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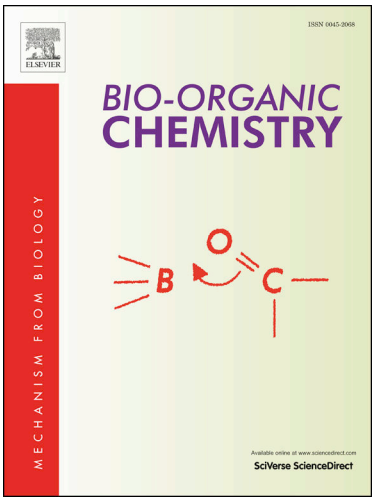

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Synthesis, biological evaluation and docking studies of a novel class of sulfur-bridged diazabicyclo[3.3.1]nonanes.

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ABSTRACT

A small library of 3-thia-7,9-diazabicyclo[3.3.1]nonanes was synthesized and their opioid receptors affinity and selectivity evaluated. Among these novel sulfur-bridged compounds, the (*E*) 9-[3'-(3-chlorophenyl)-but-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane **2i** emerged as the derivative with the highest μ receptor affinity (K_i = 85 nM) and selectivity (K_i μ/δ = 58.8, K_i μ/κ > 117.6). The antinociceptive activity of **2i** was also evaluated in acute thermal pain. Docking studies disclosed the specific pattern of interactions of these derivatives.

Keywords: 3-thia-7,9-diazabicyclo[3.3.1]nonanes; opiod receptors; binding affinities; antinociceptive activity; molecular docking

1. Introduction

Over the years many compounds, typified by a bridged piperazine structure, emerged for their interesting analgesic properties. The strong homology between the 4-anilidopiperidines, as fentanyl, deriving from structural simplification of morphine, and the diazabicycloalkane skeleton has made it an intriguing scaffold, whose pharmacomodulation could furnish molecules with marked activity and selectivity towards opioid receptors (Figure 1).

Among the variously substituted diazabicycloalkane skeletons, the 9-cinnamyl-3-propionyl-3,9-diazabicyclo[3.3.1]nonanes **1** showed an interesting affinity for μ-opioid receptors with K_i values in nM range [1,2]. In particular, we found that the 9-cinnamyl-3-propionyl-3,9-diazabicyclo[3.3.1]nonane (**1a**) and the 9-[(2*E*)-3-phenylbut-2-enyl]-3-propionyl-3,9-diazabicyclo[3.3.1]nonane (**1b**) exhibited a significant affinity and selectivity towards μ-opioid receptors with a K_i = 13 nM for **1a** [1] and 91 nM for **1b** [2] (Figure 1).

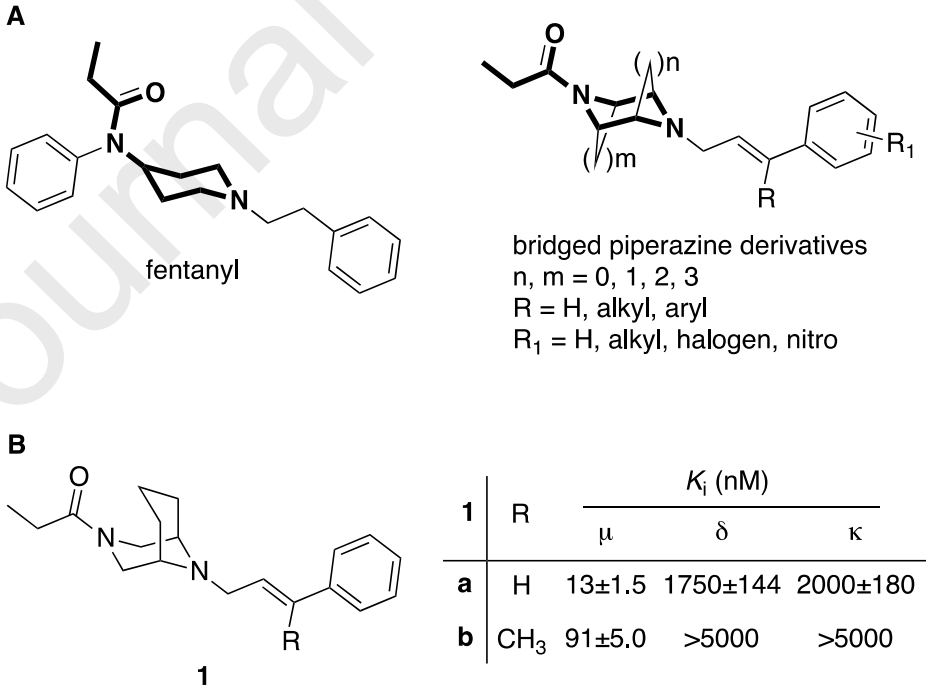
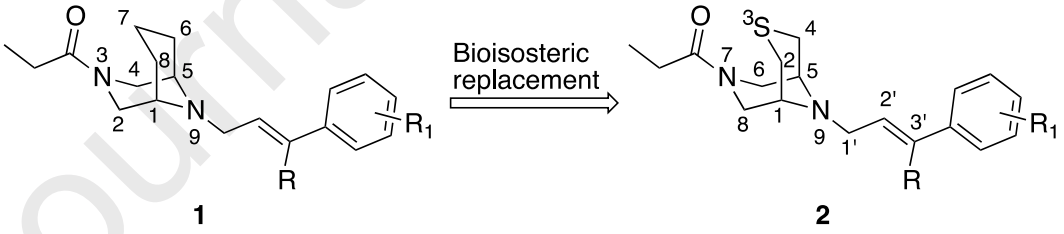


Figure 1. (A) Pharmacophore structural correlations between fentanyl and diazabicycloalkane skeleton. (B) 9-Substituted-3-propionyl-3,9-diazabicyclo[3.3.1]nonanes (DBN).

Molecular modelling studies suggest that the trimethylene loop of 3,9-diazabicyclo[3.3.1]nonane (DBN) plays an essential role in modulating μ-affinity by fitting lipophylic pockets of the receptor [1]. Therefore, with the aim of deepening the studies on the biological profile of the DBN core towards μ receptor, we designed the bioisosteric replacement [3] of methylene group at the C-7 position on the endopropylene bridge of **1** with a more lipophilic sulphur atom (Figure 2), in order to evaluate if this $–CH_2$-/-S- modification can play a role on opioid receptors selectivity. This modification led to the identification of a novel scaffold, the 3-thia-7,9-diazabicyclo[3.3.1]nonane (S-DBN) **2**, which includes similar spatial and chemical characteristics of the DBN, satisfying the peculiarities of the pharmacophore for the μ-receptor. On the other hand, the new S-DBN scaffold provides a platform that gives the chance to several structural modifications and allows the opportunity to investigate the μ-receptor functional activity.



1 **2**

Figure 2. DBN - 3-thia-7,9-diazabicyclo[3.3.1]nonanes (S-DBN) bioisosterism.

We report the synthesis and the binding data against μ-, δ- and κ-receptors of a new series of 3-thia-7,9-diazabicyclo[3.3.1]nonanes **2a-j** (Table 1) related to 3,9-diazabicyclo[3.3.1]nonanes **1**. With respect to parent nonanes **1**, compounds **2** maintain propionyl group at N_7 diazabicyclononane

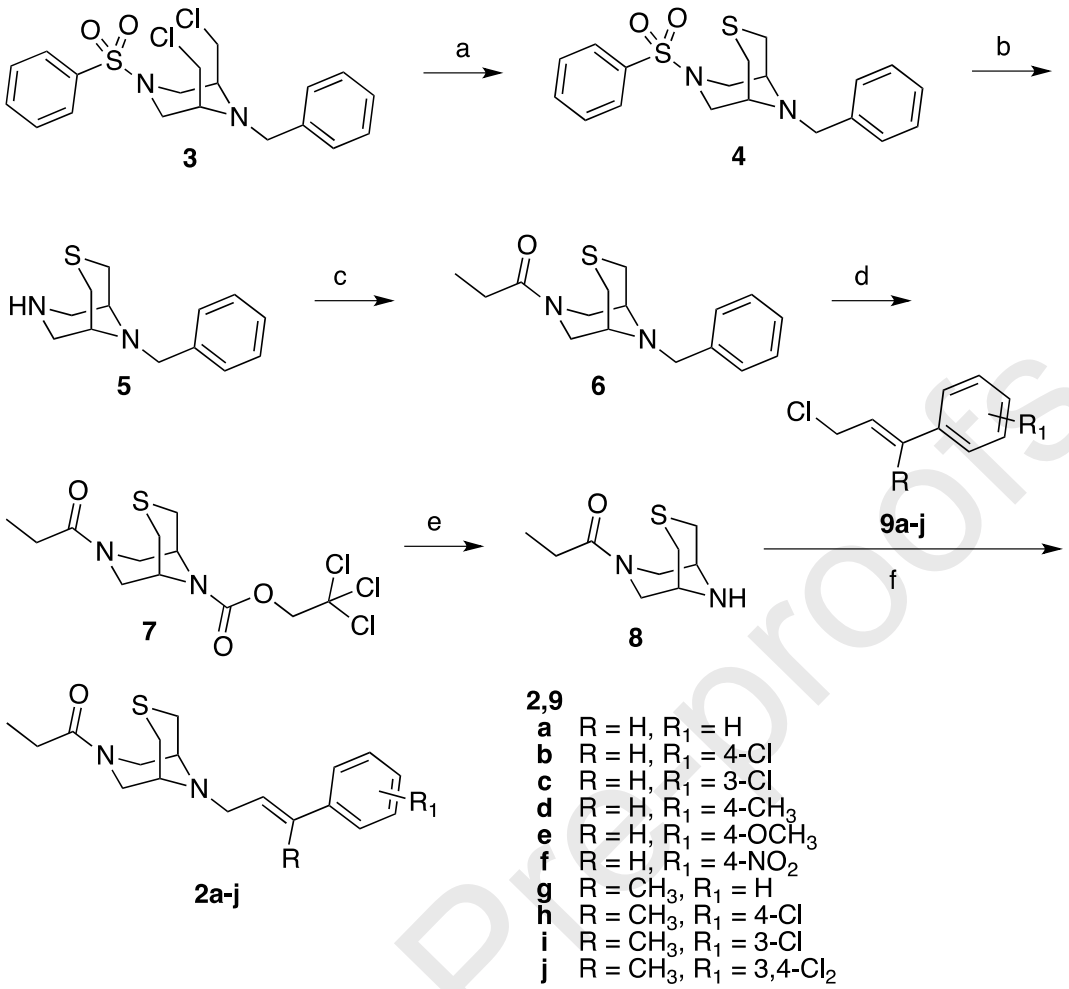
position and are functionalized with several cinnamyl substituents, in particular at the C-3' position of the allyl chain and at different positions of aromatic ring (Figure 3 and Table 1).

