the crude product purified by flash chromatography (EtOAc/$NH_4OH$, 10:0.3) to give compound **6** in 30% yield. MS (ESI): $[MH]^+ = 494$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.42 (br, 1H); 7.28–7.15 (m, 9H); 5.83 (br, 1H); 5.03 (s, 2H); 3.43 (s, 1H); 3.55–3.16 (m, 3H); 3.08–2.95 (m, 2H); 2.90–2.80 (m, 3H); 2.50–2.44 (m, 4H); 1.76–1.68 (m, 4H); 1.36 (s, 9H). $^{13}C$ NMR ($CDCl_3$): 173.02; 171.38; 144.70; 138.62; 136.946; 129.35 (2C); 128.63 (2C); 128.54; 127.83; 127.56; 126.82; 121.05 (2C); 83.92; 70.90; 60.41; 56.31; 50.498; 49.88; 39.06; 38.38; 35.82; 35.72; 28.29 (3C); 24.67. $[\alpha]_D^{20} = -8$ ($c = 0.1$ g/100 mL, chloroform).

#### 5.2.6. (L)-1-{3-[Spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]-propylcarbamoyl}-2-phenyl-ethyl)-carbamic acid-*tert*-butyl estere (7)

This product was obtained as reported for **6** using Boc-Phe-OH instead of Boc-D-Phe-OH. The crude product was purified by flash chromatography (EtOAc/$NH_4OH$, 10:0.3) to give compound **7** in 30% yield. MS (ESI): $[MH]^+ = 494$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.42 (br, 1H); 7.28–7.15 (m, 9H); 5.83 (br, 1H); 5.03 (s, 2H); 3.43 (s, 1H); 3.55–3.16 (m, 3H); 3.08–2.95 (m, 2H); 2.90–2.80 (m, 3H); 2.50–2.44 (m, 4H); 1.76–1.68 (m, 4H); 1.36 (s, 9H). $^{13}C$ NMR ($CDCl_3$): 173.02; 171.38; 144.70; 138.62; 136.946; 129.35 (2C); 128.63 (2C); 128.54; 127.83; 127.56; 126.82; 121.05 (2C); 83.92; 70.90; 60.41; 56.31; 50.498; 49.88; 39.06; 38.38; 35.82; 35.72; 28.29 (3C); 24.67. $[\alpha]_D^{20} = +8$ ($c = 0.1$ g/100 mL, chloroform).

#### 5.2.7. Benzyl-({3-[spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]-propylcarbamoil}-methyl)-carbamic acid *tert*-butyl estere (8)

To stirred solution of (benzyl-*tert*-butoxycarbonyl-amino)acetic acid (200 mg, 0.754 mmol) in DMF, at 0 °C, HOBt (138 mg, 0.905 mmol) and WSC (173 mg, 0.905 mmol) were added and the reaction stirred for 10 min. After this time compound **5** (188 mg, 0.754 mmol) dissolved in DMF (20 mL) was added and the reaction mixture stirred for 24 h. The DMF was removed in vacuo and the product partitioned between EtOAc and $NaHCO_3$ 5%. The organic phase was dried, concentrated in vacuum and the crude product purified by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9.5:0.5:0.3) to give compound **8** in 22% yield. MS (ESI): $[MH]^+ = 494$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.31–7.18 (m, 9H); 5.05 (s, 2H); 4.58–4.43 (m, 6H); 3.82–3.76 (m, 4H); 3.46–3.32 (m, 4H); 2.98–2.65 (m, 4H); 2.38 (br, 1H); 1.45 (s, 9H). $^{13}C$ NMR ($CDCl_3$): 169.96; 156.34; 143.97; 138.51; 137.65; 128.68; 128.13; 128.00; 127.76; 127.46; 127.08; 121.14 (2C); 84.25; 80.79; 79.92; 71.13; 54.86; 51.67; 50.78; 50.28; 49.56; 36.96; 34.53; 28.40 (3C); 24.46; 14.25.

#### 5.2.8. (D)-2-Amino-*N*-{3-[spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]]-propyl}-3-phenyl-propyl-amide (9)

Compound **6** (160 mg, 0.324 mmol) was dissolved in TFA (5 mL) at room temperature for 1 h, after cooling at 0 °C the reaction was basified with NaOH 2 N until pH 13 and the aqueous layer was extracted three times with EtOAc (20 mL each). The solvent was evaporated under reduced pressure and the crude material was purified by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9.5/0.5/0.3) to give compound **9** as oil in quantitative yield. MS (ESI): $[MH]^+ = 394$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.27–7.19 (m, 9H); 5.06 (s, 2H); 3.61–3.20 (m, 6H); 2.98–2.65 (m, 4H); 2.54–2.38 (m, 5H); 1.80–1.73 (m, 5H). $^{13}C$ NMR ($CDCl_3$): 174.36; 145.72; 140.72; 138.88; 138.082; 129.40; 128.73; 127.73; 127.48; 126.83; 121.18; 120.80; 84.43; 70.89; 60.48; 57.11; 56.92; 50.371; 41.42; 38.48; 36.34; 25.89; 21.15; 14.27. $[\alpha]_D^{20} = +15$ (c = 0.2 g/100 mL, chloroform). Anal. ($C_{24}H_{31}N_3O_2$) C, H, N.

#### 5.2.9. (L)-2-Amino-*N*-{3-[spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]]-propyl}-3-phenyl-propyl-amide (10)

Compound **7** was treated as for obtaining **9** to give compound **10** in quantitative yield. MS (ESI): $[MH]^+ = 394$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.27–7.19 (m, 9H); 5.06 (s, 2H); 3.61–3.20 (m, 6H); 2.98–2.65 (m, 4H); 2.54–2.38 (m, 5H); 1.80–1.73 (m, 5H). $^{13}C$ NMR ($CDCl_3$): 174.36; 145.72; 140.72; 138.88; 138.08; 129.40; 128.73; 127.77; 127.48; 126.83; 121.18; 120.80; 84.43; 70.89; 60.48; 57.11; 56.92; 50.37; 41.42; 38.48; 36.34; 25.89; 21.15; 14.27. $[\alpha]_D^{20} = -15$ ($c = 0.2$ g/100 mL, chloroform). Anal. ($C_{24}H_{31}N_3O_2$) C, H, N.

#### 5.2.10. 2-Benzylamino-*N*-{3-[spiro[isobenzofuran-1(3*H*),4'-piperidin-1-yl]]-propyl}-acetamide (11)

Compound **8** was treated as for obtaining **9** to give **11** in quantitative yield. MS (ESI): $[MH]^+ = 394$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.35–7.19 (m, 9H); 7.20–7.19 (m, 1H); 5.05 (s, 2H); 3.80 (s, 2H); 3.32–3.29 (m, 5H); 3.25–3.21 (m, 2H); 2.94–2.82 (m, 4H); 2.19–2.10 (m, 2H); 1.91–1.82 (m, 4H). $^{13}C$ NMR ($CDCl_3$): 173.72; 145.30; 140.09; 139.72; 129.68 (2C); 129.61 (2C); 129.24; 128.67; 128.55; 122.34; 121.68; 84.28; 71.90; 56.31; 54.06; 51.59; 50.92 (2C); 37.77; 35.85 (2C); 26.44. Anal. ($C_{24}H_{31}N_3O_2$) C, H, N.

