Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 1283–1291

BIOORGANIC & MEDICINAL CHEMISTRY

# Synthesis and Analgesic Activity of a Series of New Azaalkane Bis-guanidinium and Bis(2-aminoimidazolinium) Compounds

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Received 2 August 2002; accepted 29 November 2002

**Abstract**—In the present paper, we wish to report the synthesis and antinociceptive activity of a series of new azaalkane bis(2-aminoimidazolinium) compounds from which, *N*,*N*′-di(4,5-dihydro-1*H*-imidazol-2-yl)-3-aza-1,6-hexanediamine **2a** has shown the best analgesic properties in vivo in two different assays (i.e., acetic acid-induced writhing test and hot-plate test in mice), as well as oral bioavailability.


## Introduction

Pain is a problem not yet solved by Medicine. According to the World Health Organization, 90% of diseases are associated with pain. Studies from the International Association for the Study of Pain (IASP) show that pain is reported in 50% of cancer patients (all stages) and in 75% of patients with advanced tumours. In addition, according to IASP, in Denmark, researchers found that approximately 38% of the general population suffer from chronic pain,[1] and the yearly costs of back pain and sciatica in the UK are €9 billion, with €1 billion spent each year on direct health care costs.[2] Moreover, there are certain kinds of pain, such as the neuropathic pain associated to amputations, which at present have no adequate treatment. Now, if we take into account the analgesics being used nowadays, those of reference continue to be acetylsalicylic acid and morphine, both isolated in the 19th century.

The classical therapies for pain relief consist mainly in the use of Non-Steroidal Anti-inflammatory Drugs (NSAIDs) and opiates. Both families show quite serious secondary effects such as renal toxicity in the case of the NSAIDs or respiratory depression, tolerance and dependence in the case of opioids. The mechanism of pain transmission is very complex and around 10–15 neuromodulators of the pain response have been found in different studies. Thanks to these investigations, new approaches to this problem are being considered.[3]

In our search for new molecules with analgesic activity, we discovered the lead compound **2a** (Scheme 1) showing an interesting antinociceptive activity in the acetic acid-induced writhing test in mice (73.9 and 63.9% inhibition at 100 mg/kg ip and po, respectively) as well as in the hot-plate test (50% inhibition at 100 mg/kg ip). We decided to prepare a series of simple analogues of this lead in order to investigate the structure–activity relationships (SARs) of this kind of azaalkane derivatives. Thus, the cationic 2-aminoimidazolinium moiety was changed for a guanidinium cation which is very similar structurally and electronically[4] (molecule **2b**), the alkane chain was lengthened (**1a**) or shortened (**3a**) with one methylene group, the secondary amino group was replaced by a methylene group (**4a**) or substituted by a phenylethyl (**5a**) or 3(2-ethyl)indole moiety (**6a**). Moreover, a conformationally restricted analogue was prepared (**13**) in order to investigate the active conformation of this kind of molecules and finally, a mono-2-aminoimidazolinium derivative was synthesised (**14**)

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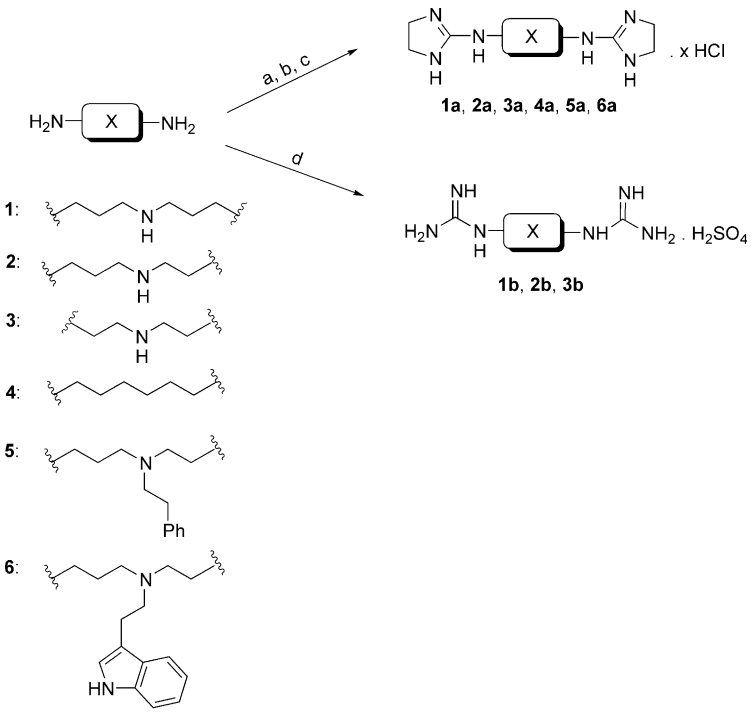
doi:10.1016/S0968-0896(02)00637-5




**Scheme 1.** (a) 2-Methylmercapto-4,5-dihydroimidazole iodide, MeOH, reflux.

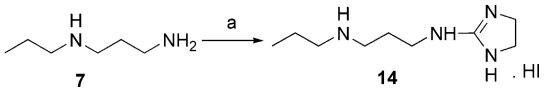
and tested to assess the importance of the two cationic groups at the extremes of the molecules.

In the present article, we report the synthesis and antinociceptive activity of the lead compound **2a** as well as the synthesis and analgesic activity (acetic-induced writhing test in mice) of seven 2-aminoimidazolinium analogues and three guanidinium analogues of this compound. Moreover, the affinity of the lead compound (**2a**) for different receptors was investigated in order to explore its mechanism of action.

## Chemistry

The guanidinium derivatives **1b** and **2b** have been described elsewhere.[5] Following the same methodology involving the nucleophilic substitution of *S*-methylisothiourea by the corresponding primary amines, we also prepared **3b** (Scheme 1). The compounds were purified by crystallisation and isolated as their sulphate salts.

For the 2-aminoimidazolinium derivatives **1a**–**6a** (Scheme 1) and **14** (Scheme 2), a similar procedure was followed using 2-methylmercapto-4,5-dihydroimidazole iodide[6] for the reaction with diamines **1**–**6** in refluxing

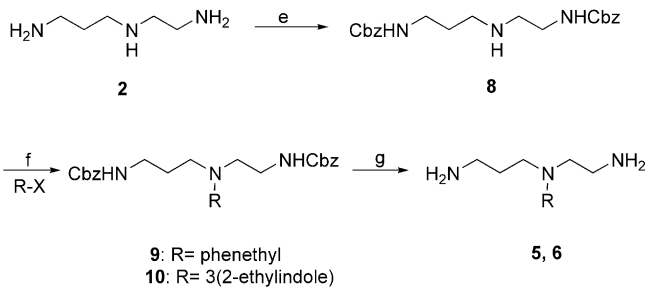



**Scheme 2.** (a) 2-methylmercapto-4,5-dihydroimidazole iodide, MeOH, reflux; (b) picric acid, $H_2O$; (c) IRA400 ($Cl^-$) basic exchange resin, $H_2O$–THF; (d) *S*-methylisothiouronium sulfate, $CH_3CN$, reflux.