Furthermore, we evaluated the *in vivo* activity of representative term **2i** to better define its pharmacological profile and performed docking studies on these new derivatives.

2. Chemistry

Compounds **2a-j** were prepared through the synthetic pathway reported in Scheme 1.

The starting dichloro derivative **3** [4] was cyclized with Na_2S to 9-benzyl-7-benzensulfonyl-3-thia-7,9-diazabicyclo[3.3.1]nonane (**4**) in refluxing AcOEt/EtOH. The chemical structure of **4** was supported by nuclear magnetic resonance (NMR), infrared (IR), mass (LC/MS), and elemental analysis data: the substitution of two chlorine atoms of **3** with a sulphur was clearly visible at ^{1}H-NMR, due to the signals of the hydrogen adjacent to the chalcogenide that appeared shielded with respect to the corresponding starting material. Finally, the mass of the sample showed the molecular ion at 375.0 m/z.



Scheme 1. Synthesis of 3-thia-7,9-diazabicyclo[3.3.1]nonanes**.** Reagents and conditions: (a) $Na_2S{\cdot}9H_2O$, EtOAc/EtOH, 80 °C, 5 h; (b) $NaAlH_2(OCH_2CH_2OCH_3)_2$, anhydrous xylene, 140 °C, 1.5 h; (c) $(CH_3CH_2CO)_2O$, CH_2Cl_2, 40 °C, 1 h; (d) Cl_3CCH_2OCOCl, CH_3CN, 80 °C, 8 h; (e) Zn, AcOH, rt, 14 h; (f) K_2CO_3, CH_3CN, rt, 24 h.

The availability of **4** allowed the synthesis of 7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane (**8**) as the key intermediate for the final compounds **2a-j**. The benzensulfonyl group of compound **4** was cleaved using sodium *bis*-(2-methoxyethoxy)aluminum hydride (Red-Al) in refluxing xylene to give the N_7-unprotected diazabicyclononane derivative **5**. Desulfonylation to amine was confirmed both by ^{1}H-NMR and IR analysis: the first evidenced the NH chemical shift at δ H 2.91, which exchange

with D_2O, whereas infrared spectroscopy showed both a characteristic absorption band of the amine at 3322 cm^{-1} and the absence of that of sulfonyl group at 1300 cm^{-1}. The N_7-propionylation of compound **5** with propionic anhydride in CH_2Cl_2 furnished the bicyclic derivative **6**. The introduction of a carbonyl on the nitrogen created a dissymmetry effect that involved the H and the C atoms immediately adjacent (positions 6 and 8 of the bicycle system). In fact, ^{1}H- ^{13}C-HSQC (Heteronuclear Single Quantum Correlation) showed resonances at δ H 3.22 and 4.65 for H-6, and δ H 3.70 and 3.84 for H-8 that crossed on δ C 43.8 and 47.8, respectively (Figure 3). Removal of the benzyl protecting group of **6**, by reaction with 2,2,2-trichloroethyl chloroformate (Troc-Cl), gave the corresponding trichloroethyl carbamate, intermediate **7**, whose treatment with zinc powder in acetic acid at room temperature, allowed to obtain the 7-propionyl-thiadiazabicyclononane **8**. The final reaction of **8** with the appropriate (*E*)-cinnamyl chloride **9a-j** [5-8] afforded the target S-DBN derivatives **2a-j**.

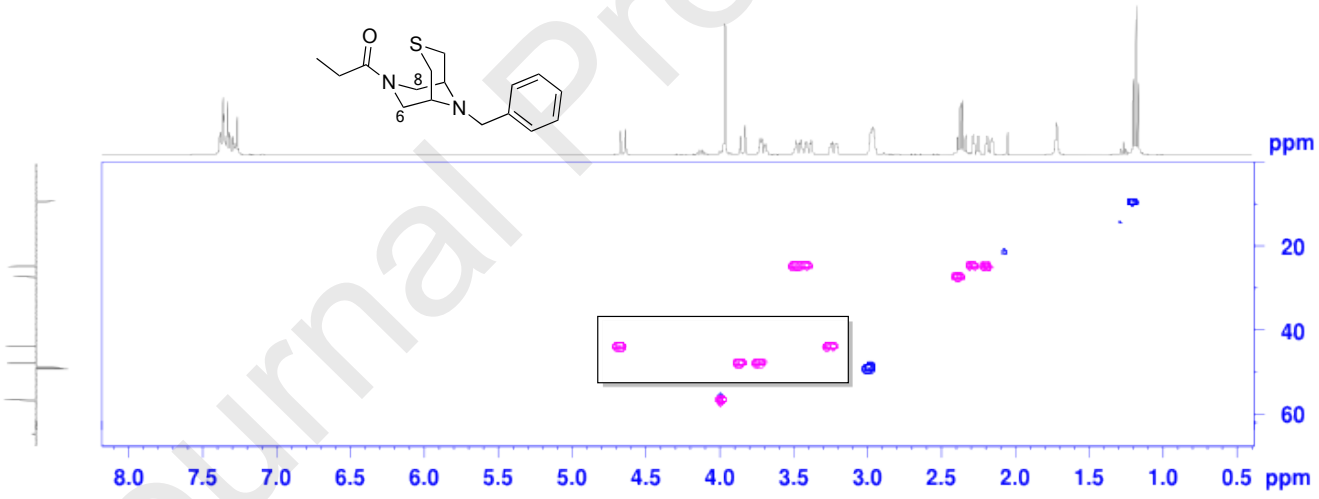


Figura 3. **^{1}H- ^{13}C**-HSQC spectrum of compound **6**.

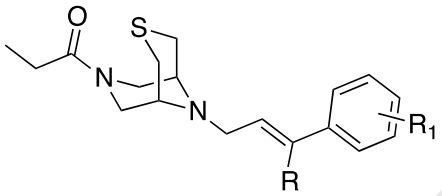
3. Results and discussion

3.1. Binding and functional assay.

The affinity of novel opioid receptor ligands was evaluated by means of radioligand binding studies on rat brain membranes by using ^{3}H-DAMGO, ^{3}H-DELTORPHINE II and ^{3}H-U69593 for μ,

δ and κ receptor subtypes, respectively. Morphine affinity was also tested and it was used as the reference compound (see Table 1).

Table 1. Binding affinity of **2a-j** and of reference opioid ligand morphine, for opioid receptors K_i (nM).[a]



2a-j

Compd[b]	**R**	**R_1**	Receptor Affinity K_i (nM) ± SEM			Selectivity	
			μ	δ	κ	K_i *ratio* δ/μ	K_i *ratio* κ/μ
2a	H	H	450±47	>10000	3250±330	>22.2	7.2
2b	H	4-Cl	4000±380	>10000	>10000	>2.5	>2.5
2c	H	3-Cl	210±23	>10000	>10000	>47.6	>47.6
2d	H	4-CH_3	850±77	>10000	>10000	>11.8	>11.8
2e	H	4-OCH_3	4500±475	>10000	>10000	>2.2	>2.2
2f	H	4-NO_2	1300±135	>10000	>10000	>7.7	>7.7
2g	CH_3	H	230±27	>10000	>10000	>43.5	>43.5
2h	CH_3	4-Cl	300±32	>10000	>10000	>33.3	>33.3
2i	CH_3	3-Cl	85±9	5000±450	>10000	58.8	>117.6
2j	CH_3	3,4-Cl_2	400±41	>10000	>10000	>25.0	>25.0
Morphine			3.35±0.30	100.2±5.1	280.8±9.2	29.9	83.8

[a]K_i values were determined by competitive displacement of radioligands [^{3}H]DAMGO, [^{3}H]Deltorphin-2, [^{3}H]U69593 for μ, δ, κ Opioid Receptors, respectively. The K_i was calculated from the IC_{50} values determined from the binding curves, using the Cheng-Prusoff equation. Values are the mean ± SEM of at least three independent experiments run in triplicate. [b]The receptor binding affinities of all compounds **2a-j** were carried on their hydrochlorides.

In general, the bioisosteric replacement of a methylene group on the endopropilenic bridge of DBN with a sulfur atom to give the thia-diazabicyclononane analogue (S-DBN) afforded compounds endowed with a lower biological profile. The pharmacomodulation of the new scaffold contemplated both the introduction of aryl-substituted cinnamyl chain on N_9 of S-DBN and the virtual substitution of the allyl H with a methyl group, with the aim to investigate the effect of such modifications on μ receptor affinity and selectivity. In general, all new compounds show moderate binding affinity to the receptor and very low binding affinity to both δ and κ receptors. Compound **2a**, bearing the cinnamyl chain on N_9, showed K_i values of 450, >10000, and 3250 nM for μ, δ, and κ opioid receptors, respectively, with a δ/μ selectivity ratio >22. Compound **2a** was assumed as the suitable lead for the synthesis of compounds reported in Table 1 and to present preliminary structure-activity relationships (SARs). The introduction of a 4- or 3-chloro atom on the phenyl ring of the cinnamyl chain, led to compounds **2b** and **2c**, respectively. Their μ receptor affinities were either decreased with *para* substitution (**2b**, K_i = 4000 nM) or increased with *meta* (**2c**, K_i = 210 nM) substitution when compared with **2a**. The 4-methyl analogue **2d** showed 1.9-fold lower affinity (K_i = 850 nM) than **2a**, whereas both the 4-methoxy (**2e**) and 4-nitro (**2f**) substituted derivatives displayed no significant μ receptor affinity, (K_i = 4500 nM and K_i = 1300 nM, respectively). These findings suggest that the binding affinity does not appear influenced by the nature of substituent (electron-withdrawing or electron-donating) on the phenyl ring on cinnamyl chain.

