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Synthesis and Antinociceptive Activity of Chimonanthines and Pyrrolidinoindoline-Type Alkaloids

L. Verotta,[a,*] F. Orsini,[a] M. Sbacchi,[b] M.A. Scheildler,[b] T.A. Amador[c] and E. Elisabetsky[c,d]

[a]*Dipartimento di Chimica Organica e Industriale, Università degli Studi di Milano,via Venezian 21, 20133 Milan, Italy*
[b]*Neurobiology Research, GlaxoSmithKline, Via Zambeletti, I-20021 Baranzate di Bollate, Milan, Italy*
[c]*Curso de Pós-graduação em Ciências Biológicas-Bioquímica, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos 2600/anexo, Porto Alegre/RS, Brazil*
[d]*Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500/202, 90040-100, Porto Alegre/RS, Brazil*

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Abstract—Hodgkinsine, a trimeric pyrrolidinoindoline type alkaloid, present as a major constituent of *Psychotria* spp. (Rubiaceae), has shown to produce dose-dependent, naloxone reversible, analgesic effect in thermal models of nociception and in the capsaicin-induced pain. SAR studies have been initiated by synthesizing the three diastereomeric dimers (chimonanthines) (**11**–**13**) which were evaluated in vitro and in vivo along with the synthetic intermediates. Strong binding affinities for μ opioid receptors were found for (−)- and (+)-chimonanthine monourethanes (**9** and **10**), whereas (−)-, (+)- and (meso)-chimonanthine (**11**–**13**) and hodgkinsine displayed low affinity. In vivo data have shown that only (+)-chimonanthine (**12**) and calycosidine resemble the analgesic profile found for hodgkinsine.

Introduction

New analgesics have been constantly and intensively sought by the pharmaceutical industry.[1] This enduring interest is related to the still significant limitations (e.g., side effects, effectiveness, tolerance and dependence) of presently available compounds. Despite of the remarkable efficacy of morphine and other opiates, chronic pain, perhaps more diffused than acute pain, cannot be effectively treated with opiates. Furthermore, neuropathic pain following injury to the nervous system responds poorly to opiates at doses that do not cause serious adverse effects. Thus, alternative management strategies need to be considered for dealing with situations where pain is unresponsive to currently available medication.

The physiological modulation of pain involves opioid receptors, exhibiting a widespread distribution in the central and peripheral nervous systems.[2] The existence of at least three types of opioid receptors, namely μ, κ and δ, has been well established.[3] Specific types of receptors mediate the pharmacological actions of opioids, either therapeutic or euphorigenic, as well as the reinforcing properties which may lead to addiction. Therefore, the detection of opioid agonists that would maintain the analgesic profile of morphine, while lacking its addictive properties, is highly desirable.

The increasing recognition of the vast chemical diversity in tropical forests,[4] associated with the development of revolutionary techniques for isolation and elucidation of chemical compounds and characterization of their pharmacological properties, justify the rekindled interest in natural products.[5,6,7] Surveys of medicinal plants used among Amazonian *caboclos* (rural peasants) of the State of Pará (Brazil) pointed to *Psychotria colorata* (Will. ex R. & S.) Muell. Arg., traditionally used for 'treatment of earache' and 'calming abdominal pain'.[8] We further reported that alkaloids present in leaves and flowers of *P. colorata* have marked analgesic activity, as evaluated through various pain models.[9,10,11] Phytochemical analyses of *P. colorata* flowers identified oligomeric pyrrolidinoindoline alkaloids as major

*Corresponding author. Tel.: +39-02-5031-4114; fax: +39-02-5031-4106; e-mail: luisella.verotta@unimi.it