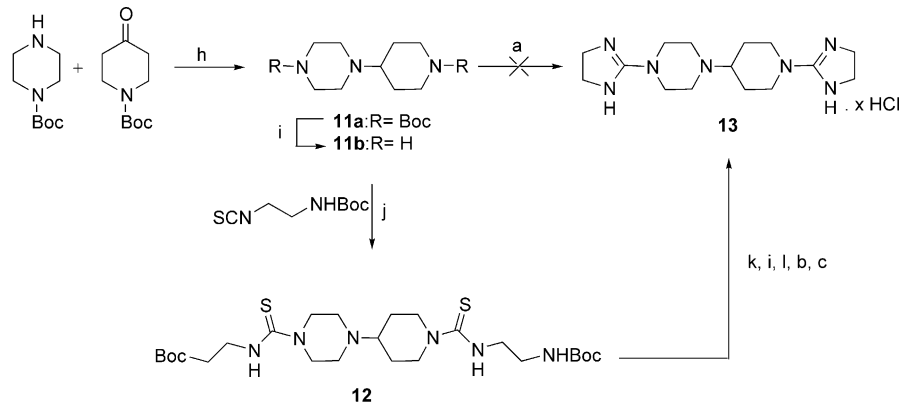
methanol. The compounds were purified either by direct crystallisation of the hydroiodide salt (**3a**, **4a**[7], **14**), or by formation of their picrate salt (**1a**, **2a**, **6a**, **13**) or oxalate salt (**5a**), when direct isolation of the iodide salt from the reaction mixture was unsuccessful. In the case of the purification by formation of the picrate salts, regeneration of the biologically compatible and water soluble hydrochloride salts was carried out with a strongly basic anion exchange resin (IRA400/$Cl^-$ form), yielding after lyophilisation the hydrochloride salts as highly hygroscopic solids.

The non-commercial *N*-substituted amines **5** and **6** were prepared as described in Scheme 3. The polyamine **2** was selectively protected on the primary amino groups with benzylcyanoformate,[8] giving **8** in good yield. The free secondary amino group of **8** was alkylated with 2-bromoethylbenzene and 3(2-bromoethyl)indole in presence of $K_2CO_3$ to give compounds **9** and **10**, respectively. Hydrogenolysis of these compounds with Pd/C 10% in MeOH yielded the corresponding amines **5** and **6**.

The synthesis of the conformationally restricted analogue **13** is depicted in Scheme 4. The 1-(4-piperidyl)piperazine skeleton was formed by reductive amination of *N*-Boc piperidone with *N*-Boc piperazine and sodium cyanoborohydride[9] at pH 7, giving **11a** in 65% yield. The Boc groups were removed easily with TFA to give **11b** as its trifluoroacetate salt quantitatively. Introduction of the imidazolinium cations into the molecule **11b** with 2-methylmercapto-4,5-dihydroimidazole iodide in refluxing ethanol as described above (Scheme 1) was not successful and only starting material




**Scheme 3.** (e) benzylcyanoformate, $CH_2Cl_2$ (79%); (f) $K_2CO_3$, $CH_3CN$, reflux; (g) $H_2$/Pd/C 10%, MeOH.




**Scheme 4.** (h) $NaBH_3CN$, AcOH, MeOH, pH 7 (65%); (i) TFA, $CH_2Cl_2$; (j) THF, rt, 2 days, (50%); (k) EtI, EtOH, 60 °C; (l) NaOEt, EtOH, rt.

was recovered. Hence, another way to generate 2-aminoimidazolinium moiety was attempted, in particular the method described by Anslyn et al.[10] that involves the five-member ring intramolecular cyclisation of a *S*-alkylisothiouronium salt with an amine. Thus, the free base of **11b** reacted with two equivalents of the commercially available [(*tert*-butoxycarbonyl)amino]ethyliso thiocyanate to yield the thiourea **12**. The next three steps were conducted in a one-pot reaction in the following manner. The first step consisted in the alkylation of the thiourea with 2-iodoethanol to form a thioethanol-leaving group. This intermediate was not isolated and was treated with TFA to remove the Boc protecting groups and generate an intramolecular nucleophile that could cyclise in basic conditions, namely with sodium ethoxide, to yield the free base of compound **13**. The product **13** was purified by formation of its picrate salt and then isolated as its hydrochloride salt.

## Pharmacology

The analgesic activity of all the compounds was evaluated with the acetic acid-induced writhing test in mice.[11] The compounds were first screened at an intraperitoneal (ip) dose of 50 mg/kg (Table 1). At this dose, four compounds (**1a**, **5a**, **6a** and **13**) were toxic (>50% death), one compound showed no analgesic activity (**14**) and five derivatives (**2a**, **2b**, **3a**, **3b**, **4a**) had a fair antinociceptive activity (>25% inhibition at 50 mg/kg).

The active molecules were further tested at different doses to assess their analgesic profile, and their $ED_{50}$ values were calculated. The results are reported in Table 2. Morphine was used as reference compound: its antinociceptive effect was evaluated (1 mg/kg: 16±2.8%, 2.5 mg/kg: 44.2±7.7%; 3.5 mg/kg: 79.6±2.8%; 5 mg/kg: 95.6±1.7%; 10 mg/kg: 100%) and its $ED_{50}$ value was calculated (Table 2, footnote).

Compounds **2a**, **2b**, **3a**, **3b** and **4a** showed a dose-dependent inhibition of the acetic acid-induced contractions at 25, 50 and 100 mg/kg, with the lead compound **2a** having a maximum effect of 100% inhibition at 150 mg/kg. The toxicity of compounds **2b** and **4a** appeared at 100 mg/kg with behavioural changes for **2b** and a death rate above 50% for **4a** at this dose. In contrast, no behavioural changes or visible signs of toxicity appeared for compounds **2a**, **3a** and **3b** at the doses tested.

Since the best activity found was that of the lead compound **2a**, the oral bio-availability of that compound

**Table 1.** Analgesic activity screening of azaalkane bis-cationic derivatives

| Compd | Acetic acid-induced writhing test (mice) % inhibition at 50 mg/kg ip |
|---|---|
| **1a** | Toxic @ 25 |
| **2a** | 60.9±2 |
| **2b** | 47.4±1.5 |
| **3a** | 50.4±1.3 |
| **3b** | 30±1.6 |
| **4a** | 24.7±2.4 |
| **5a** | Toxic @ 50 |
| **6a** | Toxic @ 50 |
| **13** | Toxic @ 25 |
| **14** | 0 |