The replacement of the allyl hydrogen with a methyl group led to aryl methylallyl derivatives **2g-j**. This small series of compounds showed a better μ receptor profile than the lead **2a** and analogues above. In fact, compound **2g**, the phenyl methylallyl analogue of **2a**, showed a 1.95-fold increase of μ receptor affinity (K_i = 230 nM) than **2a**. The same trend was observed for compound **2h**, with an increase of 13.3-fold of receptor affinity (K_i = 300 nM) with respect to 4-chloro analogue **2b**. Compound **2i**, the 3-chlorophenyl methylallyl N_9-substituted, showed the highest μ receptor affinity (K_i = 85 nM), both among the small series of four phenyl methylallyl derivatives and the previous

one, resulting an increase of 2.47- and 5.29-fold than **2c** and the lead **2a**, respectively, whereas its activity is similar to that of compound **1a**. Moreover, compound **2i** showed a good μ/δ selectivity (58.8-fold). The dichloro-substituted analogue **2j** maintained a μ receptor affinity (K_i = 400 nM) comparable to **2a**.

The capability of the compounds to activate the μ recombinant human opioid receptors stably transfected in CHO cells (CHOμ) was also evaluated. In such cells the expression of a Gαq/i hybrid mutant allows measuring receptor activation with an automated calcium mobilization assay. Dermorphin was used as standard ligand for mu receptors. In CHOμ cells the standard agonist dermorphin evoked a robust concentration-dependent stimulation of calcium release displaying high potency (pEC50 of 8.06) and maximal effects (254 ± 18% over the basal values). Morphine mimicked the effects of dermorphin showing however lower potency (pEC50 of 6.00) and maximal effects (105 ± 17% over the basal values), thus behaving as partial agonist. The new ligand 2i displayed an incomplete concentration response curve being able to stimulate calcium mobilization only at 10 and 100 micromolar concentrations, suggesting a behaviour as a low potency mu receptor agonist. The concentration response curves obtained with these ligands in CHOμ cells are displayed in Figure 4.

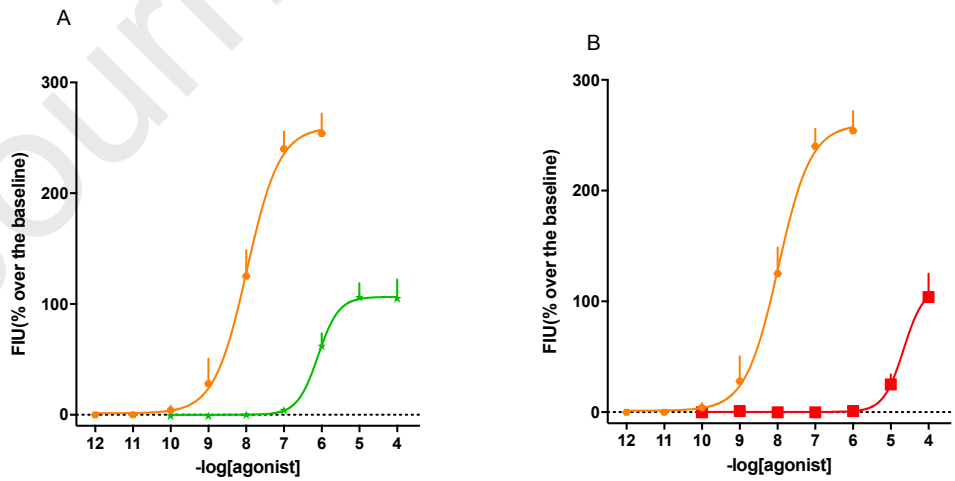


Figure 4. Calcium mobilization assay. Concentration response curves to dermorphin (orange) and morphine (green) (panel A) or **2i** (red) (panel B) in CHO_{μ} cells. Data are the mean ± sem of at least 5 separate experiments made in duplicate.

3.2. Pharmacological Evaluation.

Among the most interesting compounds found in binding assay, **2i**, **2c** and **2g** were selected for evaluation of antinociceptive properties. Compound **2i** showed a dose-dependent antinociceptive activity against a thermal stimulus. The dose of 1 mg/kg s.c. was ineffective, the dose of 10 mg/kg significantly increased the mouse pain threshold and the maximal effect was detected at 20 mg/kg (Figure 5A). Compounds **2c** (Figure 5B) and **2g** (Figure 5C) were also endowed with antinociceptive activity, but their effect appeared at a 5-time higher dose (100 mg/kg s.c.). The highest effective doses of **2i**, **2c** and **2g** produced an antinociceptive effect of intensity comparable to morphine 7 mg/kg s.c., used as reference drug (Figures 5A,B,C). The most promising molecule was compound **2i** that was selected for further investigations. Time-course studies showed that antinociceptive activity of compound **2i** reached the statistical significance 15 min after administration, peaked at 30 min and then slowly diminished disappearing at 60 min. This time-course profile paralleled that of morphine (7 mg/kg s.c.), used as reference drug (Figure 5D). Investigations into the mechanism of antinociceptive action showed that the increase of pain threshold produced by compound **2i** was completely prevented by the μ opioid receptor antagonist CTOP (0.001 μg per mouse i.c.v.) (Figure 5E). Furthermore, compound **2i**, administered at a dose 1000 times lower than the analgesic one, produced a paradoxical hyperalgesia (Figure 5F), a typical opioid effect mediated by μ receptors [9,10], further confirming a μ-mediated mechanism of action for the modulation of pain threshold.

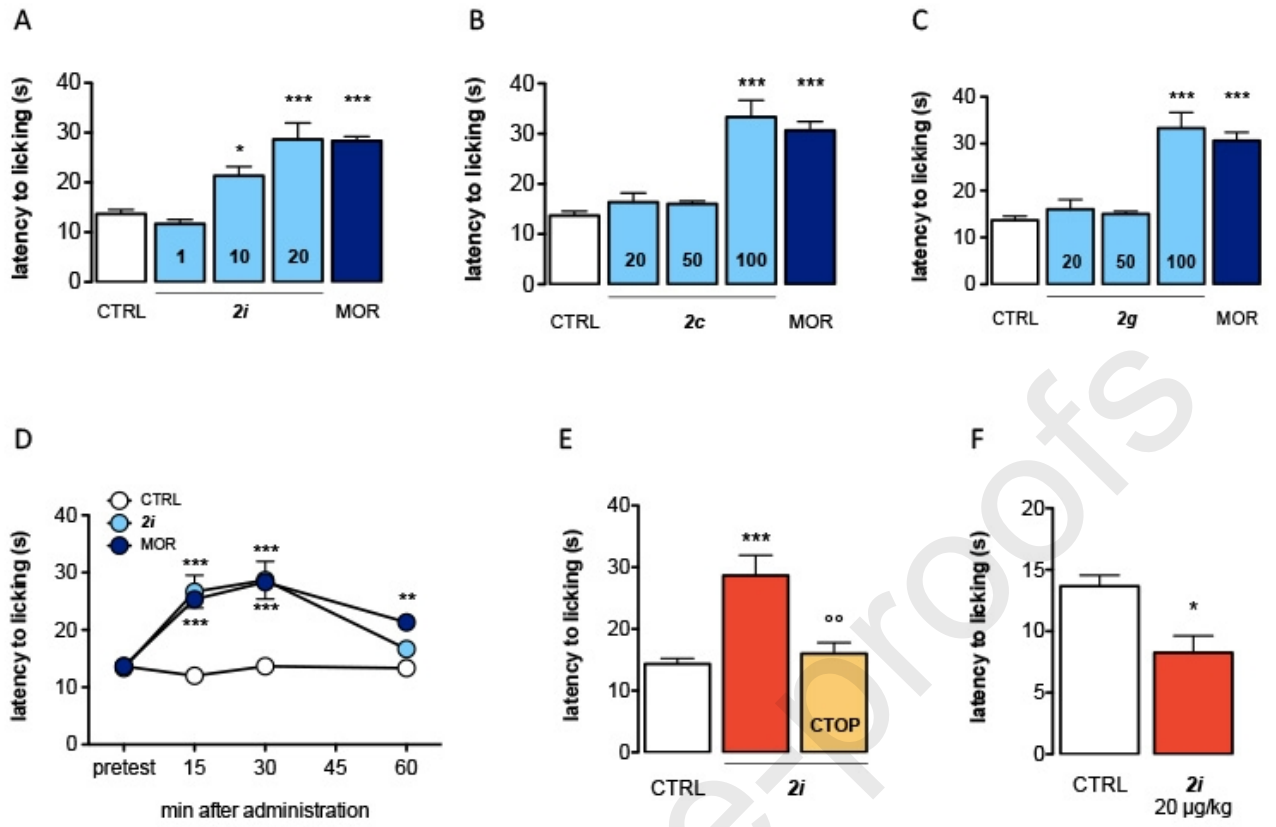


Figure 5. Antinociceptive profile of compounds **2i**, **2c** and **2g** in the mouse hot plate test (acute thermal stimulus). (**A**) Dose response curve for compound **2i** (1-20 mg/kg s.c.). (**B**) Dose response curve for compound **2c** (20-100 mg/kg s.c.). (**C**) Dose response curve for compound **2g** (20-100 mg/kg s.c.). The latency to licking values were recorded 30 min after administration. Morphine (MOR; 7 mg/kg s.c.) was used as reference drug. (**D**) Time course experiments showed that the antinociceptive effect of compound **2i** peaked 30 min after administration, similarly to the reference drug morphine (7 mg/kg s.c.). (**E**) Prevention of compound **2i**-induced antinociception by the μ opioid receptor antagonist CTOP (0.001 μg per mouse i.c.v.). (**F**) Paradoxical hypernociceptive effect induced by an extremely low dose of compound **2i** (20 μg/kg s.c.). *P<0.05, **P<0.01, ***P<0.001 vs control group (CTRL); °°P<0.01 vs compound **2i**-treated group (prestest).