#### 5.2.11. (D)-1-Benzyl-pirrolidin-2-carboxilic acid {3-[spiro[isobenzofuran-1(3*H* ),4′-piperidin-1-yl]]-propyl}-methyl-amide (12)

A solution of the Comp **24** (200 mg, 0.460 mmol) in DMF (10 mL) was added was added to a stirred suspension of sodium hydride 60% (20.276 mg, 0,5069 mmol) in DMF (5 mL) dropwise at 0 °C. The reaction mixture was left in the same conditions for half an hour, then a solution of methyl iodide (38 μl, 0.599 mmol) in DMF (1 mL) was added. After 24 h at room temperature, the reaction was monitored by TLC (EtOAc/MeOH/$NH_3$ 9.5:0.5:0.3). The DMF was removed under vacuum and the residue was diluted with Brine (5 mL) and extracted thrice with EtOAc. The organic layers were combined, dried and evaporated under reduced pressure to give a crude product as a yellow oil. This was purified by flash chromatography to obtain compound **12** (53 mg, 0.118 mmol, yield 26%). MS (ESI): $[MH]^+ = 448$. $^1H$ NMR ($CDCl_3$): $\delta$ 7.80–7.25 (m, 9H); 5.07 (s, 2H); 3.78–3.29 (m, 10H); 2.95–2.87 (m, 4H); 2.56–2.12 (m,

6H); 2.06–1.69 (m, 6H). $^{13}$C NMR ($CDCl_3$): 175.37; 171.47; 162.61; 141.82; 138.36; 138.25; 132.65; 129.390; 129.22; 128.70; 128.06; 127.54; 121.42; 81.22; 81.05; 71.47; 67.08; 66.69; 59.985; 58.04; 54.63; 45.77; 36.56; 36.25; 36.12; 31.45; 30.97; 24.18. Anal. ($C_{28}H_{37}N_3O_2$) C, H, N.

#### 5.2.12. (1-Benzyl-pirrolidin-2-yl-methyl)-{3-[spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]]-propyl}-amine (13)

In a two neck round bottom flask, under argon atmosphere, Comp 24 (150 mg, 0.346 mmol) was suspended in anhydrous THF (50 mL) and $(CH_3)_2S^*BH_3$ (100 μl, 1.038 mmol) was added drop wise. The reaction was heated at reflux overnight and monitored by TLC (EtOAc/light petroleum/$NH_3$ 9:1:0.3). The mixture was cooled at 0 °C and acidified with HCl 10%. Then it was heated again at reflux and, after 4 h, it was cooled at 0 °C and basified with NaOH 2 N. THF was evaporated under reduced pressure and the remaining aqueous layer was extracted three times with EtOAc. The organic layers were combined, dried and evaporated under vacuum to obtain the crude product, which was purified by flash chromatography to give compound **13** (20 mg, 0.239 mmol, yield 69%). MS (ESI): $[MH]^+$ = 420. $^1$H NMR ($CDCl_3$): $\delta$ 7.34–7.23 (m, 9H); 5.05 (s, 2H); 3.84–3.59 (m, 1H); 3.70–3.65 (m, 2H); 3.59 (m, 1H); 2.99–2.87 (m, 4H); 2.61–2.35 (m, 8H); 2.06–1.92 (m, 2H); 1.83–1.79 (m, 4H); 1.71–1.65 (m, 4H). $^{13}$C NMR ($CDCl_3$): 145.18; 139.28; 138.99; 128.92; 128.59; 127.81; 127.49; 127.43; 121.23; 120.69; 84.31; 70.83; 62.87; 61.88; 59.69; 57.89; 55.14; 51.69; 50.99; 49.85; 49.74; 36.28; 36.11; 29.96; 29.06; 24.14; 23.29. Anal. ($C_{27}H_{37}N_3O$) C, H, N.

#### 5.2.13. (3-Bromo-propyloxi)-*tert*-butyl-dimethyl-silane (14)

A mixture of 3-bromo-propanol (5 mL, 57.190 mmol), imidazole (11.68 g, 171.57 mmol) and TBDMS-Cl (11.206 g, 74.347 mmol) in anhydrous THF (100 mL) was stirred at room temperature for 24 h. The reaction was monitored by TLC (EtOAc/light petroleum 1:3). The solvent was removed under reduced pressure and the residue was diluted with water and extracted three times with DCM. The organic layers were combined, washed with Brine, dried and evaporated to obtain compound **14** in quantitative yield. $^1$H NMR ($CDCl_3$): $\delta$ 3.73 (t, 2H, $J$ = 2.8 Hz); 3.51 (t, 2H, $J$ = 3.2 Hz); 2.07–1.99 (m, 2H); 0.89 (s, 9H); 0.06 (s, 6H).

#### 5.2.14. 1-[3-(*tert*-Butyl-dimethyl-silan-yl-oxi)-propyl]-spiro[isobenzofuran-1(3*H*),4′-piperidine] (15)

To a stirred solution of **4** (1 g, 5.71 mmol) in DMF (50 mL) compound **14** (1.72 g, 6.8 mmol) and $K_2CO_3$ (1.58 g, 11.43 mmol) dissolved in DMF (10 mL) were added. The reaction was stirred and warmed at 60 °C for an hour and it was monitored by TLC (EtOAc/light petroleum/$NH_3$ 5:1:0.3). The solvent was removed under vacuum and the residue was dissolved in EtOAc and washed twice with saturated $NH_4Cl$ and once with Brine. The organic layer was dried and evaporated under reduced pressure to give the crude product as a red-orange oil. This was purified by flash chromatography to obtain compound **15** (1.93 g, 5.35 mmol, yield 94%). MS (ESI): $[MH]^+$ = 362. $^1$H NMR ($CDCl_3$): $\delta$ 7.28–7.13 (m, 4H); 5.06 (s, 2H); 3.67 (t, 2H, $J$ = 6 Hz); 2.91–2.83 (m, 2H); 2.53–2.36 (m, 4H); 1.99 (dt, 2H, $J$ = 6, 4 Hz); 1.81–1.73 (m, 4H); 0.89 (s, 9H); 0.05 (s, 6H). $^{13}$C NMR ($CDCl_3$): 145.77; 138.97; 127.60; 127.40; 121.08; 120.88; 84.82; 70.79 (2C); 61.68; 55.73; 50.30 (2C); 36.69 (2C); 30.32; 26.06 (3C); −5.29 (2C).