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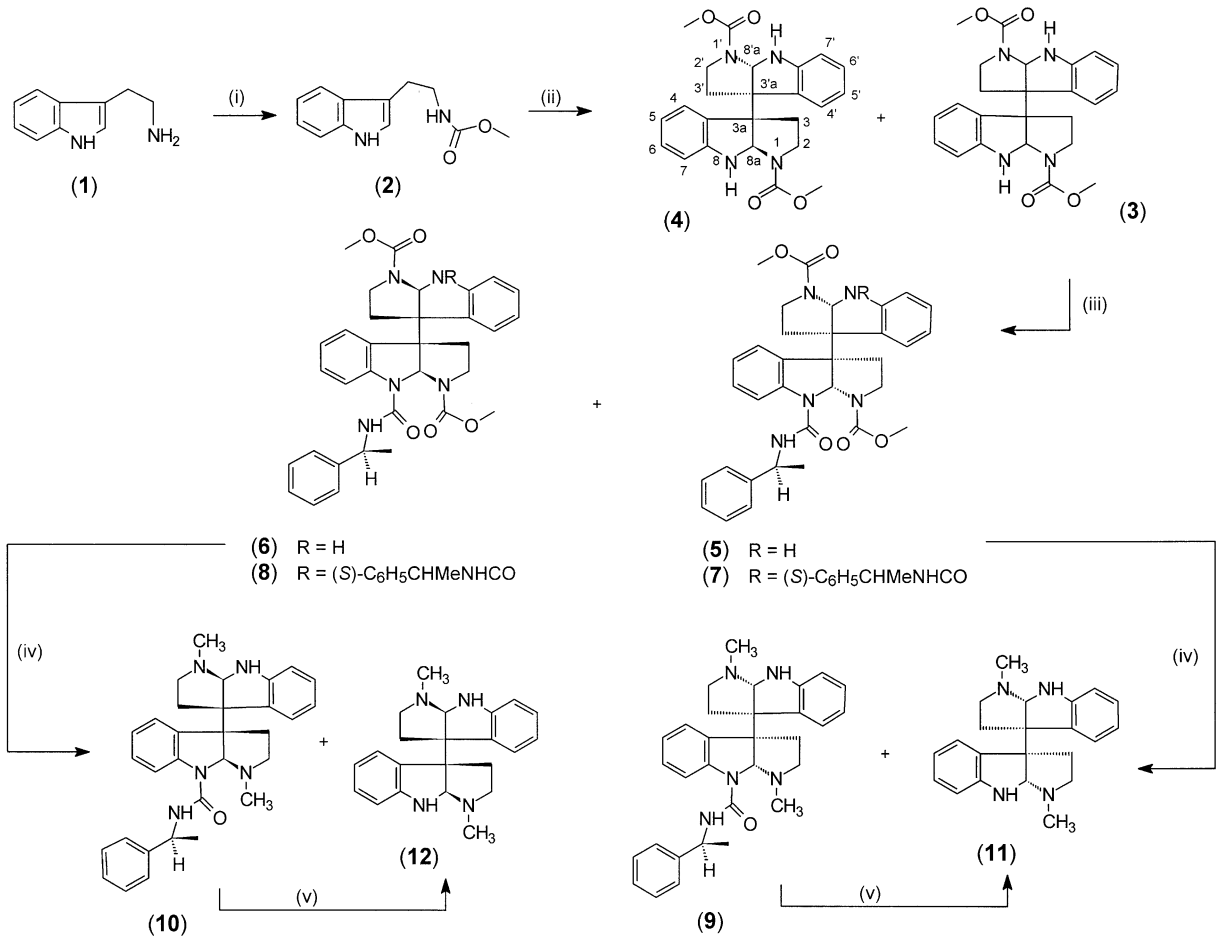


Scheme 1. (i) $ClOOCH_3$, CH_2Cl_2/Et_3N, rt: (ii) TTFA/ethereal BF_3, rt; (iii) (−)-(*S*)-(α-methyl-benzyl)isocyanate, reflux; (iv) $LiAlH_4$/THF, reflux; (v) $C_5H_{11}OH/C_5H_{11}ONa$, reflux.

components,[11,12] these alkaloids seem to be specific to *Psychotria* sps, and closely related genus.