To define the pharmacological profile of compound **2i**, the development of tolerance to the analgesic treatment was investigated. The antinociceptive responses produced by increasing doses of compound **2i**, administered twice daily for 5 days, in the hot plate test are shown in Figure 6A. The administration

of compound **2i** on day 1 produced a maximal antinociceptive effect, but successive daily injections resulted in a progressive loss of the drug-induced antinociceptive response, which approached baseline value on day 5. The progressive decrease in the antinociceptive responses produced by increasing doses of morphine, used as reference drug, was comparable to that observed with compound **2i** (Figure 6B).

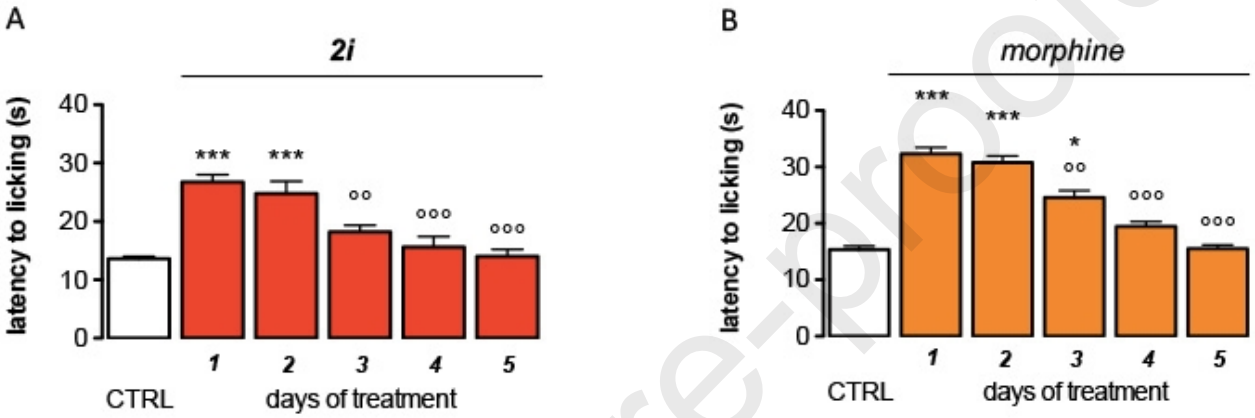


Figure 6. Tolerance to the antinociceptive effect of compound **2i**. Time course of the antinociceptive effect of repeated administration of compound **2i** (**A**) and of morphine (**B**), used as reference drug, delivered twice daily. Nociceptive testing (hot plate test) was performed 30 min after each injection. *P<0.05, ***P<0.001 versus saline-treated group; °°P<0.01, °°°P<0.001 versus morphine effect on day 1.

Compound **2i**, at the highest effective dose, did not modify animals' gross behaviour and did not produce any visible sign of toxicity. To exclude the possibility that these results were due to any motor impairment, we additionally performed behavioural measures of locomotor activity on treated mice by means of the rotarod and hole board tests to add objective measures to the study of locomotor behaviour. Rotarod performance (Figure 7A), spontaneous mobility (Figure 7B) and exploratory activity (Figure 7C) remained unaltered in compound **2i**-treated mice in comparison to control mice.

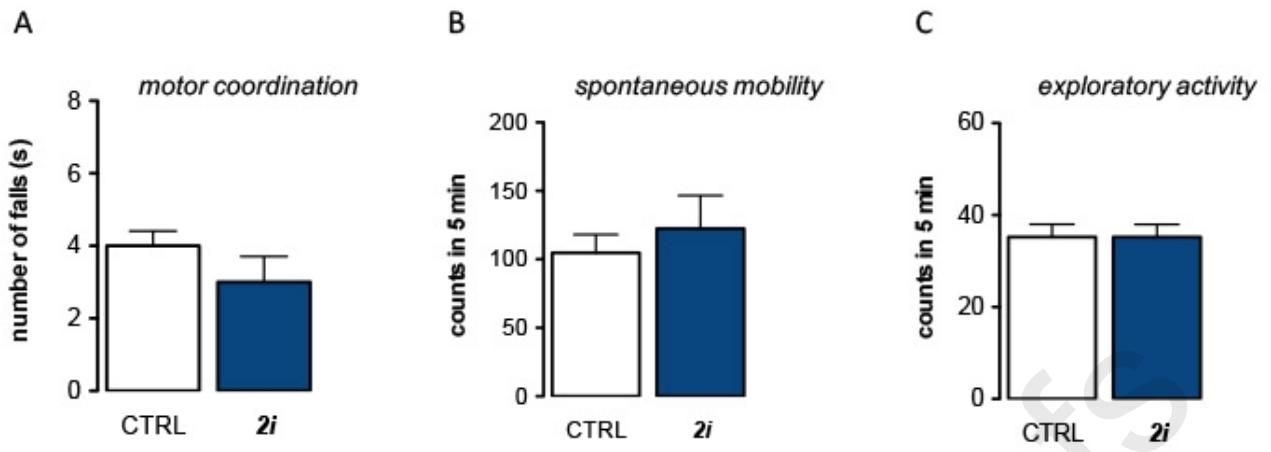


Figure 7. Lack of effect of compound **2i** on locomotor behaviour. (**A**) The motor coordination of mice, evaluated as number of falls form the rotating rod, was unaltered after administration of compound **2i**. Compound **2i** did not modify the number of movements on an open field (spontaneous mobility) (**B**) and of explorations of the holes (exploratory activity) (**C**). Compound **2i** was administered at the dose of 20 mg/kg s.c. 30 min before testing.

3.3. Molecular Docking

A docking simulation was conducted to rationalize the structure activity relationship with a possible binding mode within the μ-opoid receptor. While the automatic scoring function could not be correlated to activity, there is a clear distinction in orientation between high and low affinity binders. High affinity binders **2c**, **2g**, **2h** and **2i** are all oriented in a similar manner within the binding pocket that allows the formation of an ionic interaction the central nitrogen and Asp147, an interaction that is also shown for other reference ligands. Additionally, hydrogen bonds with water molecules 526 (for **2c**, **2g**, and **2i**) and 505 (for **2c** and **2h**) are formed. Hydrophobic interactions occur with Ile144, Trp133, and Val143 and furthermore with Tyr148 for **2i** (Figure 8A) and Ile322 for **2h**.

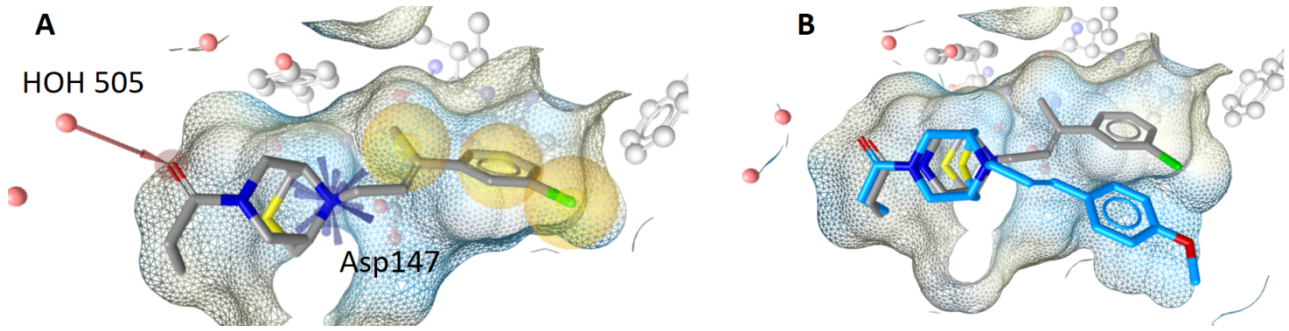


Figure 8. (**A**) Docking pose of high affinity binder **2i** within the binding pocket of the μ-opoid receptor. The red arrow indicates a hydrogen bond with the cocrystallized water molecule 526, whereas the blue star marks a positive ionisable interaction with Asp 147. (**B**) shows **2i** (grey) in comparison with the almost inactive compound **2e** (blue). The larger methoxy group causes the aromatic ring to be pushed out of the binding site.

For the lower affinity binders **2b**, **2f**, and **2e**, a hydrogen bond with the amino acids Asn127 and Tyr75 (only missing for **2e**) can be formed. Compared to the more active compounds this can cause a shift of the aromatic ring into another part of the binding pocket (Figure 8B) possibly resulting in a less favourable binding mode and consequently in a loss of activity.

4. Conclusions

In this study we investigated the binding affinities for μ, δ and κ receptor of a small series of 7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonanes. A preliminary SAR study, based on structural modification on the cinnamyl portion in N_9 of S-DBN, evidenced that all S-DBN novel derivatives had μ receptor binding affinity in the nM range although not higher than that of DBN analogues, whereas the affinity towards δ and κ receptors is negligible. This would demonstrate that the sulphur

atom is important for μ receptor selectivity in these novel derivatives. However, from these preliminary SAR studies, some conclusions can be made: the sulphur atom results an important feature for a hydrogen bond interaction with Tyr148, whereas the other two structural modifications, as the modulation of the phenyl ring in the cinnamyl chain and the virtual introduction of a methyl group in the place of the allyl H, highlighted two different aspects. Concerning the aromatic ring we can conclude that the substitution with an electron-withdrawing or electron-donating group, both in *meta*- or *para*-position, is detrimental for the binding affinity of these compounds. On the other hand the introduction of a methyl group in the cinnamyl chain leads to an increase of μ receptor affinity. Therefore, although the sulphur atom on S-DBN derivatives is a useful feature for the additional hydrogen bond interaction with the Tyr148 residue, however it is not sufficient to provide a better μ-receptor affinity with respect to the not-sulphured DBN (**1a** and **1b**). Nevertheless, the simultaneous introduction of a *m*-chlorine on the phenyl ring led to compound **2i**, endowed with the highest affinity and selectivity for μ receptor among all new synthesized derivatives, with K_i values comparable to those of compounds **1**, showing the highest analgesic efficacy in acute thermal pain assay. Furthermore, it showed no locomotor-enhancing effects, which are believed one of the main side effects of morphine-induced analgesia, neither seems to induce drug abuse due to the lack of hyperlocomotion.