#### 5.2.15. 3-{Spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]}-propan-1-ol (16)

To a solution of compound **15** (1.930 g, 5.346 mmol) in anhydrous THF (35 mL), cooled at 0 °C, TBAF (5.06 g, 16.038 mmol) was added. The reaction was stirred at room temperature for 24 h and the solvent removed under vacuum. The residue was diluted with EtOAc and washed twice with water. The organic layer was dried and concentrated under reduced pressure to give the crude product, which was purified by flash chromatography to obtain compound **16** (1.239 g, 5.015 mmol, yield 94%). MS (ESI): $[MH]^+$ = 248. $^1$H NMR ($CDCl_3$): $\delta$ 7.29–7.13 (m, 4H); 5.06 (s, 2H); 3.84 (t, 1H, $J$ = 5.2 Hz); 3.61–3.60 (m, 1H); 3.08–3.01 (m, 2H); 2.72 (t, 1H, $J$ = 5.6 Hz); 2.51–2.44 (m, 3H); 2.05–1.95 (m, 3H); 1.82–1.72 (m, 4H). $^{13}$C NMR ($CDCl_3$): 145.20; 138.87; 127.78; 127.52; 121.14; 120.89; 84.46; 70.88; 64.64; 62.85; 59.16; 50.42; 36.55; 32.83; 27.16.

#### 5.2.16. (D)-1-Benzyl-pirrolidin-2-carboxilic acid {3-[spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]-propyl}estere (17)

To a solution of 1-benzyl-pirrolidin-2-carbossilic acid (264 mg, 1.288 mmol) in DMF, cooled at 0 °C, HOBt (236 mg, 1.546 mmol) and DCC (319 mg, 1.546 mmol) were added. After 10 min compound **16** (350 mg, 1.417 mmol) dissolved in DMF was added. The reaction mixture was stirred at room temperature for 24 h and monitored by TLC (EtOAc/light petroleum/$NH_3$ 4:1:0.3). The solvent was evaporated under vacuum and the residue was basified with $NaHCO_3$ 5% and extracted with EtOAc. The organic layers were combined, dried and concentrated under reduced pressure to obtain the crude product, which was purified by flash chromatography to obtain compound **17** (170 mg, 0.392 mmol, yield 30%). MS (ESI): $[MH]^+$ = 435. $^1$H NMR ($CDCl_3$): $\delta$ 7.31–7.23 (m, 9H); 5.04 (s, 2H); 4.12 (dt, 2H, $J$ = 6.6, 2.4 Hz); 3.91 (d, 1H, $J$ = 12.6 Hz); 3.57 (d, 1H, $J$ = 12.8 Hz); 3.27–3.20 (m, 2H); 3.03–2.95 (m, 2H); 2.86–2.81 (m, 2H); 2.51–2.33 (m, 5H); 1.90–1.72 (m, 8H). $^{13}$C NMR ($CDCl_3$): 174.21; 145.52; 138.85; 138.41; 129.177 (2C); 128.19 (2C); 127.61; 127.39; 127.09; 121.06; 120.83; 84.62; 70.76; 65.27; 63.10; 58.65; 55.29; 53.20; 50.16 (2C); 36.51 (2C); 29.38; 26.26; 23.03. $[\alpha]_D^{20}$ = +23 (c = 0.1 g/100 mL, Chloroform). Anal. ($C_{27}H_{34}N_2O_3$) C, H, N.

#### 5.2.17. 1-(3-Chloro-propyl)-4-spiro[isobenzofuran-1(3*H*),4′-piperidine] (18)

In a two neck round bottom flask compound **16** (200 mg, 0.81 mmol) was dissolved in dry DCM (10 mL) and cooled at 0 °C, tosylchloride (170 mg, 0.89 mmol) and $Et_3N$ (90 mg, 0.89 mmol) were added drop wise. The reaction was stirred at room temperature for 24 h and after evaporation, the crude material was dissolved in 20 mL of EtOAc. The organic phase was washed twice with $NaHCO_3$ saturated solution (20 mL each), the combined organic phases were concentrated to dryness and the product purified by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9:1:0.3) to obtain compound **18** (85 mg, 0.320 mmol, yield 40%). MS (ESI): $[MH]^+$ = 266. $^1$H NMR ($CDCl_3$): $\delta$ 7.27–7.14 (m, 4H); 5.05 (s, 2H); 3.61 (t, 2H, $J$ = 6.6 Hz); 2.85–2.80 (m, 2H); 2.55 (t, 2H, $J$ = 7.2 Hz); 2.40 (dt, 2H); 2.04–1.95 (m, 4H); 1.79–1.78 (m, 2H).

#### 5.2.18. 1-[3-(1-Benzyl-pirrolidin-2-ylmetoxy)-propyl]-4-spiro-[isobenzofuran-1(3*H*),4′piperidine] (20)

To a stirred suspension of sodium hydride (14 mg, 0.34 mmol) in anhydrous THF at 0 °C, a solution of **19** (60 mg, 0.31 mmol) in THF was added. After 30 minutes, a catalytic amount of TBAI was added followed by drop wise addiction of compound **18** (85 mg, 0.31 mmol) dissolved in THF. After 24 h at room temperature, the reaction was heated at reflux for 12 h, the solvent was removed in vacuo, diluted with $Et_2O$ and the resulting precipitate filtrated on Celite pad. The solvent was concentrated to dryness and the crude material purified by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9.5:0.5:0.3) to give compound **20** in 56% yield. MS (ESI): $[MH]^+$ = 421. $^1$H NMR ($CDCl_3$): $\delta$ 7.32–7.21 (m, 9H); 5.03 (s, 2H); 4.12 (dt, 2H, $J$ = 6.6, 2.4 Hz); 3.91 (d, 1H, $J$ = 12.6 Hz); 3.57 (d, 1H, $J$ = 12.8 Hz); 3.45–3.21 (m, 2H); 3.27–3.20 (m, 2H); 3.03–2.95 (m, 1H); 2.86–2.81 (m, 2H); 2.51–2.33 (m, 5H); 1.90–1.72 (m, 8H).

$^{13}$C NMR ($CDCl_3$): 145.52; 138.85; 138.41; 129.17 (2C); 128.19 (2C); 127.61; 127.39; 127.09; 121.06; 120.83; 84.62; 74.34; 70.76; 65.27; 63.10; 58.65; 55.29; 53.20; 50.16 (2C); 36.51 (2C); 29.38; 26.26; 23.03. $[\alpha]_D^{20}$ = +21 ($c$ = 0.2 g/100 mL, chloroform). Anal. ($C_{27}H_{36}N_2O_2$) C, H, N.