**Table 2.** Analgesic activity profile (ip) and $ED_{50}$ values of compounds **2a**, **2b**, **3a**, **3b** and **4a**

| Compd | % Inhibition of acetic acid-induced writhes in mice | | | | | $ED_{50}$[c] |
|---|---|---|---|---|---|---|
| | 10 mg/kg | 25 mg/kg | 50 mg/kg | 100 mg/kg | 150 mg/kg | mg/kg (confidence interval) |
| **2a** | 30±1.4 | 34.8 | 60.9±2 | 73.9±1.8 | 100 | 30.69 (16.47–57.19) |
| **2b** | —[a] | 12.8±4.1 | 47.4±1.5 | 82.2 ±1.4 | —[a] | 54.91 (7.897–381.8) |
| **3a** | —[a] | 40.2±1 | 50.4±1.3 | 55.3±1.6 | —[a] | 53.14 (19.72-143.20) |
| **3b** | —[a] | 8.4±4.2 | 30±1.6 | 53 ±1.9 | 55±1.7[b] | 110.0 (67.54–179.20) |
| **4a** | −18.5±1.8 | 25.7±1.7 | 24.7±2.4 | 15.5 ±2.9 | —[a] | |

[a]Dosis not tested for these compounds.
[b]Dosis of 125 mg/kg.
[c]Reference: morphine: $ED_{50}$ = 1.8 mg/kg (0.53–6.25).

was tested. Compound **2a**, when orally administered (po), showed a dose-dependent inhibition of writhes of 14% at 25 mg/kg, 30.9% at 50 mg/kg, and 63.9% at 100 mg/kg, with a similar profile to that of the ip administration and a maximum effect of 75.5% inhibition at 150 mg/kg.

The most active compound **2a** was also tested using a second test: the hot-plate test in mice. The mean percentage of the Maximum Possible Effect (M.P.E.; the formula for the calculation of the M.P.E. is defined in the Experimental) induced by the ip administration of **2a** was: 25±4.2% at 50 mg/kg and 50±9.7% at 100 mg/kg. As a comparison, the mean percentage of M.P.E. for morphine was 41±3.2% at 5 mg/kg in this assay.

In order to investigate the possible mechanism of action of **2a**, the compound was screened according to standard protocols for binding and inhibition of a range of targets considered to be involved in pain,[12] discarding any affinity for Cyclooxygenase-2 (COX-2), Nitric Oxide Synthase inducible (iNOS), Bradykinin $B_2$, Dopaminergic $D_{4.2}$, Glutamate NMDA (agonist and phencyclidine), Muscarinic ($M_1$, $M_2$, and $M_4$), Nicotinic Acetylcholine (central), Serotonin 5-$HT_{1A}$ and Tachykinin $NK_1$ receptors. Compound **2a** was also tested in a functional assay with guinea pig ileum (GPI) preparations. This molecule did neither provoke any contractile response of the GPI, nor antagonize the contractile response induced by acetylcholine, serotonine, histamine, bradykinin or substance P. These results suggest that the analgesic effect of **2a** is not due to a direct interaction with these receptors. Moreover, the absence of action mediated by opioid receptors or by $\alpha_1$-, $\alpha_2$-adrenergic receptors was confirmed by functional tests on isolated mouse vas deferens (MVD) preparations; **2a** did not elicit contractile responses in this tissue and was not able to modify the contraction induced by electrical stimulation or by noradrenaline.

## Discussion

From these results, a few conclusions can be drawn regarding the SAR of compound **2a** (Fig. 1). First of all, in general, it seems that only few changes can be made to the structure without losing the analgesic activity or increasing considerably the toxicity of the molecule. Regarding the length of the alkane chain, the addition of one methylene group (**1a**) provokes a high toxicity whereas the shortening in one methylene carbon (**3a**) reduces slightly the activity without inducing toxicity. Regarding the nature of the cationic group, the 2-aminoimidazolinium moiety seems to give slightly better activity than the guanidinium one (Table 1, **2a** vs **2b** and **3a** vs **3b**). The presence of two terminal cationic groups seems to be indispensable as reflected by the lack of activity of the mono-2-aminoimidazolinium compound **14**. Finally, the presence of a non-substituted secondary amino group in the alkylic linker appears to be important to avoid the toxicity in this family of azaalkane compounds (see the toxicity of derivatives **4a**, **5a**, **6a** and **13**).

Concerning the mechanism underlying the analgesic effect of **2a**, functional tests and binding assays permit to discard direct interaction with a wide number of receptors enumerated above. It is interesting to remark that interactions with opioid receptors could be discarded because μ and κ activation induces dose-dependent inhibition of the contractile electrically-induced contractile response in GPI[13] and δ activation inhibits the electrically induced contraction in MVD.[14] This is of great importance because activation of the opioid system is one of the main and most common analgesic mechanisms.

## Conclusion

In this series of bis-guanidinium and bis(2-aminoimidazolinium) azaalkane derivatives synthesised and tested for their antinociceptive activity, the *N*,*N*′-di(4,5-dihydro-1*H*-imidazol-2-yl)-3-aza-1,6-hexanediamine **2a** has shown to be the most potent compound. One of the structural elements that seems to be important is the nature of the cation, because the bis-guanidinium analogue is toxic producing conduct changes in mice. Moreover, the length of the aliphatic chain is rather important. Thus, shortening in one C atom diminishes the activity, whereas the lengthening in one methylene unit increases considerably the toxicity. Finally, the secondary amine group seems to be essential because its absence produces high toxicity. Besides, the introduction over such amine group of aromatic groups such as phenethyl or 3(2-ethylindole) produces very high toxicity.

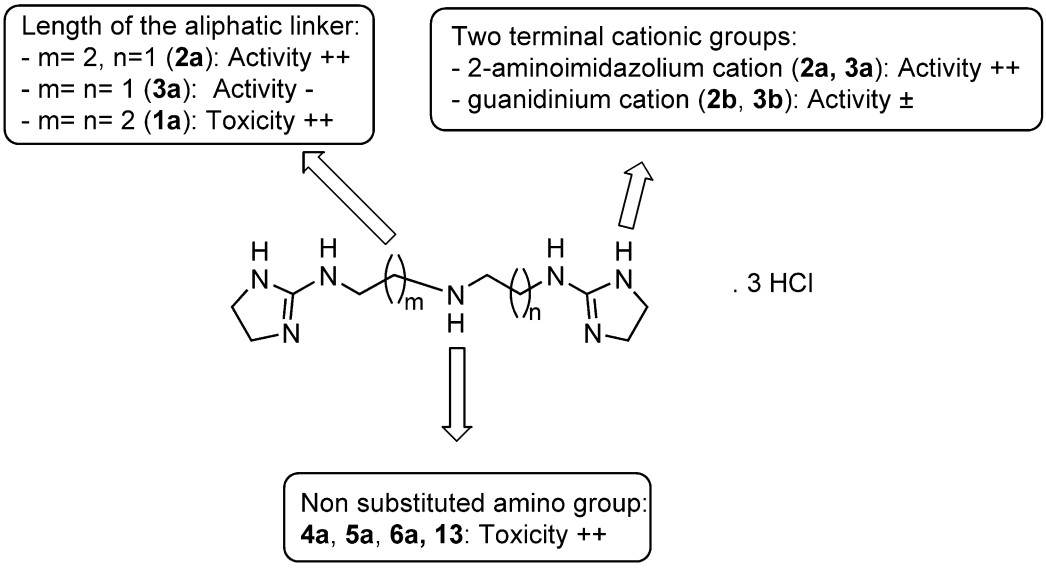



**Figure 1.** SAR studies on compound **2a**.

Concluding, compound **2a** has shown the best analgesic properties in vivo, as well as very good oral bio-availability. This derivative possesses potent non-opioid antinociceptive activity the mechanism action of which still remains to be determined.