These preliminary findings encourage us to continue to focus our efforts on further SAR on N_7- and N_9-positions of S-DBN.

5. Experimental procedures

5.1. General procedures

All reactions involving air or moisture-sensitive compounds were performed under nitrogen atmosphere. Unless otherwise specified, all materials, solvents, reagents, and (*E*)-cinnamyl chloride

9a were purchased from commercial suppliers and were used without further purification. The compound **3**[4] and the requisite arylalkenylchloride **9b-j** [5-8] were prepared as reported in literature.

Flash column chromatography was performed automatically on Flash-master (Biotage®) with pre-packed Biotage® SNAP silica gel cartridges or manually on silica gel (Kieselgel 60, 0.040−0.063 mm, Merck®). Thin layer chromatography (TLC) was performed with Polygram SIL N-HR/HV_{254} pre-coated plastic sheets (0.2 mm) on aluminum sheets (Kieselgel 60 F254, Merck®). Melting points were obtained on a Köfler melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded as thin films (for oils) or nujol mulls (for solids) on NaCl plates with a Jasco FT/IR 460 plus spectrophotometer and are expressed in ν (cm^{-1}). NMR experiments were run on a Bruker Avance III Nanoboy 400 system (400.13 MHz for ^{1}H, and 100.62 MHz for ^{13}C). Spectra were acquired using deuterated chloroform (Chloroform-*d*) or deuterated dimethylsulfoxide (DMSO-d_6) as solvents. Chemical shifts (δ) for ^{1}H- and ^{13}C-NMR spectra are reported in parts per million (ppm) using the residual non-deuterated solvent resonance as the internal standard (for Chloroform-*d*: 7.26 ppm, ^{1}H and 77.16 ppm, ^{13}C; for DMSO-d_6: 2.50 ppm, ^{1}H, 39.52 ppm, ^{13}C). Data are reported as follows: chemical shift (sorted in descending order), multiplicity (s for singlet, br s for broad singlet, d for doublet, dd for double doublet, t for triplet, dt for double triplet, q for quadruplet, m for multiplet), coupling constants (*J*) in Hertz (Hz), and integration. LC/MS analyses were run on an Agilent 1100 LC/MSD system consisting of a single quadrupole detector (SQD) mass spectrometer (MS) equipped with an electrospray ionization (ESI) interface and a photodiode array (PDA) detector; PDA range was 120−550 nm. ESI in positive mode was applied.

Purity of compounds. All final compounds purity was determined by elemental analysis on a PerkineElmer 240- B analyser, for C, H, and N. All of the final compounds were found to be >95% when analysed.

The general procedure for conversion of compounds **2a-j** to their hydrochloride salts was the addition of excess ethereal HCl solution to a solution of the opportune compound **2a-j** in ethanol or diethyl

ether. The solvent was evaporated and the resulting salt was triturated with anhydrous ether and dried on vacuum.

5.1.2. 7-Benzensulfonyl-9-benzyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(4)***.

$Na_2S{\cdot}9H_2O$ (1.16 g, 4.84 mmol) was added to a solution of **3** [4] (0.40 g, 0.97 mmol) in EtOH (3.3 mL) and EtOAc (2.2 mL) at room temperature under nitrogen atmosphere. The reaction mixture was heated for 5 h at 80 °C, then allowed to cool to room temperature, diluted with water, and extracted with CH_2Cl_2. The organic layer was separated, dried (Na_2SO_4), and evaporated under reduced pressure. The residue was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford pure **4** as white solid. Yield 98%; R_f 0.40 (petroleum ether/EtOAc 8:2); mp 107-108 °C; IR (nujol): *v* 1360 (SO_2), 695 (C–S); ^{1}H-NMR ($CDCl_3$): δ 7.80 (d, J = 7.6 Hz, 2H), 7.64-7.52 (m, 3H), 7.32-7.22 (m, 5H), 3.88 (s, 2H), 3.72 (d, J = 9.6 Hz, 2H), 3.43-3.37 (m, 2H), 3.04-2.97 (m, 4H), 2.26 (d, J = 13.6 Hz, 2H); ^{13}C-NMR ($CDCl_3$): δ 137.8 (C), 136.2 (C), 132.7 (CH), 129.1 (CH x 2), 128.6 (CH x 2), 128.6 (CH x 2), 127.8 (CH x 2), 127.6 (CH), 56.7 (CH_2), 48.6 (CH x 2), 48.0 (CH_2 x 2), 23.8 (CH_2 x 2); MS (ESI): $C_{19}H_{22}N_2O_2S_2$ requires m/z 374.1, found 375.0 $[M+H]^+$. Anal. calcd for $C_{19}H_{22}N_2O_2S_2$: C, 60.93; H, 5.92; N, 7.48. Found: C, 60.78; H, 5.90; N, 7.45.

5.1.3. 9-Benzyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(5)***.

To a stirred suspension of **4** (8.10 g, 21.63 mmol) in anhydrous xylene (159 mL) under inert atmosphere, was added dropwise a 65% solution of Red-Al in toluene (18.82 mL, 62.94 mmol). The obtained yellow solution was refluxed at 140 °C for 2 h, then allowed to cool to room temperature, and poured onto ice and acidified with 2N HCl. The mixture was washed three times with EtOAc. Then, the acid acqueous layer was separed, neutralized with 40% NaOH, and extracted with a solution of EtOAc/EtOH 50:1. The combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc/MeOH/NH_4OH 8:2:0.1) to obtain the diazabicyclononane **5** as light yellow oil. Yield 78%; R_f 0.20 (EtOAc/MeOH/NH_4OH 8:2:0.4); IR (nujol): *v* 3323 (NH), 697 (C–S); ^{1}H-NMR ($CDCl_3$): δ 7.38 (d, J = 7.2 Hz, 2H), 7.34-

7.29 (m, 2H), 7.27-7.22 (m, 1H), 3.97 (s, 2H), 3.54-3.48 (m, 2H), 3.44-3.38 (m, 2H), 3.14 (d, J = 13.6 Hz, 2H), 2.88 (br s, 1H), 2.71-2.69 (m, 2H), 2.22 (d, J = 13.6 Hz, 2H); ^{13}C-NMR ($CDCl_3$): δ 138.6 (C), 128.6 (CH x 2), 128.5 (CH x 2), 127.3 (CH), 56.8 (CH_2), 50.5 (CH_2 x 2), 48.9 (CH x 2), 23.5 (CH_2 x 2); Anal. calcd for $C_{13}H_{18}N_2S$: C, 66.62; H, 7.74; N, 11.95. Found: C, 66.43; H, 7.72; N, 11.92.

5.1.4. 9-Benzyl-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(6)****.*

To a solution of **5** (0.10 g, 0.43 mmol) in CH_2Cl_2 (3 mL) at 0 °C was added a solution of propionic anhydride (0.19 mL, 1.49 mmol) in CH_2Cl_2 (1 mL). When addition was complete, the mixture was refluxed for 1 h. After cooling at room temperature, the mixture was made alkaline with 40% NaOH, and stirred overnight. The reaction mixture was extracted with CH_2Cl_2, the organic layers dried over Na_2SO_4, evaporated, and the residue was purified by FC eluting with petroleum ether: EtOAc 4:6, to afford pure **6** as light yellow oil. Yield 81%; R_f 0.15 (petroleum ether: EtOAc 4:6); IR (nujol): ν 1639 (CO), 698 (C–S); ^{1}H-NMR ($CDCl_3$): δ 7.38-7.25 (m, 5H), 4.65 (d, J = 13.4 Hz, 1H), 3.96 (s, 2H), 3.84 (d, J = 13.2 Hz, 1H), 3.70 (dd, J = 2.5 and 13.0 Hz, 1H), 3.49-3.36 (m, 2H), 3.22 (dd, J = 3.4 and 13.2 Hz, 1H), 2.99-2.92 (m, 2H), 2.36 (q, J = 7.4 Hz, 2H), 2.26 (d, J = 13.2 Hz, 1H), 2.17 (d, J = 12.8 Hz, 1H), 1.18 (t, J = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (C), 137.9 (C), 128.5 (CH x 2), 128.4 (CH x 2), 127.4 (CH), 56.4 (CH_2), 49.0 (CH), 48.7 (CH), 47.6 (CH_2), 43.6 (CH_2), 27.0 (CH_2), 24.7 (CH_2), 24.5 (CH_2), 9.1 (CH_3); Anal. calcd for $C_{16}H_{22}N_2OS$: C, 66.17; H, 7.64; N, 9.65. Found: C, 65.99; H, 7.61; N, 9.62.