#### 5.2.19. 5-(1-Benzyl-pirrolidin-2-yl)-pent-4-enoic acid (22)

In a two neck round bottom flask, under argon atmosphere, was placed NaH 60% (12.57 g, 64.31 mmol) and washed five times with anhydrous pentane (20 mL each). The resulting white solid was dried flushing argon and anhydrous DMSO (50 mL) was added; the mixture was heated at 77 °C for 40 min. At this time, the reaction was cooled at 0 °C and the (3-carboxy-propyl)-triphenyl-phosphonium bromide **21** (12.55 g, 29.23 mmol) dissolved in DMSO was added drop wise. When the reaction becomes a brown-red color, 1.105 g (5.85 mmol) of 1-benzyl-pirrolidine-2-carbaldheyde were added drop wise. The reaction was checked by TLC (EtOAc/MeOH/$NH_4OH$, 9:1:0.3), the DMSO was removed under vacuum and the crude material dissolved in EtOAc (60 mL) and washed with a 10% solution of citric acid (30 mL). The organic phases were dried and evaporated to dryness, the compound was purified by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9:1:0.3) to give compound **22** (350 mg, 1.135 mmol, yield 23%). MS (ESI): $[MH]^+$ = 260. $^1$H NMR ($CD_3OD$): $\delta$ 7.76–7.32 (m, 5H); 5.95–5.86 (m, 1H); 5.60–5.50 (m, 1H); 4.45–4.39 (m, 2H); 4.16–4.10 (m, 1H); 3.34–3.29 (m, 6H); 2.40–2.39 (m, 4H). $^{13}$C NMR ($CD_3OD$): 177.93; 139.63; 132.54; 131.91; 131.02; 130.47; 124.63; 66.22; 57.71; 53.82; 50.47; 40.62; 35.48; 31.32; 25.01; 22.35. $[\alpha]_D^{20}$ = −7.14 ($c$ = 0.21 g/100 mL, ethanol). IR: 3398.72; 2993.34; 1658.97; 1560.22; 1435.77; 1405.21; 1315.37; 1211.62; 1159.59; 1124.14; 1013.97; 952.12; 752.60; 701.61.

#### 5.2.20. 5-(1-Benzyl-pirrolidin-2-yl)-1-[spiro[isobenzofuran-1(3*H*), 4′-piperidin-1-yl]]-pent-4-en-1-one (23)

Compound **22** (590 mg, 2.278 mmol) was dissolved in DMF (50 mL) and at 0 °C HOAt (372 mg, 2.773 mmol), WSC (524 mg, 2.733 mmol) and compound **4** (430 mg, 2.278 mmol) were added. After 12 h at room temperature, the solvent was removed by evaporation under reduced pressure and the residue diluted with EtOAc (50 mL); the organic layer washed with $NaHCO_3$ 5% (10 mL) and the aqueous phase extracted three times with EtOAc (30 mL each). The combined organic phases were dried and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc/light petroleum/$NH_4OH$, 1:1:0.3) to obtain compound **23** (783 mg, 1.822 mmol, yield 80%). MS (ESI): $[MH]^+$ = 431. $^1$H NMR ($CDCl_3$): $\delta$ 7.2967.22 (m, 9H); 5.67–5.58 (m, 1H); 5.51–5.44 (m, 1H); 5.08 (s, 2H); 4.67–4.61 (m, 1H); 4.04–3.98 (m, 1H); 3.86–3.80 (m, 1H); 3.67 (t, 1H, $J$ = 6.4 Hz); 3.57 (t, 1H, $J$ = 6.4 Hz); 3.22–2.89 (m, 4H); 2.55–2.44 (m, 4H); 1.95–1.55 (m, 8H). $^{13}$C NMR ($CDCl_3$): 170.73; 144.70; 139.43; 138.86; 133.47; 130.75; 129.11; 128.19; 127.97; 127.58; 126.85; 121.30; 120.69; 84.76; 71.03; 61.94; 58.52; 53.28; 44.99; 42.61; 38.73; 37.09; 36.19; 33.39; 31.40; 29.97; 23.84; 22.10. $[\alpha]_D^{20}$ = +18 ($c$ = 0.1 g/100 mL, chloroform).

#### 5.2.21. (D)-1-Benzyl-pirrolidin-2-carboxilic acid {3-[spiro[isobenzofuran-1(3*H*),4′-piperidin-1-yl]]-propyl}-amide (Comp 24)

To a stirred solution of *N*-Bn-D-Pro (860 mg, 3.496 mmol) in DMF (20 mL) cooled at 0 °C, HOBt (642 mg, 4.195 mmol), WSC (804 mg, 4.195 mmol) were added and the reaction stirred for 10 min. After this time compound **5** (716 mg, 3.496 mmol) dissolved in DMF (20 mL) was added and the reaction mixture was stirred for 24 h. The DMF was removed in vacuo and the product partitioned between EtOAc and $NaHCO_3$ 5%. The organic phase was dried, concentrated in vacuum and the crude product purified by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9.5:0.5:0.3) to give Comp 24 in 37% yield. MS (ESI): $[MH]^+$ = 434. $^1$H NMR ($CDCl_3$): $\delta$ 7.60 (br, 1H); 7.30–7.20 (m, 9H); 5.05 (s, 2H); 3.87 (d, 1H, $J$ = 12.8 Hz); 3.48 (d, 1H, $J$ = 12.8 Hz); 3.42–3.16 (m, 4H); 3.09–3.00 (m, 1H); 2.87–2.80 (m, 2H); 2.48–2.29 (m, 5H); 2.23–2.13 (m, 3H); 2.03–1.87 (m, 2H); 1.77–1.65 (m, 4H). $^{13}$C NMR ($CDCl_3$): 174.62; 145.61; 138.94; 138.64; 128.81 (2C); 128.56 (2C); 127.62; 127.38 (2C); 121.10; 120.79; 84.69; 70.79; 67.61; 60.02; 56.76; 54.07; 50.54; 50.21; 37.67; 36.60 (2C); 30.78; 26.99; 24.09. $[\alpha]_D^{20}$ = +43 ($c$ = 0.1 g/100 mL, chloroform). Anal. ($C_{27}H_{35}N_3O_2$) C, H, N.

#### 5.2.22. 1-[5-(1-Benzyl-pirrolidin-2-yl)-pent-4-enyl]-spiro[isobenzofuran-1(3*H*),4′-piperidine] (25)

In a round-bottomed flask under argon atmosphere, to a stirred solution of **23** (140 mg, 0.325 mmol) in anhydrous THF (10 mL) cooled at 0 °C, was added drop wise to a solution of borane dimethylsulfide complex (0.094 mL, 0.977 mmol) dropwise. The solution was heated at reflux overnight, after this time, cooled at 0 °C and HCl 10% was added until pH 2. The resulting solution was stirred at reflux for 4 h, basified with NaOH 2 N and the solvent was removed under vacuum. The crude product was purified by flash chromatography (EtOAc/light petroleum/$NH_4OH$, 2:1:0.3) to give **25** (35 mg, 0.084 mmol, yield 26%). $^1$H NMR ($CDCl_3$): $\delta$ 7.326–7.216 (m, 9H); 5.42–5.37 (m, 2H); 5.06 (s, 2H); 4.09 (t, 1H, $J$ = 6.4 Hz); 3.77 (s, 1H); 3.67 (t, 1H, $J$ = 6.4 Hz); 2.91–2.86 (m, 2H); 2.68–2.34 (m, 4H); 2.09–1.96 (m, 4H); 1.80–1.50 (m, 8H); 1.24–1.15 (m, 2H). $^{13}$C NMR ($CDCl_3$): 145.70; 141.23; 138.93; 130.53; 130.06; 128.44; 128.19; 127.63; 127.43; 126.95; 121.09; 120.92; 84.77; 70.83; 64.34; 62.38; 59.00; 54.08; 50.27; 49.80; 36.60; 32.51; 30.40; 29.87; 27.88; 27.71; 26.48; 25.143. $[\alpha]_D^{20}$ = +1.67 ($c$ = 0.31 g/100 mL, chloroform). Anal. ($C_{28}H_{36}N_2O$) C, H, N.