## Experimental

### Chemistry

Reagents were used as received. 2-methylmercapto-4,5-dihydroimidazole iodide was synthesised according to the procedure described in ref 6. Reaction solvents were purchased anhydrous and used as received. Chromatography was performed either with silica gel 60 $PF_{254}$ (Merck, particle size 40–63 μm) or with the Biotage™ 'Flash 40' system using KP-Sil™ or 40M cartridges (particle size 32–63 μm, 60 Å). **CAUTION**: the noxious gas $CH_3SH$ is evolved during the synthesis and it should be trapped with a concentrated aqueous solution of NaOH and then destroyed with sodium hypochlorite. Other solvents used were reagent grade. $^1H$ and $^{13}C$ NMR spectra were recorded at 200 and 50 MHz, respectively, on a Varian Gemini 200 spectrometer. Chemical shifts of the $^1H$ NMR spectra were internally referenced to the residual proton resonance in $D_2O$ (δ 4.6 ppm) and in DMSO (δ 2.49 ppm). Chemical shifts of the $^{13}C$ NMR spectra were referenced with a capilar of DMSO-$d_6$ (δ 39.5 ppm). IR spectra were recorded on a Perkin–Elmer 681 FTIR spectrophotometer as KBr pellets. Melting points were determined with a Reichert-Jung Thermovar apparatus and are uncorrected. Mass spectra were recorded on a Hewlett Packard Series 1100 MSD spectrometer (ES, APCI) and on a VG Autospec spectrometer (FAB). Elemental analysis was performed on a Heraeus CHN-O Rapid analyser. Analytical results were within ±0.4% of the theoretical values unless otherwise noted.

### Method A. Preparation of the bis(2-aminoimidazolinium) compounds 1a–6a

A solution of 4.75 mmol of amine (**1**–**6**) and 2-methylmercapto-4,5-dihydroimidazole iodide (10 mmol) of in dry MeOH (15 mL) was heated under reflux for 2.5 h. A current of nitrogen was bubbled into the solution to remove $CH_3SH$. The solvent was removed in vacuo and the product was purified by formation of the picrate salt. The crude residue dissolved in water (50 mL) was treated with a hot aqueous solution of picric acid (10 mmol). The yellow picrate salt that precipitated was washed successively with $H_2O$, hexane, $Et_2O$ and crystallized from hot $CH_3CN$. The hydrochloride salts were regenerated with Amberlyte IRA400 ($Cl^-$ form) exchange resin. For this purpose, the picrate salt was dissolved in $H_2O$/THF (300 mL) and stirred with the resin (previously washed) overnight. The resin was removed by filtration and the solution concentrated in vacuo. The oily residue was taken up in a little water and washed twice with EtOAc. The product was lyophilised to afford the pure chloride salt.

***N,N′*-Di(4,5-dihydro-1*H*-imidazol-2-yl)-4-aza-1,7-heptanediamine (1a). Picrate salt**: yellow solid (26% yield); mp 188–189 °C; IR (KBr) 3420, 3320, 3100, 1680, 1640, 1610, 1340, 1280, 795, 755, 720 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) δ: 8.59 (s, 2H), 8.4–8.0 (m, 4H), 3.6 (s, 8H), 3.25–3.1 (m, 4H), 3.0–2.8 (m, 4H), 1.76 (quint, 4H). Anal. calcd for $C_{30}H_{34}N_{16}O_{21}$: C, 37.74; H, 3.59; N, 23.47. Found: C, 37.97; H, 3.65; N, 23.65. **Hydrochloride salt**: slightly orange hygroscopic oily solid (33%); IR (KBr): 1670, 1605, 1470, 1290, 1050 $cm^{-1}$; $^1H$ NMR ($D_2O$) δ: 3.5 (s, 8H); 3.11 (t, 4H, $J$ = 6.8 Hz); 2.89 (t, 4H, $J$ = 7.6 Hz), 1.77 (quint 4H, $J$ = 7.1 Hz); $^{13}C$ NMR ($D_2O$) δ: 158.9, 44.1, 41.7, 38.5, 24.5; MS ($ES^+$) $m/e$: 268 ($MH^+$, 100%), 303 (M + HCl).

***N,N′*-Di(4,5-dihydro-1*H*-imidazol-2-yl)-3-aza-1,6-hexanediamine (2a). Picrate salt**: yellow solid; mp 210–212 °C; IR (KBr) 3440, 3360, 3260,1660, 1620, 1575, 1535, 1345, 1315, 1255, 890, 770, 725, 690 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) δ: 8.59 (s, 2H), 8.5–8.0 (m, 8H), 3.6 (d, 8H), 3.4-2.8 (m, 8H), 1.77 (quint, 2H); $^{13}C$ NMR (DMSO-$d_6$) δ: 160.9; 159.3; 159.3; 141.8; 125.3; 124.4; 45.7; 44.6; 42.6; 42.5; 25.5; MS ($ES^+$) 254 ($MH^+$). Anal. calcd for $C_{29}H_{36}N_{16}O_{21}$: C, 36.87; H, 3.84; N, 23.72. Found: C, 36.64; 3.55; 23.48. **Hydrochloride salt**: yellowish hygroscopic solid (66%); mp 194–197 °C; $^1H$ NMR ($D_2O$) δ: 3.59 (s, 4H); 3.56 (s, 4H); 3.48 (t, 2H, $J=5.9$ Hz), 3.3–3.1 (m, 4H); 1.88 (quint, 2H, $J=7.8$ Hz); $^{13}C$ NMR ($D_2O$) δ: 159.25, 159.13, 45.6, 44.7, 42.2, 42.1, 38.9, 38.3, 24.7; MS ($FAB^+$) *m/e*: 254 ($MH^+$, 100%), 290.2 (M + HCl, 32%), 322 (M + 2HCl). Anal. calcd for $C_{11}H_{26}N_7Cl_3/H_2O$: C, 34.70; H, 7.41; N, 25.75. Found: C, 35.11; H, 7.25; N, 25.72.