5.1.5. 7-Propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(8)****.*

To a stirred solution of **6** (1.70 g, 5.85 mmol) in CH_3CN (45 mL) was added 2,2,2-trichloroethyl chloroformate (0.82 mL, 5.97 mmol) at room temperature. The reaction mixture was stirred for 8 h at 80 °C. Then, the solution was allowed to cool to room temperature, diluted with water, and extracted with EtOAc. The organic layer was washed with water and 15% citric acid, separated, dried (Na_2SO_4), and evaporated under reduced pressure. The crude carbamate intermediate

7 was dissolved in AcOH (29 mL). To solution was added zinc powder (3.83 g, 58.53 mmol) at room temperature. After being stirred for 14 h, insoluble substance was removed by filtration. The filtrate was treated with 25% NH_4OH and extracted with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by flash chromatography ($CHCl_3$/MeOH 9.5:0.5) to obtain **8** as light yellow oil. Yield 56%; R_f 0.31 ($CHCl_3$/MeOH 9.5:0.5); IR (nujol): *v* 1625 (CO); ^{1}H-NMR ($CDCl_3$): δ 4.76 (d, J = 13.2 Hz, 1H), 3.94 (d, J = 13.2 Hz, 1H), 3.82 (d, J = 13.2 Hz, 1H), 3.52-3.35 (m, 4H), 3.28 (d, J = 13.6 Hz, 1H), 3.21 (d, J = 13.6 Hz, 1H), 2.57 (d, J = 11.2 Hz, 1H), 2.53 (d, J = 11.2 Hz, 1H), 2.28 (q, J = 7.2 Hz, 2H), 1.10 (t, J = 7.2 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.6 (CO), 47.6 (CH_2), 44.8 (CH), 44.4 (CH), 43.5 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 26.9 (CH_2), 9.0 (CH_3); Anal. calcd for $C_9H_{16}N_2OS$: C, 53.97; H, 8.05; N, 13.99. Found: C, 53.83; H, 8.03; N, 13.96.

5.1.6. General procedure for preparation of compounds **2a-j**.

A mixture of propionyl bicyclononane **8** (0.50 g, 2.50 mmol), opportune (*E*)-cinnamyl chloride **9a-j** [5-8] (3.00 mmol, 1.2 eq), and anhydrous K_2CO_3 (1.21 g, 8.75 mmol, 3.5 eq) in 15 mL of anhydrous acetonitrile under inert atmosphere, was allowed to stir at room temperature for 24 h. The solution was filtered and the volatiles were evaporated under reduced pressure. The residue was purified by flash chromatography to afford the desired pure target compounds **2a-j** as oils All final compounds were converted into the HCl salts.

5.1.7. 9-Cinnamyl-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2a)***.

General procedure was used to convert **8** and commercially available (*E*)-cinnamyl chloride (**9a**) into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2a**. Yield 35%; R_f 0.31 (EtOAc); mp 157-159 °C (as hydrochloride salt); IR (nujol): *v* 1630 (CO); ^{1}H-NMR ($CDCl_3$): δ 7.38 (d, J = 7.4 Hz, 2H), 7.35-7.30 (m, 2H), 7.28-7.22 (m, 1H), 6.59 (d, J = 15.7 Hz, 1H), 6.18 (dt, J = 6.5 and 15.9 Hz, 1H), 4.67 (d, J = 13.3 Hz, 1H), 3.86 (d, J = 13.3 Hz, 1H), 3.74-3.66 (m, 1H), 3.60 (d, J = 6.6 Hz, 2H), 3.48-3.33 (m, 2H), 3.25-3.18 (m, 1H),

3.10-3.04 (m, 2H), 2.36 (q, *J* = 7.3 Hz, 2H), 2.27 (d, *J* = 13.5 Hz, 1H), 2.18 (d, *J* = 12.7 Hz, 1H), 1.18 (t, *J* = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (CO), 136.6 (C), 133.0 (CH), 128.7 (CH x 2), 128.1 (CH), 127.8 (CH), 126.4 (CH x 2), 54.8 (CH_2), 49.0 (CH), 48.9 (CH), 47.6 (CH_2), 43.7 (CH_2), 27.0 (CH_2), 24.5 (CH_2), 24.3 (CH_2), 9.1 (CH_3); MS (ESI): $C_{18}H_{24}N_2OS$ requires m/z 316.2, found 317.2 $[M+H]^+$. Anal. calcd for $C_{18}H_{24}N_2OS$: C, 68.32; H, 7.64; N, 8.85. Found: C, 68.14; H, 7.62; N, 8.82.

5.1.8. (E) 9-[3'-(4-Chlorophenyl)-prop-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2b)****.*

General procedure was used to convert **8** and (*E*)-4-chlorocinnamyl chloride (**9b**)[5] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2b**. Yield 39%; R_f 0.33 (EtOAc); mp 175-177 °C (as hydrochloride salt); IR (nujol): *v* 1627 (CO); ^{1}H-NMR ($CDCl_3$): δ 7.35-7.25 (m, 4H), 6.55 (d, *J* = 16.0 Hz, 1H), 6.17 (dt, *J* = 6.5 and 15.7 Hz, 1H), 4.67 (d, *J* = 13.6 Hz, 1H), 3.87 (d, *J* = 13.6 Hz, 1H), 3.75-3.68 (m, 1H), 3.60 (d, *J* = 6.5 Hz, 2H), 3.48-3.33 (m, 2H), 3.25-3.18 (m, 1H), 3.09-3.04 (m, 2H), 2.36 (q, *J* = 7.4 Hz, 2H), 2.27 (d, *J* = 13.0 Hz, 1H), 2.20 (d, *J* = 14.1 Hz, 1H), 1.17 (t, *J* = 7.3 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.6 (CO), 135.0 (C), 133.4 (C), 131.9 (CH), 128.8 (CH x 2), 127.8 (CH), 127.6 (CH x 2), 54.7 (CH_2), 49.1 (CH x 2), 47.6 (CH_2), 43.6 (CH_2), 27.0 (CH_2), 24.6 (CH_2), 24.4 (CH_2), 9.1 (CH_3); MS (ESI): $C_{18}H_{23}ClN_2OS$ requires m/z 350.1, found 351.1 $[M+H]^+$. Anal. calcd for $C_{18}H_{23}ClN_2OS$: C, 61.61; H, 6.61; N, 7.98. Found: C, 61.50; H, 6.60; N, 7.96.

5.1.9. (E) 9-[3'-(3-Chlorophenyl)-prop-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2c)****.*

General procedure was used to convert **8** and (*E*)-3-chlorocinnamyl chloride (**9c**) [5] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2c**. Yield 31%; R_f 0.33 (EtOAc); mp 170-172 °C (as hydrochloride salt); IR (nujol): *v* 1625 (CO); ^{1}H-NMR ($CDCl_3$): δ 7.39-7.19 (m, 4H), 6.54 (d, *J* = 15.8 Hz, 1H), 6.19 (dt, *J* = 6.5 and 15.7 Hz, 1H), 4.66 (d, *J* = 13.5 Hz, 1H), 3.86 (d, *J* = 13.2 Hz, 1H), 3.73-3.66 (m, 1H), 3.59 (d, *J* =

6.3 Hz, 2H), 3.46-3.32 (m, 2H), 3.24-3.17 (m, 1H), 3.07-3.02 (m, 2H), 2.36 (q, *J* = 7.4 Hz, 2H), 2.26 (d, *J* = 13.5 Hz, 1H), 2.19 (d, *J* = 13.3 Hz, 1H), 1.17 (t, *J* = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (CO), 138.4 (C), 134.6 (C), 131.5 (CH), 129.8 (CH x 2), 127.7 (CH), 126.3 (CH), 124.6 (CH), 54.6 (CH_2), 49.1 (CH), 49.0 (CH), 47.6 (CH_2), 43.6 (CH_2), 27.0 (CH_2), 24.5 (CH_2), 24.3 (CH_2), 9.1 (CH_3); MS (ESI): $C_{18}H_{23}ClN_2OS$ requires m/z 350.1, found 351.1 $[M+H]^+$. Anal. calcd for $C_{18}H_{23}ClN_2OS$: C, 61.61; H, 6.61; N, 7.98. Found: C, 61.47; H, 6.59; N, 7.95.

5.1.10. (E) 9-[3'-(4-Methylphenyl)-prop-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2d)****.*

General procedure was used to convert **8** and (*E*)-4-methylcinnamyl chloride (**9d**) [6] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2d**. Yield 40%; R_f 0.30 (EtOAc); mp 162-164 °C (as hydrochloride salt); IR (nujol): *v* 1629 (CO); ^{1}H-NMR ($CDCl_3$): δ 7.27 (d, *J* = 8.0 Hz, 2H), 7.13 (d, *J* = 7.9 Hz, 2H), 6.55 (d, *J* = 15.7 Hz, 1H), 6.11 (dt, *J* = 6.6 and 15.7 Hz, 1H), 4.66 (d, *J* = 13.8 Hz, 1H), 3.85 (d, *J* = 13.0 Hz, 1H), 3.73-3.66 (m, 1H), 3.58 (d, *J* = 6.6 Hz, 2H), 3.47-3.33 (m, 2H), 3.24-3.17 (m, 1H), 3.09-3.04 (m, 2H), 2.36 (q, *J* = 7.4 Hz, 2H), 2.34 (s, 3H), 2.26 (d, *J* = 13.3 Hz, 1H), 2.17 (d, *J* = 13.3 Hz, 1H), 1.18 (t, *J* = 7.3 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (CO), 137.6 (C), 133.7 (C), 132.9 (CH), 129.3 (CH x 2), 126.2 (CH x 2), 125.3 (CH), 54.8 (CH_2), 49.0 (CH), 48.8 (CH), 47.6 (CH_2), 43.6 (CH_2), 27.0 (CH_2), 24.5 (CH_2), 24.3 (CH_2), 21.2 (CH_3), 9.1 (CH_3); MS (ESI): $C_{19}H_{26}N_2OS$ requires m/z 330.2, found 331.2 $[M+H]^+$. Anal. calcd for $C_{19}H_{26}N_2OS$: C, 69.05; H, 7.93; N, 8.48. Found: C, 68.89; H, 7.91; N, 8.45.