#### 5.2.23. [3-(4-Oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-8-yl)-propyl]-carbamic acid *tert*-butyl estere (27)

To a stirred solution of **26** (500 mg, 2.62 mmol) in DMF, was added *N*-Boc-3-bromo-propyl-amine (612 mg, 2.57 mmol) in 10 mL of DMF and $K_2CO_3$ (596 mg, 4.32 mmol), the reaction was warmed at 60 °C for 1 h. Most of the solvent was removed and the residue was diluted with EtOAc and washed twice with $NH_4Cl$ saturated solution (30 mL each) and brine (30 mL). The organic layer was dried and evaporated under reduced pressure. The crude product was crystallized from $Et_2O$ to give the final compound (535 mg, 1.38 mmol, yield 64%). mp = 163–165 °C. MS (ESI): $[MH]^+$ = 389. $^1$H NMR ($CDCl_3$): $\delta$ 7.31–7.23 (m, 3H); 6.90–6.84 (m, 2H); 5.38 (br, 2H); 4.73 (s, 2H); 3.23–3.19 (m, 2H); 2.82–2.45 (m, 8H); 1.75–1.66 (m, 4H); 1.41 (s, 9H). $^{13}$C NMR ($CDCl_3$): 178.14; 156.20; 143.21; 129.37 (2C); 119.02 (2C); 115.50 (2C); 79.00; 59.50; 59.38; 56.98; 49.84 (2C); 40.16; 29.31; 28.55 (3C); 26.91.

#### 5.2.24. Acid-(D)-1-benzyl-pirrolidin-2-carboxilic-[3-(4-oxo-1-fenyl-1,3,8-triaza-spiro[4.5]dec-8-yl)-propyl]-amide (28)

Compound **27** (535 mg, 1.37 mmol) was treated with TFA (5 mL) at 0 °C for 1 h. NaOH 2 N was added until pH 12–14, the aqueous phase was extracted several times with EtOAc and the organic phases were combined, dried and evaporated under vacuum to give the free amine (395 mg, 1.37 mmol) in 100% yield. This product was condensed with *N*-Bn-D-Pro in a similar manner as reported for Comp **24**. The crude product was purified by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9.5:0.5:0.3) to give **28** in 56% yield. MS (ESI): $[MH]^+$ = 476. $^1$H NMR ($CDCl_3$): $\delta$ 7.94–7.83 (m, 2H); 7.35–6.86 (m, 10H); 4.72 (s, 2H); 3.84 (d, 1H, $J$ = 13 Hz); 3.51 (d, 1H, $J$ = 13 Hz); 3.27–3.19 (m, 3H); 3.07–2.81 (m, 6H); 2.76–2.47 (m, 2H); 2.36–1.66 (m, 10H). $^{13}$C NMR ($CDCl_3$): 178.00; 175.06; 143.32; 138.80; 129.52 (2C); 128.91 (2C); 128.74 (2C); 127.54; 125.82; 119.61; 116.19 (2C); 115.30; 67.58; 60.17; 59.59; 56.04; 54.33; 49.86; 37.55; 30.98; 29.90; 29.34; 27.22;

24.37. $[\alpha]_D^{20}$ = +24 ($c$ = 0.1 g/100 mL, chloroform). Anal. ($C_{28}H_{37}N_5O_2$) C, H, N.

#### 5.2.25. 2-(2,6-Dichloro-phenyl)-4-hydroxy-4-methyl-6-oxo-cyclohexane-1,3-dicarboxylic acid diethyl ester (30)

Compound **29** (5 g, 28.57 mmol) and ethyl aceto-acetate (7.22 mL, 57.14 mmol) were dissolved in 20 mL of ethanol 96%. At this stirred solution was added piperidine (0.5 mL) and the reaction mixture was stirred at room temperature overnight.

The solvent was removed under reduced pressure and the product was crystallized from $Et_2O$ to give compound **30** with a quantitative yield. mp = 112–115 °C. MS (ESI): $[MH]^+$ = 418. $^1$H NMR ($CDCl_3$): $\delta$ 12.50 (s, 1H); 7.28–7.18 (m, 2H); 7.05 (t, 1H, 8 Hz); 5.01 (d, 1H, $J$ = 11 Hz); 4.05–3.83 (m, 5H); 3.10 (d, 1H, $J$ = 11.4 Hz); 2.48 (s, 2H); 1.32 (s, 3H); 0.98 (t, 3H, 7.4 Hz); 0.85 (t, 3H, 7 Hz). $^{13}$C NMR ($CDCl_3$): 174.77; 171.11; 170.52; 138.03; 137.03; 134.28; 130.16; 128.04; 127.90; 98.09; 69.35; 61.36; 60.29; 50.85; 41.72; 38.28; 28.05; 13.83; 13.39.

#### 5.2.26. 3-(2,6-Dichloro-phenyl)-pentane-dioic acid (31)

Compound **30** (7 g, 16.79 mmol) dissolved in ethanol 96% (55 mL), NaOH 35% (40.75 mL) and of water (16.3 mL) were added. The reaction mixture was stirred at reflux for 3 h. The solvent was removed under reduced pressure, the resulting aqueous layer was acidified at 0 °C with HCl 20% and extracted with ethyl acetate. The organic layers were combined, dried and evaporated under vacuum to obtain the crude product, which was crystallized from ethyl ether to give **31** (4.026 g, 14.53 mmol, yield 87%). mp = 153–155 °C. MS (ESI): $[MH]^+$ = 278. $^1$H NMR ($CDCl_3$): $\delta$ 12.20 (s, 2H); 7.41 (t, 2H, $J$ = 8 Hz); 7.23 (t, 1H, $J$ = 8 Hz); 3.43–3.32 (m, 1H); 2.92–2.70 (m, 4H). IR: 2918.07; 1713.09; 1560.58; 1438.02; 1310.02; 1226.35; 1202.58; 1085.05; 903.48; 779.13.