***N,N′*-Di(4,5-dihydro-1*H*-imidazol-2-yl)-3-aza-1,5-pentanediamine (3a).** The product was isolated directly as the hydroiodide salt from the reaction mixture in the following manner: the solvent was removed in vacuo and the resulting thick oily residue dissolved in hot $^i$PrOH (5 mL) was triturated with a glass rod. The yellow solid that formed was recrystallized successively from $^i$PrOH and EtOH to give **3a** as a white solid (20%); mp 145–146 °C. **Hydroiodide salt**: IR (KBr) 3300–3000, 1640, 1560, 1265 $cm^{-1}$; $^1H$ NMR ($D_2O$) δ: 3.61 (s, 8H,); 3.24 (t, 4H, $J=6.2$ Hz); 2.7 (t, 4H, $J=6.2$ Hz); $^{13}C$ NMR ($D_2O$) δ: 162.63, 47.35, 43.13, 42.32. Anal. calcd for $C_{10}H_{23}N_7I_2$: C, 24.26; H, 4.68; N, 19.80. Found: C, 24.50; H, 4.69; N, 19.54.

***N,N′*-Di(4,5-dihydro-1*H*-imidazol-2-yl)-1,6-hexanediamine (4a).**[7] The product was isolated as the hydroiodide salt by crystallisation from MeOH/$Et_2O$: white solid (26%); mp 197–199 °C; $^1H$ NMR ($D_2O$) δ: 3.48(s, 8H); 2.99 (t, 4H, $J=6.8$ Hz); 1.5–1.25 (m, 4H); 1.25–1.00 (m, 4H); $^{13}C$ NMR ($D_2O$) δ: 158.9, 41.7, 41.5, 27.2, 24.5; MS ($APCI^+$) *m/e*: 253 ($MH^+$). Anal. calcd for $C_{12}H_{26}N_6I_2$: C, 28.36; H, 5.16; N, 16.54. Found: C, 28.43; H, 5.02; N, 16.64.

**3-(2-Aminoethylamino-2-phenethyl)propylamine (5).** A mixture of **9** (590 mg, 1.2 mmol) and Pd/C 10% (55 mg) in MeOH (50 mL) was shaken under $H_2$ (20 Psi) for 16 h. After filtration and evaporation of the solvent, **5** was obtained as a colourless oil (272 mg, 100%); $^1H$ NMR ($CDCl_3$) δ: 7.3–7.1 (m, 5H); 4.19 (br, 4H, $NH_2$); 2.85–2.40 (m, 12H); 1.65 (quint, 2H); $^{13}C$ NMR ($D_2O$) δ: 141.0; 129.5; 129.4; 127.0; 55.7; 55.3; 51.7; 39.4; 38.1; 32.3; 27.5. Anal. calcd for $C_{13}H_{23}N_3/1.6H_2O$: C, 61.94; H, 10.52; N, 16.67. Found: C, 62.20; H, 9.29; N, 16.67.

***N,N′*-Di-(4,5-dihydro-1*H*-imidazol-2-yl)-3-phenethyl-3-aza-1,6-hexanediamine (5a).** Following method A, from **5** (270 mg, 1.2 mmol) and 2-methylmercapto-4,5-dihydroimidazole iodide (617 mg, 2.53 mmol) in MeOH (10 mL) for 3.5 h. The solvent was removed in vacuo and oxalic acid (108 mg, 1 equiv) was added to the residue dissolved in a small quantity of MeOH. EtOAc was added and the product precipitated as a thick yellow oil. The solvent was removed from the flask, $Et_2O$ was added and the residue was triturated with a glass rod until a solid crystallised. The product was recrystallised from MeOH/$Et_2O$ following the same procedure, and dried in vacuo (45 °C) to afford **5a** as an hygroscopic yellow solid (50%); **Dihydroiodide monooxalate**: Pf > 90 °C (dec); IR (KBr): 1643, 1575, 1260, 695, 668 $cm^{-1}$; $^1H$ NMR ($D_2O$) δ: 7.5–7.2 (m, 5H); 3.70 (s, 4H); 3.68 (s, 4H); 3.6–3.0 (m, 12H); 2.0 (m, 2H); $^{13}C$ NMR ($D_2O$) δ: 168.9; 160.3; 136.5; 129.8; 129.5; 128.1; 54.5; 52.3; 51.4; 43.4; 43.3; 40.1; 38.0; 37.1; 30.0; LRMS ($ES^+$) *m/e*: 358 ($MH^+$); 486 ([M + HI]). Anal. calcd for $C_{19}H_{31}N_7I_2 \cdot 1/2C_2H_2O_4$: C, 36.60; H, 4.76; N, 14.94. Found: C, 36.53; H, 4.96; N: 12.85.

**3-(2-Ethylindol-3-yl)-3-aza-1,6-hexanediamine (6).** A mixture of **10** (830 mg, 1.57 mmol), Pd/C 10% (360 mg) in MeOH (50 mL) was shaken under $H_2$ (33 Psi) for 18 h. After filtration and evaporation of the solvent, **6** was obtained as a colourless oil (408 mg, 100%); $^1H$ NMR ($D_2O$) δ: 7.5–7.2 (m, 2H); 7.1–6.8 (m, 3H); 2.9–2.1 (m, 12H); 1.56 (quint, 2H); $^{13}C$ NMR ($D_2O$) δ: 154.9; 141.3; 131.9; 128.5; 127.1; 124.4; 123.8; 117.2; 58.3; 55.3; 54.7; 42.98; 41.5; 28.6; 26.3; LRMS ($ES^+$) *m/e*: 261 ($MH^+$).

***N,N′*-Di(4,5-dihydro-1*H*-imidazol-2-yl)-3-(2-ethylindol-3-yl)-3-aza-1,6-hexanediamine (6a).** Method A, starting from **6** (220 mg, 0.85 mmol), 2-methylmercapto-4,5-dihydroimidazole iodide (413 mg, 1.69 mmol) in EtOH (3 mL) for 24 h; **Monopicrate monoiodide salt**: yellow solid (573 mg; 92%); mp 95–105 °C (> 118 dec.); $^1H$ NMR (DMSO-$d_6$) δ: 11.01 (s, 1H): 9.45 (bs, 1H); 8.59 (s, 2H); 8.4–8.0 (m, 3H); 7.56 (d, 1H, $J=6.8$ Hz); 7.38 (d, 1H, $J=7.4$ Hz); 7.26 (s, 1H); 7.07 (q, 2H); 3.62 (bd, 8H); 3.5–2.8 (m, 14H), 1.91 (m, 2H); LRMS ($ES^+$) *m/e*: 397 ($MH^+$/100%). Anal. calcd for $C_{27}H_{34}$ I $N_{11}O_6$: C, 44.08; H, 4.66; N, 20.95. Found: C, 43.35; H, 4.31; N, 20.81. **Trihydrochloride salt**: yellow hygroscopic solid (74%); IR (KBr): 1633; 1017; 992; 970 $cm^{-1}$; $^1H$ NMR ($D_2O$) δ: 7.61 (d, 1H, $J=7.4$ Hz); 7.45 (d, 1H, $J=7.6$ Hz); 7.28 (s, 1H); 7.14 (q, 2H); 3.7–3.0 (m, 20H); 1.92 (br, 2H); $^{13}C$ NMR ($D_2O$) δ: 160.1; 137.0; 126.7; 124.8; 122.9; 120.2; 118.6; 112.7; 108.8; 53.7; 52.4; 51.6; 43.3; 40.0; 37.9; 23.6; 20.6. Anal. calcd for $C_{21}H_{34}N_8Cl_3/4.5H_2O$: C, 42.97; H, 7.55; N, 19.09. Found: C, 42.90; H, 7.27, N, 18.26.