5.1.11. (E) 9-[3'-(4-Methoxyphenyl)-prop-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2e)****.*

General procedure was used to convert **8** and (*E*)-4-methoxycinnamyl chloride (**9e**) [5] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2e**. Yield 38%; R_f 0.27 (EtOAc); mp 72-74 °C (as hydrochloride salt); IR (nujol): *v* 1632

(CO); ^{1}H-NMR ($CDCl_3$): δ 7.32 (d, J = 7.8 Hz, 2H), 6.86 (d, J = 7.6 Hz, 2H), 6.53 (d, J = 15.6 Hz, 1H), 6.03 (dt, J = 6.5 and 16.0 Hz, 1H), 4.66 (d, J = 14.0 Hz, 1H), 3.86 (d, J = 13.2 Hz, 1H), 3.81 (s, 3H), 3.73-3.66 (m, 1H), 3.57 (d, J = 6.7 Hz, 2H), 3.47-3.33 (m, 2H), 3.24-3.17 (m, 1H), 3.10-3.05 (m, 2H), 2.36 (q, J = 7.4 Hz, 2H), 2.26 (d, J = 13.3 Hz, 1H), 2.17 (d, J = 13.6 Hz, 1H), 1.18 (t, J = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (CO), 159.3 (C), 132.5 (CH), 129.3 (C), 127.5 (CH x 2), 124.1 (CH), 114.0 (CH x 2), 55.3 (CH_3), 54.9 (CH_2), 49.0 (CH), 48.8 (CH), 47.6 (CH_2), 43.6 (CH_2), 27.0 (CH_2), 24.5 (CH_2), 24.3 (CH_2), 9.1 (CH_3); MS (ESI): $C_{19}H_{26}N_2O_2S$ requires m/z 346.2, found 347.2 $[M+H]^+$. Anal. calcd for $C_{19}H_{26}N_2O_2S$: C, 65.86; H, 7.56; N, 8.08. Found: C, 65.71; H, 7.54; N, 8.06.

*5.1.11. (E) 9-[3'-(4-Nitrophenyl)-prop-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane **(2f)**.*

General procedure was used to convert **8** and (*E*)-4-nitrocinnamyl chloride (**9f**) [7] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2f**. Yield 28%; R_f 0.15 (EtOAc); mp 164-166 °C (as hydrochloride salt); IR (nujol): v 1627 (CO), 1536 (NO_2), 1376 (NO_2); ^{1}H-NMR ($CDCl_3$): δ 8.18 (d, J = 8.6 Hz, 2H), 7.51 (d, J = 8.6 Hz, 2H), 6.68 (d, J = 15.9 Hz, 1H), 6.38 (dt, J = 6.4 and 15.8 Hz, 1H), 4.67 (d, J = 13.3 Hz, 1H), 3.88 (d, J = 13.2 Hz, 1H), 3.75-3.68 (m, 1H), 3.64 (d, J = 6.2 Hz, 2H), 3.48-3.32 (m, 2H), 3.25-3.18 (m, 1H), 3.08-3.01 (m, 2H), 2.37 (q, J = 7.4 Hz, 2H), 2.29 (d, J = 13.6 Hz, 1H), 2.21 (d, J = 12.8 Hz, 1H), 1.18 (t, J = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (CO), 147.0 (C), 138.2 (C), 130.6 (CH), 128.7 (CH x 2), 126.9 (CH x 2), 124.0 (CH), 54.5 (CH_2), 49.2 (CH x 2), 47.6 (CH_2), 43.6 (CH_2), 27.0 (CH_2), 24.6 (CH_2), 24.4 (CH_2), 9.1 (CH_3); MS (ESI): $C_{18}H_{23}N_3O_3S$ requires m/z 361.1, found 362.1 $[M+H]^+$. Anal. calcd for $C_{18}H_{23}N_3O_3S$: C, 59.81; H, 6.41; N, 11.63. Found: C, 59.63; H, 6.39; N, 11.60.

*5.1.12. (E) 9-(3'-Phenyl-but-2'-en-1'-yl)-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane **(2g)**.*

General procedure was used to convert **8** and (*E*)-(4-chlorobut-2-en-2-yl)benzene (**9g**) [8] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure

compound **2g**. Yield 33%; R_f 0.32 (EtOAc); mp 159-161 °C (as hydrochloride salt); IR (nujol): *v* 1625 (CO); ^{1}H-NMR ($CDCl_3$): *δ* 7.40 (d, *J* = 8.0 Hz, 2H), 7.36-7.30 (m, 2H), 7.29-7.23 (m, 1H), 5.79 (t, *J* = 6.6 Hz, 1H), 4.67 (d, *J* = 13.6 Hz, 1H), 3.86 (d, *J* = 13.6 Hz, 1H), 3.73-3.67 (m, 1H), 3.61 (d, *J* = 6.8 Hz, 2H), 3.47-3.33 (m, 2H), 3.25-3.18 (m, 1H), 3.09-3.04 (m, 2H), 2.37 (q, *J* = 7.4 Hz, 2H), 2.28 (d, *J* = 13.3 Hz, 1H), 2.20 (d, *J* = 14.6 Hz, 1H), 2.12 (s, 3H), 1.18 (t, *J* = 7.5 Hz, 3H); ^{13}C-NMR ($CDCl_3$): *δ* 172.5 (CO), 143.0 (C), 138.2 (C), 128.3 (CH x 2), 127.2 (CH), 125.7 (CH x 2), 124.8 (CH), 50.5 (CH_2), 49.2 (CH), 49.1 (CH), 47.6 (CH_2), 43.7 (CH_2), 27.0 (CH_2), 24.8 (CH_2), 24.6 (CH_2), 16.4 (CH_3), 9.1 (CH_3); MS (ESI): $C_{19}H_{26}N_2OS$ requires m/z 330.2, found 331.2 $[M+H]^+$. Anal. calcd for $C_{19}H_{26}N_2OS$: C, 69.05; H, 7.93; N, 8.48. Found: C, 68.89; H, 7.92; N, 8.46.

5.1.13. (E) 9-[3'-(4-Chlorophenyl)-but-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2h)****.*

General procedure was used to convert **8** and (*E*)-1-chloro-4-(4-chlorobut-2-en-2-yl)benzene (**9h**) [8] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2h**. Yield 45%; R_f 0.35 (EtOAc); mp 57-59 °C (as hydrochloride salt); IR (nujol): *v* 1630 (CO); ^{1}H-NMR ($CDCl_3$): *δ* 7.32 (d, *J* = 8.7 Hz, 2H), 7.28 (d, *J* = 8.7 Hz, 2H), 5.77 (t, *J* = 6.6 Hz, 1H), 4.67 (d, *J* = 13.3 Hz, 1H), 3.87 (d, *J* = 13.0 Hz, 1H), 3.73-3.66 (m, 1H), 3.60 (d, *J* = 6.5 Hz, 2H), 3.47-3.32 (m, 2H), 3.24-3.17 (m, 1H), 3.07-3.02 (m, 2H), 2.36 (q, *J* = 7.4 Hz, 2H), 2.29 (d, *J* = 13.0 Hz, 1H), 2.20 (d, *J* = 13.8 Hz, 1H), 2.09 (s, 3H), 1.18 (t, *J* = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): *δ* 172.5 (CO), 141.3 (C), 137.1 (C), 133.0 (C), 128.4 (CH x 2), 127.0 (CH x 2), 124.7 (CH), 50.5 (CH_2), 49.2 (CH x 2), 47.6 (CH_2), 43.6 (CH_2), 27.0 (CH_2), 24.8 (CH_2), 24.6 (CH_2), 16.3 (CH_3), 9.1 (CH_3); MS (ESI): $C_{19}H_{25}ClN_2OS$ requires m/z 364.1, found 365.1 $[M+H]^+$. Anal. calcd for $C_{19}H_{25}ClN_2OS$: C, 62.53; H, 6.90; N, 7.68. Found: C, 62.36; H, 6.88; N, 7.65.

5.1.14. (E) 9-[3'-(3-Chlorophenyl)-but-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2i).***

General procedure was used to convert **8** and (*E*)-1-chloro-3-(4-chlorobut-2-en-2-yl)benzene (**9i**) [8] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2i**. Yield 47%; R_f 0.34 (EtOAc); mp 53-55 °C (as hydrochloride salt); IR (nujol): *v* 1626 (CO); ^{1}H-NMR ($CDCl_3$): δ 7.39-7.35 (m, 1H), 7.30-7.21 (m, 3H), 5.80 (t, *J* = 6.5 Hz, 1H), 4.68 (d, *J* = 13.5 Hz, 1H), 3.87 (d, *J* = 13.3 Hz, 1H), 3.74-3.66 (m, 1H), 3.60 (d, *J* = 6.5 Hz, 2H), 3.46-3.32 (m, 2H), 3.24-3.17 (m, 1H), 3.07-3.01 (m, 2H), 2.37 (q, *J* = 7.3 Hz, 2H), 2.29 (d, *J* = 13.8 Hz, 1H), 2.20 (d, *J* = 13.2 Hz, 1H), 2.09 (s, 3H), 1.18 (t, *J* = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (CO), 144.8 (C), 137.0 (C), 134.3 (C), 129.5 (CH), 127.2 (CH), 126.1 (CH), 125.9 (CH), 123.8 (CH), 50.5 (CH_2), 49.2 (CH x 2), 47.7 (CH_2), 43.7 (CH_2), 27.1 (CH_2), 24.7 (CH_2), 24.5 (CH_2), 16.3 (CH_3), 9.1 (CH_3); MS (ESI): $C_{19}H_{25}ClN_2OS$ requires m/z 364.1, found 365.1 $[M+H]^+$. Anal. calcd for $C_{19}H_{25}ClN_2OS$: C, 62.53; H, 6.90; N, 7.68. Found: C, 62.41; H, 6.88; N, 7.66.