#### 5.2.27. 4-(2,6-Dichloro-phenyl)-piperidin-2,6-dione (32)

Compound **31** (4.026 g, 15.60 mmol) was dissolved in $NH_4OH$ (40 mL). The solvent was evaporated under vacuum and the solid residue was heated at 200 °C for 6 h. At the crude product was added DCM (150 mL) and was washed twice with $Na_2CO_3$ 0.1 M. The organic layer was dried and concentrated in vacuum to obtain a crude product, which was crystallized from ethyl ether to give **32** with a quantitative yield. mp = 155–157 °C. MS (ESI): $[MH]^+$ = 259. $^1$H NMR ($CDCl_3$): $\delta$ 8.20 (br, 1H); 7.36–7.13 (m); 4.43–4.30 (m, 1H); 3.60 (dd, 2H, $J$ = 18, 13.5 Hz); 2.69 (dd, 2H, $J$ = 17.7, 4.6 Hz). IR: 3208.19; 3078.49; 2921.50; 2385.98; 2342.76; 1696.60; 1560.07; 1437.89; 1361.16; 1271.72; 1150.48; 1079.12; 770.20; 731.23.

#### 5.2.28. 4-(2,6-Dichloro-phenyl)-piperidine (33)

Compound **32** (4 g, 15.5 mmol) was suspended, under argon atmosphere, in anhydrous THF (150 mL) and $(CH_3)_2S^*BH_3$ (14.9 mL, 155 mmol) added at 0 °C under stirring. The reaction was heated at reflux overnight. The solution was cooled at 0 °C, HCl 10% (66.6 mL) was added until pH of the solution was strongly acidic and the solution was left at reflux for 4 h. The reaction mixture was cooled again at 0 °C, basified with NaOH 2 N and monitored by TLC (EtOAc/light petroleum 3:2). The THF was evaporated under vacuum and the aqueous layer was extracted with ethyl acetate. The organic layers were combined, dried and concentrated under vacuum to obtain the crude product, which was crystallized with ethyl ether to give **33** (1.23 g, 5.348 mmol, yield 35%). mp = 210–215 °C. MS (ESI): $[MH]^+$ = 231. $^1$H NMR ($CDCl_3$): $\delta$ 9.77 (br, 1H); 7.28–7.25 (m, 2H); 7.089 (t, 1H, $J$ = 8 Hz); 3.78–3.72 (m, 1H); 3.67–3.64 (m, 2H); 3.13–3.02 (m, 4H); 1.82–1.79 (m, 2H). $^{13}$C NMR ($CDCl_3$): 137.08; 135.21; 130.67; 128.65 (2C); 44.73 (2C); 38.30 (2C); 24.80 (2C).

#### 5.2.29. 3-[4-(2,6-Dichloro-phenyl)-piperidin-1-yl]-propylamine (34)

To a stirred solution of **33** (284 mg, 1.236 mmol) in DMF (5 mL), *N*-Boc-3-bromo-propyl-amine (350 mg, 1.471 mmol) and $K_2CO_3$ (341.77 mg, 2.473 mmol) were added. The reaction mixture was stirred at 60 °C for 1 h and than evaporated under vacuum, the residue was dissolved in EtOAc and washed twice with a saturated solution of $NH_4Cl$ and once with Brine. The organic layer was dried and concentrated under reduced pressure to obtain the product as a red-orange oil. This crude product was then purified by flash chromatography (EtOAc/light petroleum/$NH_4OH$ 5:1:0.3) (yield 94%). At the product so obtained (1.163 mmol), cooled at 0 °C, was added drop wise 1 mL of TFA. The reaction was monitored by TLC (EtOAc/light petroleum 5:1) and after about 1 h was cooled at 0 °C and basified with NaOH 2 N. The aqueous layer was extracted three times with EtOAc and the organic layers were combined, dried and evaporated under vacuum to give compound **34** with a quantitative yield. MS (ESI): $[MH]^+$ = 288. $^1$H NMR ($CDCl_3$): $\delta$ 7.25–7.00 (m, 3H); 3.53–3.46 (m, 1H); 3.36 (br, 2H); 3.116 (dd, 2H, $J$ = 9.6, 4 Hz); 2.90 (t, 2H, $J$ = 6.8 Hz); 2.67–2.56 (m, 2H); 2.50 (t, 2H, $J$ = 6.8 Hz); 2.05 (dt, 2H, $J$ = 10, 2.4 Hz); 1.75 (t, 2H, $J$ = 6.4 Hz); 1.58 (d, 2H, $J$ = 12.4 Hz).

#### 5.2.30. (D)-1-Benzyl-pirrolidin-2-carbossilic acid {3-[4-(2,6-dichloro-phenyl)-piperidin-1-yl]-propyl}-amide (35)

*N*-Bn-D-Pro and compound **34** were condensed in the same experimental condition as for Comp 24 yielding **35** (48 mg, 0.101 mmol, yield 65%). MS (ESI): $[MH]^+$ = 475. $^1$H NMR ($CDCl_3$): $\delta$ 7.56–7.52 (br, 1H); 7.35–7.24 (m, 7H); 7.03 (t, 1H, $J$ = 8 Hz); 3.86 (d, 1H, $J$ = 13.2 Hz); 3.50 (d, 1H, $J$ = 13.2 Hz); 3.28–3.17 (m, 2H); 3.07–3.03 (m, 3H); 2.69–2.64 (m, 2H); 2.47–2.34 (m, 3H); 2.27–2.19 (m, 2H); 2.06–2.01 (m, 2H); 1.92–1.90 (m, 2H); 1.75–1.72 (m, 4H); 1.56 (d, $J$ = 12.8 Hz). $^{13}$C NMR ($CDCl_3$): 174.71; 138.67; 130.41; 128.76 (2C); 128.57 (2C); 127.78 (2C); 127.35 (2C); 67.52; 59.95 (2C); 56.41; 54.64; 54.10; 40.57; 37.43 (2C); 30.78 (2C); 27.64; 26.80; 24.14 (2C). $[\alpha]_D^{20}$ = +27 ($c$ = 0.1 g/100 mL, chloroform). Anal. ($C_{26}H_{33}Cl_2N_3O$) C, H, N, Cl.

#### 5.2.31. (L)-1-Benzyl-pirrolidin-2-carboxilic acid {3-[4-(2,6-dichloro-phenyl)-piperidin-1-yl]-propyl}-amide (36)

*N*-Bn-Pro and compound **34** were condensed in the same experimental condition as for Comp 24. yielding **36** (48 mg, 0.101 mmol, yield 35%). MS (ESI): $[MH]^+$ = 475. $^1$H NMR ($CDCl_3$): $\delta$ 7.53 (br, 1H); 7.33–7.25 (m, 7H); 7.03 (t, 1H, $J$ = 8 Hz); 3.86 (d, 1H, $J$ = 13.2 Hz); 3.50 (d, 1H, $J$ = 13.2 Hz); 3.30–3.17 (m, 3H); 3.07–3.03 (m, 2H); 2.67–2.64 (m, 2H); 2.42–2.32 (m, 3H); 2.24–2.19 (m, 2H); 2.07–2.01 (m, 3H); 1.88–1.85 (m, 2H); 1.78–1.67 (m, 3H); 1.57–1.54 (m, 2H). $^{13}$C NMR ($CDCl_3$): 174.69; 138.69; 130.40; 128.73 (2C); 128.57 (2C); 127.74 (2C); 127.35 (2C); 67.53; 59.95 (2C); 56.51; 54.68; 54.09; 40.64; 37.47 (2C); 30.78 (2C); 27.74; 26.86; 24.14 (2C). $[\alpha]_D^{20}$ = −27 ($c$ = 0.1 g/100 mL, chloroform). Anal. ($C_{26}H_{33}Cl_2N_3O$) C, H, N, Cl.