***N,N″*-Ddi(benzyloxycarbonyl)-3-aza-1,6-hexanediamine (8).** A two-neck flask was charged with 3-(2-aminoethylamino)propylamine **2** (1.814 g, 15.48 mmol) dissolved in dry $CH_2Cl_2$ (40 mL). Caution: the reaction should be carried out with a positive pressure of nitrogen and one neck of the flask connected to a trap containing a concentrated aqueous NaOH solution to trap cyanhydric acid that is evolved during the reaction. Benzyl cyanoformate (5 g, 31 mmol) dissolved in dry $CH_2Cl_2$ (100 mL) was added dropwise at room temperature over a 7 h period. The reaction was stirred another 15 h at room temperature. The solvent was removed in vacuo and the product was purified by flash chromatography on silica with $CHCl_3$/MeOH (3/1). **8** was dissolved in a little $CH_2Cl_2$ and filtered on a path of Celite to remove traces of silica. White solid (4.59 g,

79%); mp 96–97 °C ($^i$PrOH); IR (KBr): 3280, 1665, 1515, 1255, 1225 cm$^{-1}$; $^1$H NMR ($CDCl_3$) δ: 7.38–7.26 (m, 10H); 5.4 (b, NH); 5.33 (b, NH); 5.08 (s, 2H); 5.07 (s, 2H); 3.25 (bm, 4H); 2.69 (b, 2H); 2.63 (t, 2H, $J=6.2$ Hz); 1.62 (quint, 2H); 1.54 (b, NH); $^{13}$C NMR ($CDCl_3$) δ: 156.6; 156.5; 136.7; 136.6; 128.4; 128.0; 66.6; 66.5; 48.9; 46.8; 40.5; 39.3; 29.8; LRMS ($FAB^+$) *m*/*e*: 386 ($MH^+$/100%). Anal. calcd for $C_{21}H_{27}N_3O_4$: C, 65.43; H, 7.06; N, 10.90. Found: C, 65.72; H, 7.14; N, 11.14.

***N,N′*-Di(benzyloxycarbonyl)-3-phenylethyl-3-aza-1,6-hexanediamine (9).** A solution of **8** (1 g, 2.6 mmol), bromoethylbenzene (0.5 mL, 3.6 mmol) and $K_2CO_3$ (180 mg, 1.3 mmol) in $CH_3CN$ (25 mL) was refluxed for 43 h. The reaction mixture was filtered on Celite and the filter cake was rinsed with $CH_2Cl_2$. The solvent was removed in vacuo and the product was purified by chromatography on silica using $CHCl_3$/MeOH (gradient 70/1 to 20/1); colourless oil (1.062 g, 83%); IR (neat): 3300; 1680; 1500; 1432; 1230; 715; 675 cm$^{-1}$; $^1$H NMR ($CDCl_3$) δ: 7.4–7.1 (m, 15H); 5.68 (br, NH); 5.42 (br, NH); 5.14 (s, 4H); 3.21 (m, 4H); 2.71 (s, 4H); 2.55 (m, 4H); 1.61 (quint, 2H); $^{13}$C NMR ($CDCl_3$) δ: 156.92; 156.86; 140.6; 137.1; 129.1; 128.8; 128.77; 128.4; 126.4; 66.8; 66.7; 55.9; 53.6; 51.9; 39.9; 39.1; 33.8; 27.39. Anal. calcd for $C_{29}H_{35}N_3O_4$: C, 71.14; H, 7.20; N, 8.58. Found: C, 71.35; H, 7.04; N, 9.02.

***N,N′*-Di(benzyloxycarbonyl)-3-(2-ethylindol-3-yl)-3-aza-1,6-hexanediamine (10).** A solution of **8** (1.108 g, 2.88 mmol), 3(2-bromoethyl)indole (537 mg, 2.4 mmol) and $K_2CO_3$ (331 mg, 2.4 mmol) in $CH_3CN$ (15 mL) was refluxed for 21 h. The reaction mixture was filtered on Celite and the filter cake was rinsed with $CH_2Cl_2$. The solvent was removed in vacuo and flash chromatography on silica using a 40S cartridge ($CH_2Cl_2$/MeOH 5%) afforded **10** as an orange oil (1.035 g, 82%); IR (neat): 3365; 3300; 3020; 2990; 2910; 1680; 1500; 1235; 1215; 720; 675 cm$^{-1}$; $^1$H NMR ($CDCl_3$) δ: 8.66 (br, NH); 7.58 (d, 1H, $J=7.2$ Hz); 7.4–7.0 (d, 1H, $J=1.6$ Hz); 5.65 (br, NH); 5.47 (br, NH); 5.07 (s, 2H); 5.05 (s; 2H); 3.2–2.9 (m, 4H); 2.9–2.6 (m, 4H); 2.5–2.3 (m, 4H); 1.5 (m, 2H); $^{13}$C NMR ($CDCl_3$) δ: 156.96; 156.90; 137.0; 136.6; 128.7; 128.2; 127.6; 122.3; 121.9; 119.2; 118.8; 113.8; 111.7; 66.7; 66.6; 54.5; 53.5; 52.0; 39.9; 39.1; 27.1; 23.2; LRMS ($ES^+$) *m*/*e*: 529 ($MH^+$/100%). Anal. calcd for $C_{31}H_{36}N_4O_4/0.5H_2O$: C, 69.25; H, 6.75; N, 10.42. Found: C, 69.06; H, 6.73; N, 10.35.

***N*-Propyl-*N′*-(4,5-dihydro-1*H*-imidazol-2-yl)-1,3-propanediamine (14).** Following method A with *N*-propyl-1,3-propanediamine (994 mg, 8.57 mmol) and 2-methylmercapto-4,5-dihydroimidazole iodide (2.3 g, 9.42 mmol) in MeOH (15 mL). The product was isolated as the oxalate salt. After the reaction finished, the solvent was removed in vacuo and the crude residue dissolved in $^i$PrOH was treated with 2 equiv of oxalic acid. The product was allowed to crystallize in the fridge overnight. The crystals were washed with $^i$PrOH and ether, yielding **14** as a white solid (65%); **Monoiodide monooxalate salt**: mp 184–190 °C; IR (KBr) 1650; 1580; 1290; 1270 cm$^{-1}$; $^1$H NMR ($D_2O$) δ: 3.63 (s, 4H); 3.24 (t, 2H, $J=6.8$ Hz); 3.03 (t, 2H, $J=7.7$ Hz); 2.95 (t, 2H, $J=7.5$ Hz); 1.91 (quint, 2H, $J=7.5$ Hz); 1.6 (sext, 2H); 0.89 (t, 3H, $J=7.5$ Hz); $^{13}$C NMR ($D_2O$) δ: 174.2; 162.6; 52.3; 47.7; 45.7; 42.6; 28.3; 22.2; 13.4; LRMS ($ES^+$) *m*/*e*: 185 ($MH^+$/100%). Anal. calcd for $C_{10}H_{22}N_4O_2I$: C, 33.61; H, 6.20; N, 15.68. Found: C, 33.98; H, 5.78; N, 15.26.