5.1.15. (E) 9-[3'-(3,4-Dichlorophenyl)-but-2'-en-1'-yl]-7-propionyl-3-thia-7,9-diazabicyclo[3.3.1]nonane ***(2j).***

General procedure was used to convert **8** and (*E*)-1,2-dichloro-4-(4-chlorobut-2-en-2-yl)benzene (**9j**) [8] into the title product. The crude product was purified by flash chromatography (EtOAc) to afford pure compound **2j**. Yield 37%; R_f 0.37 (EtOAc); mp 76-78 °C (as hydrochloride salt); IR (nujol): *v* 1620 (CO); ^{1}H-NMR ($CDCl_3$): δ 7.50 (d, *J* = 2.4 Hz, 1H), 7.42 (d, *J* = 8.4 Hz, 1H), 7.25 (dd, *J* = 2.0 and 8.4 Hz, 1H), 5.89 (t, *J* = 8.4 Hz, 1H), 4.67 (d, *J* = 13.2 Hz, 1H), 3.86 (d, *J* = 13.0 Hz, 1H), 3.75-3.64 (m, 1H), 3.60 (d, *J* = 6.6 Hz, 2H), 3.46-3.31 (m, 2H), 3.24-3.16 (m, 1H), 3.08-3.00 (m, 2H), 2.37 (q, *J* = 7.4 Hz, 2H), 2.29 (d, *J* = 13.4 Hz, 1H), 2.20 (d, *J* = 13.6 Hz, 1H), 2.10 (s, 3H), 1.18 (t, *J* = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 172.5 (CO), 144.9 (C), 137.0 (C), 134.4 (C), 133.9 (C), 128.6 (CH), 126.8 (CH), 124.9 (CH), 124.7 (CH), 50.5 (CH_2), 49.2 (CH x 2), 47.6 (CH_2), 43.7 (CH_2), 27.0 (CH_2), 24.8 (CH_2), 24.6 (CH_2), 16.3 (CH_3), 9.1 (CH_3); MS (ESI): $C_{19}H_{24}Cl_2N_2OS$ requires m/z 398.1, found 399.1 $[M+H]^+$. Anal. calcd for $C_{19}H_{24}Cl_2N_2OS$: C, 57.14; H, 6.06; N, 7.01. Found: C, 56.99; H, 6.04; N, 7.00.

5.2. Receptor binding and functional assays.

Binding studies in rat brain membranes were carried out as previously described [11], with slight modifications. Briefly, membranes were freshly prepared from whole brains minus cerebellum. ^{3}H-DAMGO (1 nM), DELTORPHINE II (1 nM) and U69593 (1 nM) were used to label μ-, δ and κ-receptors. Membranes were incubated with the appropriate ^{3}H-ligand in 50 mM Tris HCl pH 7.4 at 25 °C for 60 min in the absence or in the presence of 10 μM Naloxone. Final protein concentration was determined by the method of Biorad, with bovine serum albumin as standard.

Functional assays were carried out in CHO cells lines permanently co-expressing μ receptors with the C-terminally modified Gαqi5 as described in [12]. Cells were cultured in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) / HAMS F12 (1:1) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml), geneticin (G418; 200 μg/ml) and hygromycin B (100 μg/ml). Cell cultures were kept at 37 °C in 5% CO_2 / humidified air. When confluence was reached (3-4 days), cells were sub-cultured as required using trypsin / EDTA and used for experimentation. Cells were seeded at a density of 50,000 cells / well into 96-well black, clear-bottom plates. After 24 hours incubation the cells were loaded with Hank's Balanced Salt Solution (HBSS) supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM, 0.01% pluronic acid and 20 mM HEPES (pH 7.4) for 30 min at 37 °C. Afterwards the loading solution was aspirated and a washing step with 100 μl / well of HBSS, HEPES (20 mM, pH 7.4), 2.5 mM probenecid and 500 μM Brilliant Black was carried out. Subsequently 100 μl / well of the same buffer was added. After placing cell culture and compound plates into the FlexStation II (Molecular Devices, Sunnyvale, CA, USA), changes in fluorescence of the cell-loaded calcium sensitive dye Fluor-4 AM were measured. On-line additions were carried out in a volume of 50 μl/well. Brilliant black, bovine serum albumin (BSA), 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and probenecid were from Sigma Aldrich (St. Louis, MO, USA). Pluronic acid and Fluo-4 AM were from Thermo Fisher Scientific (Waltham, US). All cells

culture media and supplements were from Euroclone (Milano, Italy). Morphine was dissolved in distilled water (10 mM), while 2i compound was dissolved in dimethyl sulfoxide (DMSO, 10 mM). The stock solutions were kept at -20 °C until use.

5.3. Animals

Male CD1 mice (Harlan Laboratories, Bresso, Italy) were used. Mice were randomly assigned to standard cages, with four to five animals per cage. The cages were placed in the experimental room 24 h before behavioural test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. All tests were conducted during the light phase. Mice weighed between 23 and 40 g. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee of the University of Florence, Italy, under license from the Italian Department of Health (54/2014-B) and in compliance with international laws and policies (Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). All studies involving animals are reported in accordance with the ARRIVE guidelines for experiments involving animals [13]. All effort was taken to minimize the number of animals used and their suffering.
Mice were sacrificed by cervical dislocation for removal of spinal cord and sciatic nerve for in vitro analyses. The number of animals per experiment was based on a power analysis [14]. For antinociceptive and locomotor assays all tested groups comprised ten animals.

5.4. Reagents for behavioural testing

D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NHh2 (CTOP) (Sigma Chemicals St Louis, MO, USA) and morphine hydrochloride (SALARS, Milan, Italy) were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use and administered in a volume of 5 µl per mouse by intracerebroventricular (i.c.v.) injection, performed as previously described [15], or 10 ml/kg by subcutaneous (s.c.) administration. Doses and administration schedules of substances applied in this study were based on doses shown to be effective in previous reports.

5.5. Behavioural testing

Animals were habituated to the testing environment daily for at least 2 days before baseline testing and randomly assigned to each treatment group by an individual other than the operator. All testing was performed with a blind procedure.

5.6. Hot-plate test

The hot plate test was performed as previously described [16]. Mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1 °C in a precision water-bath. The hot-plate apparatus (Ugo Basile Biological Research Apparatus, Varese, Italy) was 25 x 37 x 47(h) cm. Reaction times (s) were measured with a stopwatch before and 15, 30, 45, 60 min after administration of morphine. The endpoint used was the licking of the fore or hind paws. Those mice scoring less than 12 and more than 18 s in the pre-test were rejected (30%). An arbitrary cut-off time of 60 s was adopted.

5.7. Rotarod test

Potential motor incoordination/ataxia caused by compound injection was evaluated using a rota-rod apparatus in which animals were required to walk against the motion of a rotating rod for 30 s. The rod, 30 cm in length, was placed at a height of 15 cm from the base and divided into 5 equal sections by 6 disks. Thus, up to 5 mice were tested simultaneously on the apparatus, with a rod-rotation speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s.

5.8. Hole-board test

The spontaneous locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 10 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane

(spontaneous mobility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

5.9. Tolerance to analgesic treatment

Morphine tolerance was induced with a 5-day scheme. The mice received 2 daily injections of morphine at 10:00 am and 8:00 pm. The individual doses were 10 mg/kg s.c on day 1, 15 mg/kg s.c. on day 2, 20 mg/kg s.c. on day 3, and 30 mg/kg s.c. on day 4 and 5. Similarly, ***2i*** tolerance was induced with a 5-day scheme. The mice received 2 daily injections of ***2i*** at 10:00 am and 8:00 pm. The individual doses were 20 mg/kg s.c on day 1, 25 mg/kg s.c. on day 2, 30 mg/kg s.c. on day 3, and 40 mg/kg s.c. on day 4 and 5.

5.10. Statistical analysis

All experimental results are given as the mean ± s. e. mean. Data were analysed using one-way or two-way ANOVA. Tukey's test was used for *post hoc* analysis following a significant one-way ANOVA. Multiple comparisons following two-way ANOVA were conducted with Bonferroni *post hoc* comparison. A *P*-value of <0.05 was considered statistically significant. The computer programme GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used in all statistical analyses. All data were analyzed using Graph Pad Prism 6.0 (La Jolla, CA, USA). Functional data are expressed as mean ± sem of *n* experiments performed in duplicate. Agonist effects were expressed as maximum change in percent over the baseline fluorescence. Baseline fluorescence was measured in wells treated with vehicle. Agonist potency was expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. Concentration response curve to agonists were fitted with the four parameter logistic nonlinear regression model:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1+10^{(LogEC_{50} - Log_{[compound]})Hillslope})}$$

5.11. Molecular Docking

All docking studies were performed with GOLD version 5.2. This program uses a genetic algorithm to calculate up to ten docking poses per input-ligand. The scoring function GoldScore which takes into account hydrogen bonding, ligand internal strains, and steric aspects of the receptor-ligand complex was selected.

The crystal structure of the μ- opioid receptor bound to the agonist BU72 (PDB-entry 5c1m) [17] was used for the docking studies. In the course of the protein preparation, hydrogens were added, and all water molecules except for 505, 526 and 538 were deleted. The remaining two water molecules were set to "toggle and spin". This allowed the program to automatically decide whether or not a water molecule is included during docking, and to optimize the orientation of the water molecule. The area of 6 Å around the co-crystallized ligand was defined as binding site. ChemPLP was used as a scoring function. A redocking of the original ligand was conducted to validate the workflow and achieved an RMSD of 1.057.

Docking poses and interaction patterns were analysed in LigandScout 4.4 (www.inteligand.com)

Appendix A. Supplementary data

Supporting Information is available.

Conflict of interest

The authors declare no competing financial interest.

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Dedication

This paper is dedicated to the memory of Professor Giorgio Cignarella.

Abbreviation used

DBN: 7,9-diazabicyclo[3.3.1]nonane, (S-DBN): 3-thia-7,9-diazabicyclo[3.3.1]nonane, Red-Al: *bis*-(2-methoxyethoxy)aluminum hydride, HSQC (Heteronuclear Single Quantum Correlation), Troc-Cl: 2,2,2-trichloroethyl chloroformate.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

NONE

GRAPHICAL ABSTRACT

bridged piperazines
n, m = 0-3
R = H, alkyl, aryl
R_1 = H, EWD, EDG

- Synthesis and characterization of novel sulphur-bridged diazabicyclo[3.1.1]nonanes.
- μ, δ and κ ligands binding studies.
- Antinociceptive activity.