#### 5.2.32. 4-(2-Oxo-2,3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid *tert*-butyl ester (38)

To a stirred solution of compound **37** (1 g, 4.60 mmol) in DCM (50 mL) at 0 °C, a catalytical amount of DMAP and $(Boc)_2O$ (1.57 g, 6.9 mmol) were added. After 6 h at room temperature, water (100 mL) was added and the organic layer washed with citric acid 10% (10 mL × 3), $NaHCO_3$ saturated solution (10 mL × 3) and brine (10 mL × 3). The organic phase was dried and concentrated in vacuo to give compound **38** in quantitative yield pure enough to be used in the next step without further purification.

#### 5.2.33. 1-Ethyl-3-piperidin-4-yl-1,3-dihydro-benzoimidazol-2-one (39)

To a suspension of sodium hydride (92 mg, 3.79 mmol) in anhydrous DMF (5 mL), compound **38** was added dropwise at 0 °C. After

30 minutes, ethyl bromide (413 mg, 3.79 mmol) was added, and the reaction mixture was allowed to stir at room temperature overnight. The DMF was removed in vacuo and the salts were dissolved in water, the aqueous layer was extracted with EtOAc (25 mL × 3), dried and concentrated in vacuo. The crude material was dissolved in 5 mL of TFA and stirred for 1 h; after this time, the TFA was evaporated and the solution was basified with NaOH 20% until pH 12. The aqueous layer was extracted twice with EtOAc (30 mL each), concentrated to dryness and purified by flash chromatography using EtOAc/light petroleum/$NH_4OH$: 2:1:0.3 as eluent to give 0.84 g of desired compound (yield 75%). MS (ESI): $[MH]^+$ = 246.3 $^1H$ NMR ($CDCl_3$): $\delta$ 7.25 (m, 1H); 7.07–7.00 (m, 3H); 4.43 (m, 1H); 3.98–3.87 (q, 2H, $J$ = 6 Hz); 3.22 (br s, 2H); 2.79 (dt, 2H, $J$ = 15.6 Hz, $J$ = 2.4 Hz); 2.37–2.30 (dq, 2H, $J$ = 8.4 Hz, $J$ = 4.2 Hz); 1.85 (bs, 2H); 1.32 (t, 3H, $J$ = 7.4 Hz).

#### 5.2.34. 1-[1-(3-Amino-propyl)-piperidin-4-yl]-3-ethyl-1,3-dihydro-benzoimidazol-2-one (40)

To a stirred solution of **39** (92 mg, 0.375 mmol) in DMF (5 mL), *N*-Boc-3-bromo-propyl-amine (106 mg, 0.447 mmol) and $K_2CO_3$ (103 mg, 0.750 mmol) were added. After 1 h at 60 °C, most of the solvent was removed and the residue diluted with EtOAc and washed twice with $NH_4Cl$ saturated solution (30 mL each) and brine (30 mL). The organic layer was dried and evaporated under reduced pressure. The crude material was treated at 0 °C with TFA (5 mL) for 1 h and then treated with NaOH 2 N until pH 12–14. The aqueous phase was extracted several times with EtOAc and the organic phases were combined, dried and evaporated under vacuum to give compound **40** in 80% yield. The product was enough pure to be used in the next step without further purification.

#### 5.2.35. 1-Benzyl-pyrrolidine-2-carboxylic acid {3-[4-(3-ethyl-2-oxo-2,3-dihydro-benzoimidazol-1-yl)-piperidin-1-yl]-propyl}-amide (41)

*N*-Bn-D-Pro and compound **40** were condensed in the same experimental condition as for Comp 24 yielding **41** in 24% yield after purification by flash chromatography (EtOAc/MeOH/$NH_4OH$, 9.5:0.5:0.3). MS (ESI): $[MH]^+$ = 490. $^1H$ NMR ($CDCl_3$): $\delta$ 7.58–7.52 (m, 1H); 7.29–7.25 (m, 5H); 7.04–7.01 (m, 4H); 4.58–4.42 (m, 1H); 3.92 (q, 2H, $J$ = 7.2 Hz); 3.866–3.560 (dd, a–b sistem, 2H, $J$ = 13 Hz); 3.24–3.09 (m, 5H); 2.59–2.19 (m, 9H); 1.87–1.72 (m, 7H); 1.315 (t, 3H, $J$ = 7.2 Hz). $^{13}C$ NMR ($CDCl_3$): 175.07; 153.40; 138.58; 129.14; 128.71; 128.586; 127.87; 127.39; 121.06; 120.98; 109.80; 107.67; 67.41; 59.95; 55.44; 54.17; 53.00; 52.92; 49.83; 36.93; 35.95; 30.77; 28.11; 26.36; 24.13; 13.62. Anal. ($C_{29}H_{39}N_5O_2$) C, H, N.

### 5.3. Cell culture and cell membrane preparation

$CHO_{hNOP}$ cells were cultured in DMEM and Hams F-12 (1:1) supplemented with 5% foetal calf serum, penicillin (100 IU/mL), Streptomycin (100 μg/mL) and Fungizone (2.5 μg/mL). Stock cultures were further supplemented with geneticin (G418, 200 μg/mL) and Hygromycin B (200 μg/mL) as described previously.[25] CHO cell lines stably expressing the human MOP, DOP, KOP and NOP receptors and the C-terminally modified $G\alpha_{qi5}$[26] were generated as described previously[8] and maintained in Dulbecco Minimum Essential Medium (DMEM) and Hams F-12 (1:1) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 200 μg/mL Geneticin and 100 μg/mL Hygromicin B. Cells were cultured at 37 °C in 5% carbon dioxide humidified air, and used when confluent.

For binding experiments membranes were prepared from freshly harvested cell suspensions in Tris–HCl (50 mM), $Mg^{++}$ (5 mM) pH 7.4 ([$^3$H]N/OFQ binding experiments) or in Tris–HCl (50 mM), EGTA (0.2 mM) pH 7.4 ([$^{35}$S]GTPγS binding experiments) via homogenisation and centrifugation at 13,500 rpm for 10 min at 4 °C. The final protein concentration was determined according to Lowry.[27]

### 5.4. Receptor binding

[$^3$H]N/OFQ binding experiments. 5 μg of $CHO_{hNOP}$ homogenate protein was incubated in 0.5 mL volumes of Tris-HCl (50 mM) buffer supplemented with 10 μM peptidase inhibitors (amastatin, bestatin, captopril and phosphoramidon), 0.5% bovine serum albumin (BSA), increasing concentrations of compounds under study and approximately 200 pM [$^3$H]-N/OFQ. Total radiolabel bound was ≪10%. Non-specific binding was determined in the presence of 1 μM unlabeled N/OFQ. In all experiments Comp 24 was included as a reference ligand. Reactions were incubated for 1 h at room temperature and terminated by vacuum filtration (Brandel Harvester) through Whatman GF/B filters soaked at 0.5% polyethylenimine (PEI). Radioactivity was determined after 8 h extraction in scintillation cocktail. [$^3$H]-Diprenorphine binding experiments. 50 μg ($CHO_{hMOP}$), 25 μg ($CHO_{hDOP}$) and 40 μg ($CHO_{hKOP}$) membrane protein were incubated in 0.5 ml buffer containing Tris-HCl (50 mM) pH 7.4, BSA (0.5%), ∼0.7 nM [$^3$H]Diprenorphine and increasing concentrations of naloxone and compound **35**. Non-specific binding was determined in the presence of 10 μM naloxone. Reactions were incubated at room temperature for 1 h, harvesting and determination of radioactivity were as for [$^3$H]N/OFQ binding.