### Method B. Preparation of bisguanidinium compounds 1b, 2b, 3b, 5b

Following the procedure of Villaroya et al.[5] A solution of 3 mmol of diamine (**1**–**3**) and *S*-methylisothiouronium sulfate (3 mmol) of in 10 mL of dry $CH_3CN$ was heated under reflux for 20 h. A white solid precipitated from the solution. The solid was washed with cold $CH_3CN$ and recrystallised from EtOH/$H_2O$ (1/5).

**1,7-Diguanidinium-4-azaheptane sulfate (1b).** White amorphous solid (24% yield); mp 275–279 °C (Lit.[5] 280–283 °C dec). Anal. calcd for $C_8H_{26}N_7O_7S_{1.5}$: C, 25.25; H, 6.89; N, 25.77; S, 12.63. Found: C, 25.13; H, 6.87; N, 25.06; S, 11.75.

**1,6-Diguanidinium-3-azahexane sulfate (2b).** White amorphous solid (30% yield); mp: 244–248 °C (Lit.[5] 192 °C dec). Anal. calcd for $C_7H_{21}N_7O_4S$: C, 28.09; H, 7.07; N, 32.75; S, 10.71. Found: C, 28.35; H, 7.27; N, 32.77; S, 10.43.

**1,5-Diguanidinium-3-azapentane sulfate (3b).** White solid (12%); mp: 229–232 °C; $^1$H NMR ($D_2O$/TFA) δ: 3.09 (t, 4H, $J=6$ Hz); 2.58 (t, 4H, $J=6$ Hz); $^{13}$C NMR ($D_2O$/TFA) δ: 150.8, 46.0, 40.0; MS ($ES^+$) *m*/*e*: 286 ($M+H_2SO_4$, 100%), 188 (M + H). Anal. calcd for $C_6H_{19}N_7O_4S$. $H_2O$: C, 23.75; H, 6.98; N, 32.32; S, 10.57. Found: C, 23.90; H, 7.52; N, 32.21; S, 10.54.

**1-(*tert*-Butoxycarbonyl)-4-[(1-*tert*-butoxycarbonyl)piperidin-4-yl]piperazine (11a).** Sodium cyanoborohydride (880 mg, 14 mmol) was added at once to a stirred solution of *N*-Boc-piperidone (1.407 g, 7 mmol) and *N*-Boc-piperazine (1.447 g, 7.8 mmol) in MeOH (30 mL). The pH of the solution was adjusted with $CH_3CO_2H$ at ca. 7 (and checked during the course of the reaction) and the reaction mixture was stirred at room temperature until the *N*-Boc piperidone was consumed (48 h). The solvent was removed in vacuo and the crude residue was partitioned between $CH_2Cl_2$ and water. The aqueous phase was extracted three times with $CH_2Cl_2$. Combined organic extracts were washed with brine, dried ($Na_2SO_4$) and concentrated in vacuo to afford **11a** as a white solid. The product was crystallised from hexane/EtOAc at −20 °C and cold filtered; white solid (1.669 g; 65%); mp 134–139 °C; IR (KBr): 1685; 1670; 1410; 1355; 1230; 1155; 1110 cm$^{-1}$; $^1$H NMR ($CDCl_3$) δ: 4.3–4.1 (br, 2H); 3.48 (br s, 4H); 2.7 (br t, 2H, $J=12$ Hz); 2.6–2.45 (m, 5H); 1.9–1.7 (m, 2H); 1.46 (br s, 20H); $^{13}$C NMR ($CDCl_3$/100 MHz) δ: 154.7; 79.7; 79.5; 62.2; 49.1; 43.9 (br); 43.5 (br); 43.3 (br); 28.6; 28.2; LRMS ($ES^+$) *m*/*e*: 370 ($MH^+$). Anal. calcd for $C_{19}H_{35}N_3O_4$: C, 61.76; H, 9.55; N, 11.37. Found: C, 61.90; H, 9.53; N, 11.12.

**1-(4-Piperidyl)piperazine (11b).** A solution of **11a** (291 mg, 0.79 mmol) in 50% TFA/$CH_2Cl_2$ was stirred 2.5 h at room temperature. The solvent was evaporated to dryness and the residue was triturated in ether with a spatula until a white solid crystallised. The solid was washed with ether and dried in vacuo (282 mg); **Trifluoroacetate salt** of **11b**: 92%; mp 140–150 °C; $^1$H NMR ($D_2O$) δ: 3.4–3.1 (m, 2H); 3.0 (br s, 4H); 2.9–2.3 (br m, 7H); 2.1–1.75 (br m, 2H); 1.6–1.25 (br m, 2H); $^{13}$C NMR ($D_2O$) δ: 162 (q, TFA); 116.2 (q, TFA); 61.8; 46.5; 43.1; 41.5; 23.8; LRMS ($ES^+$) *m/e*: 170 ($MH^+$). Anal. calcd for $C_{15}H_{22}F_9N_3O_6$: C, 35.23; H, 4.36; N, 8.21. Found: C, 35.25; H, 4.79; N, 8.30.

**1-[[2-[(1,1-Dimethylethoxy)carbonyl]amino]ethyl]amino]-thioxomethyl]-amino]-4-[[[2-[(1,1-dimethylethoxy)carbonyl]amino]ethyl]amino]thioxomethyl] - amino]piperidin - 4-yl]piperazine (12).** The trifluoroacetate salt of **11b** (2.473 g) was dissolved in water (200 mL) and stirred with 65 mL of IRA 400 ($OH^-$ form) to liberate the freebase of the amine. After 5 h, the pH of the aqueous solution was about 8–9. The resin was filtered off and the solution concentrated in vacuo to afford the free base of **11b** as a white solid (648 mg). In a flask flushed with nitrogen, a solution of [(*tert*-butoxycarbonyl)amino]ethylisothiocyanate (1.244 g, 6.16 mmol) in THF (20 mL) was added dropwise to a solution of **11b** in a mixture of dry THF (25 mL) and DMF (5 mL added to help solubilisation of **11b**). The reaction was stirred 2 days at room temperature and the solvent was removed in vacuo. The yellow residue was purified by chromatography on silica with $CHCl_3$/MeOH 5% to yield **12** as a white foam (804 mg, 50%); mp 147–149 °C; $^1$H NMR ($CDCl_3$) δ: 7.7 (br, 1H); 7.1 (br, 2H); 5.77 (br, 2H); 4.45–4.2 (m, 2H); 3.52 (br, 3H); 3.34 (br, 3H); 3.05 (br, 3H); 2.68 (s, 4H); 2.56 (s, 3H); 2.26 (br, 4H); 1.52 (m, 2H); 1.12 (s, 18H); $^{13}$C NMR ($CDCl_3$) δ: 181.3; 180.8; 162.6; 158.0; 79.5; 74.2; 61.0; 48.6; 47.3; 46.6; 39.5; 36.5; 31.4; 28.3; 27.8; LRMS ($ES^+$) *m/e*: 574 ($MH^+$/100%). Anal. calcd for $C_{25}H_{47}N_7O_4S_2$: C, 52.33; H, 8.26; N, 17.09; 11.17. Found: C, 52.04; H, 8.40; N, 17.31; S, 10.98.