### 5.5. [$^{35}$S]GTPγS binding

20 μg of $CHO_{hNOP}$ membranes were incubated in 0.5 mL buffer containing Tris–HCl (50 mM), EGTA (0.2 mM) $MgCl_2$ (1 mM), NaCl (100 mM), bacitracin (0.15 mM) peptidase inhibitors (as above), GDP (100 μM) and approximately 150 pM [$^{35}$S]GTPγS. Compound **35** was pre-incubated for 15 min at 30 °C. Non-specific binding was determined in the presence of unlabeled 10 μM GTPγS. The reaction was incubated for 1 h with increasing concentration of N/OFQ at 30 °C with gentle shaking and terminated by filtration through Whatman GF/B filters using a Brandel Harvester. PEI was not used.

### 5.6. Calcium mobilization experiments

$CHO_{hMOP}$, $CHO_{hDOP}$, $CHO_{hKOP}$ and $CHO_{hNOP}$ stably expressing the $G\alpha_{qi5}$ protein were seeded at a density of 40,000 cells/well into 96-well black, clear-bottom plates. After 24 h incubation the cells were loaded with medium supplemented with 2.5 mM of probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. Afterwards, the loading solution was aspirated and 100 μL/well of assay buffer: Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM Brilliant Black (Aldrich) was added. Stock solutions (1 mM) of ligands were prepared in distilled water and stored at −20 °C. Serial dilutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction V). After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA), fluorescence changes were measured at room temperature. On-line additions were carried out in a volume of 50 μL/well.

### 5.7. Electrically stimulated isolated tissues

Tissues were taken from male Swiss mice (30–35 g), albino guinea pigs (300–350 g) and Sprague-Dawley rats (300–350 g). The mouse and the rat vas deferens and the guinea pig ileum were prepared as previously described.[28] Tissues were continuously stimulated through two platinum ring electrodes with supramaximal

rectangular pulses of 1 ms duration and 0.05 Hz frequency. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006, UgoBasile s.r.l., Varese, Italy) and recorded with the PC based acquisition system Power Lab (ADInstrument, USA).

Following an equilibration period of 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration–response curves to N/OFQ were performed (0.5 log unit steps) in the absence or presence of compound **35** (15 min pre-incubation time). For selectivity studies, in some experiments the DOP selective agonist DPDPE was used in the mouse vas deferens and the MOP selective agonist dermorphin was used in the guinea pig ileum.

### 5.8. Mouse tail withdrawal assay

Male Swiss albino mice weighing 25–30 g were used. Animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and Italian national regulations (D.L. 116/92). They were housed in 425 × 266 × 155-mm cages (Techniplast, Milan, Italy), fifteen animals/cage, under standard conditions (22 °C, 55% humidity, 12-h light/dark cycle, light on at 7:00 am) with food (MIL, standard diet; Morini, Reggio Emilia, Italy) and water ad libitum for at least 5 days before experiments began. Each mouse was used only once. Icv (2 μl/mouse) or i.t (5 μl/mouse) injections were given according to the procedure described by Laursen and Belknap[29] and Hylden and Wilcox[30], respectively.

All experiments were started at 10:00 am and performed according to the procedure described previously in detail.[22] Briefly, the mice were placed in a holder and the distal half of the tail was immersed in water at 48 °C. Withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut-off time of 20 s was chosen to avoid tissue damage. For each experiment sixteen mice were used by randomly assigning four animals to each treatment group. The experiment was repeated four times; therefore, each experimental point shown in Figure 2 is the mean of the results obtained in 16 mice. Tail-withdrawal latency was determined immediately before and 5, 15, 30, and 60 min after icv or i.t. injection of vehicle (saline) or N/OFQ (1 nmol). Compound **35** (10 mg/kg) or its vehicle (2% DMSO and 10% encapsin) were given i.p. 30 min before icv injection. Increased and decreased tail withdrawal latencies compared with baseline indicated antinociceptive and pronociceptive effects, respectively.

### 5.9. Data analysis and terminology

All data are expressed as means ± standard error of the mean (sem) of *n* experiments. For potency values confidence limits 95% were indicated. Data have been analyzed statistically using one-way ANOVA followed by Dunnett's test for multiple comparisons or the Student's t test. In competition binding studies (Tables 2 and 3), the log concentration of competitor producing 50% inhibition of specific binding ($pIC_{50}$) was corrected for the competing mass of radiolabel according to Cheng and Prusoff[31] to yield $pK_i$ values. $K_D$ values for [$^3$H]N/OFQ in $CHO_{hNOP}$ was 83 pM and for [$^3$H]diprenorphine in MOP, DOP and KOP were 125, 323, and 134 pM, respectively.

[$^{35}$S]GTPγS data are expressed as a stimulation factor that is, the ratio between agonist-stimulated [$^{35}$S]GTPγS specific (minus NSB) binding and basal specific binding. Calcium mobilization data are expressed as fluorescence intensity units (FIU) in percent over the baseline. Isolated tissue data are expressed as percent of the twitch response to electrical field stimulation.

Agonist potencies are given as $pEC_{50}$ = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. Antagonist potencies have been evaluated (i) using $pA_2$ derived from the classical Schild protocol in [$^{35}$S]GTPγS binding and mouse vas deferens experiments (Fig. 1 and Table 2), (ii) using $pK_b$ derived from the Gaddum Schild equation:

$$K_b = ((CR - 1)/[\text{antagonist}])$$

assuming a slope value equal to unity, where CR indicate the ratio between agonist potency in the presence and in the absence of the antagonist, in rat vas deferens and guinea pig ileum experiments (Table 2). Finally, (iii) using $pK_b$ values derived from the following equation:[32]

$$K_b = IC_{50}/([2 + ([A]/EC_{50})^n]^{1/n} - 1$$

where $IC_{50}$ is the concentration of antagonist that produces 50% inhibition of the agonist response, [*A*] is the concentration of the agonist, $EC_{50}$ is the concentration of agonist producing a 50% maximal response and *n* is the Hill coefficient of the concentration response curve to the agonist in inhibition response experiments measuring calcium mobilization (Tables 2 and 4).

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.05.068.

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