**1-(4,5-Dihydro-1*H*-imidazol-2-yl)-4-[1-(4,5-dihydro-1*H*-imidazol-2-yl)piperidin-4-yl] piperazine (13).** A solution of **12** (140 mg, 0.24 mmol) and iodoethane (3 mL) in EtOH (2 mL) was heated 22 h (bath temperature: 60 °C) under a nitrogen atmosphere. The solvent was evaporated in vacuo to give an orange foam. The product was not isolated and was dissolved in 3 mL of a TFA/$H_2O$ solution (2/1). This mixture was stirred 3.5 h at room temperature and the solvent was removed in vacuo. Lyophilization of the residue afforded an orange solid that was dissolved in a mixture of MeOH (5 mL) and EtOH (10 mL). This solution was added dropwise, under nitrogen, to a sodium ethoxide solution (25 mg of sodium in 3 mL of absolute EtOH). The reaction was stirred 24 h at room temperature. The solvent was removed in vacuo and the product was isolated by formation of its picrate salt (method B). **Picrate salt**. Yellow solid (31 mg); mp 204–207 °C and 220–225 °C; $^1$H NMR (DMSO) δ: 8.54 (s, 2H); 8.49 (s, 1H); 8.33 (s, 1H); 3.62 (s, 4H); 3.60 (s, 4H); 4.0–2.9 (br m, 13H); 2.07 (br d, 2H); 1.59 (br d, 2H); $^{13}$C NMR (DMSO) δ: 160.7, 158.8; 158.6; 141.8; 125.1; 124.3; 61.4; 47.1; 44.5; 43.2; 42.8; 42.7; 25.3; **Monohydrochloride monoiodide salt**: white hygroscopic solid; 13%; $^1$H NMR ($D_2O$) δ: 3.8–3.4 (br m, 5H); 3.65 (s, 4H); 3.61 (s, 4H); 3.4–2.9 (br m, 8H); 2.2–2.0 (br m, 2H); 1.8–1.5 (br m, 2H); $^{13}$C NMR ($D_2O$) δ: 159.1 ; 62.4; 48.2; 45.6; 44.4; 43.6; 43.5; 26.4; LRMS ($ES^+$) *m/e*: 306 ($MH^+$/100%). Anal. calcd for $C_{15}H_{30}N_7ClI/2H_2O$: C, 33.22; H, 6.32; N, 18.08. Found: C, 33.39; H, 6.03; N, 17.49.

## Pharmacology

**Functional activity. In vivo assays.** CD1 male mice in the weight range of 27–30 g were used for the study. All the animals were supplied with food and water 'ad libitum' and were housed in a temperature-controlled room at 21 °C. Lighting was on a 12/12-h light/dark cycle. The mice were housed for a least 1 day in the test-room before experimentation.

To detect antinociceptive activity, several doses (25–100 mg/kg) of the tested compounds or saline solution were ip administered to separated groups of mice ($n \geq 10$) 30 min before the analgesic effect was recorded. In order to compare the antinociception level with that of a reference compound, the effect of ip administration of morphine (3.5 mg/kg) was also tested. Each mouse was used only once and an observer who was unaware of the treatment performed the testing and data recording.

**Writhing test.** The mice were ip injected with a 2% acetic acid solution to produce the typical writhing reaction[15] which is characterised by a wave of contraction of the abdominal musculature followed by extension of the hinds limbs. After the acetic acid administration, the mice were placed in individual transparent containers and, 5 min later, the number of writhes was counted during a 10-min period.

To test the antinociceptive effect, writhing test was carried out 30 min after the administration of separate doses of: compound **1a**, **2a–b**, **3a–b**, **4a**, **5a**, **6a**, **13** and **14**.

The effect of **2a** was also tested 30 min after po (intragastric cannula) administration. SS (saline solution) of one dose (25, 50, 100 and 150 mg/kg) of **2a** were given 30 min before testing.

**Hot plate test.** This test was carried out with a hot plate at 55 °C as a nociceptive stimulus.[16] The control reaction latency of the animals was measured before the treatment. The time of licking of the front paws was taken as an index of nociception. The latency was measured before drug administration (control) and 30 min after treatment. The cut-off time was 30 s and analgesia was quantified with the formula of the % M.P.E.:

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

To test the antinociceptive effect, hot plate test was carried out 30 min after the administration of SS or

separated doses of: compound **2a** (50 and 100 mg/kg) and morphine (5 mg/kg).

### In vitro assays

**Isolated tissues.** Male guinea-pigs weighing 300–450 g were used for this study. Myenteric plexus-longitudinal muscle strips (MP-LM) were isolated from guinea-pig ileum (GPI) as described by Ambache.[17] Tissues were suspended in a 10 mL organ bath containing Krebs solution (NaCl 118, KCl 4.75; $CaCl_2$ 2.54; $KH_2PO_4$ 1.19; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11 mM). This solution was continuously gassed with 95% $O_2$ and 5% $CO_2$. Tissues were kept under 1 g of resting tension at 32 °C and were electrically stimulated through two platinum ring electrodes with rectangular pulses of 70 V, 0.1 ms duration and 0.3 Hz frequency. The isometric force was recorded on a Grass model 7A polygraph.

In order to detect agonistic or antagonist effects mediated through the receptors presents in GPI, cumulative administration of increasing concentrations of **2a** were carried out in a step by step manner, being the interval between applications 15 min. The effect of **2a** was tested in electrically stimulated tissues as well as in non stimulated tissues.

Finally, to test antagonistic activity, separated doses of **2a**, adequate to reach $10^{-8}$, $5\times10^{-8}$, $10^{-7}$ and $5\times10^{-6}$ M concentration in the organ bath, were administered. Then, GPI was stimulated by addition of acetylcholine ($5\times10^{-8}$ M), serotonine ($2\times10^{-7}$ M), histamine ($10^{-5}$ M), bradykinin ($5\times10^{-7}$ M) or substance P ($10^{-6}$ M), and MVD with noradrenaline ($5\times10^{-7}$ M). The concentration of the agonists was those needed to approximately reach a contractile response of 1 g in control tissues.

The general receptor and enzyme screenings were performed by MDS Pharmacology Services, Bothell, WA 98011-8890, USA.[12]

## Acknowledgements

Financial support was provided by the Spanish DGICYT (Project SAF 97-0044-C02). C.D was a holder of a Marie Curie Research Training Grant for which he thanks the European Commission, Directorate General XII (Science, Research and Development).

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