The trimer hodgkinsine is one of the most abundant pyrrolidinoindoline alkaloids present in extracts of *Psychotria* species collected in different places and seasons.[13] Pharmacological analyses revealed that hodgkinsine produces a dose-dependent, naloxone reversible, analgesic effect in thermal models of nociception, suggesting that activation of opioid receptors participates in its mode of action. Hodgkinsine is likewise a potent dose-dependent analgesic in the capsaicin-induced pain, suggesting that the glutamate NMDA receptors are also likely to be involved.[14]

The synthesis of hodgkinsine is complicated by the number of existing stereocenters. A number of other trimers are present in the extracts,[13] nevertheless, the difficulties in defining their absolute configurations limit a rational approach to a possible drug development.

The first members of the oligomeric pyrrolinoindoline alkaloids series, namely the three diastereomeric dimers, (+)-, (−)-, and (meso)-chimonanthine can be easily prepared following the protocol here described (Scheme 1). From these only (+)- and (meso)-chimonanthine are found in the alkaloid extracts of the *Psychotria* sps we have studied.[13] Because these compounds are present in the extracts in low concentrations, it is expected that they do not significantly contribute to the overall activity of the traditional plant-based preparations. Nevertheless, a sustainable supply of all the three chimonanthines could represent a valid starting point for the structure–activity studies.[15]

The purpose of this study is to generate preliminary data relevant to the analgesic activity of chimonanthines and the intermediates obtained during the synthetic pathway in choosen models of analgesia.

Synthesis

The oxidative dimerization of carbomethoxytryptamine (**2**) with thallium trifluoroacetate (TTFA) allowed to obtain (±)-carbomethoxychimonanthine (**3**) and (meso)-carbomethoxychimonanthine (**4**). The (meso)-derivative was directly reduced with $LiAlH_4$ to give (meso)-chimonanthine (**13**).

The racemate (**3**) was resolved by treatment with (−)-(*S*)-(α-methyl-benzyl) isocyanate. Mono-urethanes of (−)- and (+)-carbomethoxychimonanthine (**5**–**6**) were obtained, accompanied by a 20% of the corresponding

diurethanes (**7**–**8**). Monourethanes of carbomethoxychimonanthines (**5**) and (**6**) were separately reduced with $LiAlH_4$ to give monourethanes of (−)- and (+)-chimonanthine (**9**)-(**10**) along with a 20% of, respectively, (−)- and (+)-chimonanthine (**11**–**12**). (−)- and (+)-chimonanthine were finally prepared by alkaline hydrolysis of the corresponding monourethanes (**9**–**10**) (see Scheme 1).

In an alternative pathway, a first reduction of **3**, followed by racemate resolution through (−)-(*S*)-(α-methyl-benzyl) isocyanate led to lower yields, probably due to the formation of N1-formyl and N1-hydroxymethylene derivatives as by products, which were not obtained when a N8-protecting group is present.

The oxidative dimerization of carbomethoxytryptamine[16] is the limiting factor for obtaining high yields from the reported sequence of reactions, but it easily allowed preparation in one step of the three chimonanthines. The reaction is sensitive to the N1 acyl derivative; in fact, the same protocol applied to the product obtained from tryptamine and menthylchloroformiate was completely unsuccessful.

Initially, the racemate resolution was tentatively approached through the preparation of (+)-*O*,*O*′-dibenzoyl-D-tartrates, but the low yields of crystalline derivatives led us to prepare the diastereomeric carbamates (**5**–**6**).

Results and Discussion

Chimonanthines and their precursors, obtained through the synthetic pathway shown in Scheme 1, were tested on μ- and κ-opioid binding assay, and on the tail-flick and the capsaicin-induced pain models.

Opioid binding

The binding affinities of chimonantines and pyrrolidinoindoline-like analogues for the μ- and κ-opioid receptors are reported in Table 1. Strong binding affinities (low nanomolar range) towards μ receptor were found for both (+)- and (−)-chimonanthine monourethanes (**9**) and (**10**), whereas (−)-chimonanthine (**11**), meso-chimonanthine (**13**) and hodgkinsine displayed much lower affinities to μ receptor than morphine or the monourethanes. Even lower affinity was found for (+)-chimonanthine (**12**), whereas no affinity for carbomethoxychimonanthine (**4**) could be detected.

All these compounds, tested in a selective κ-opioid binding assay, showed poor affinities with K_i values in the micromolar range.

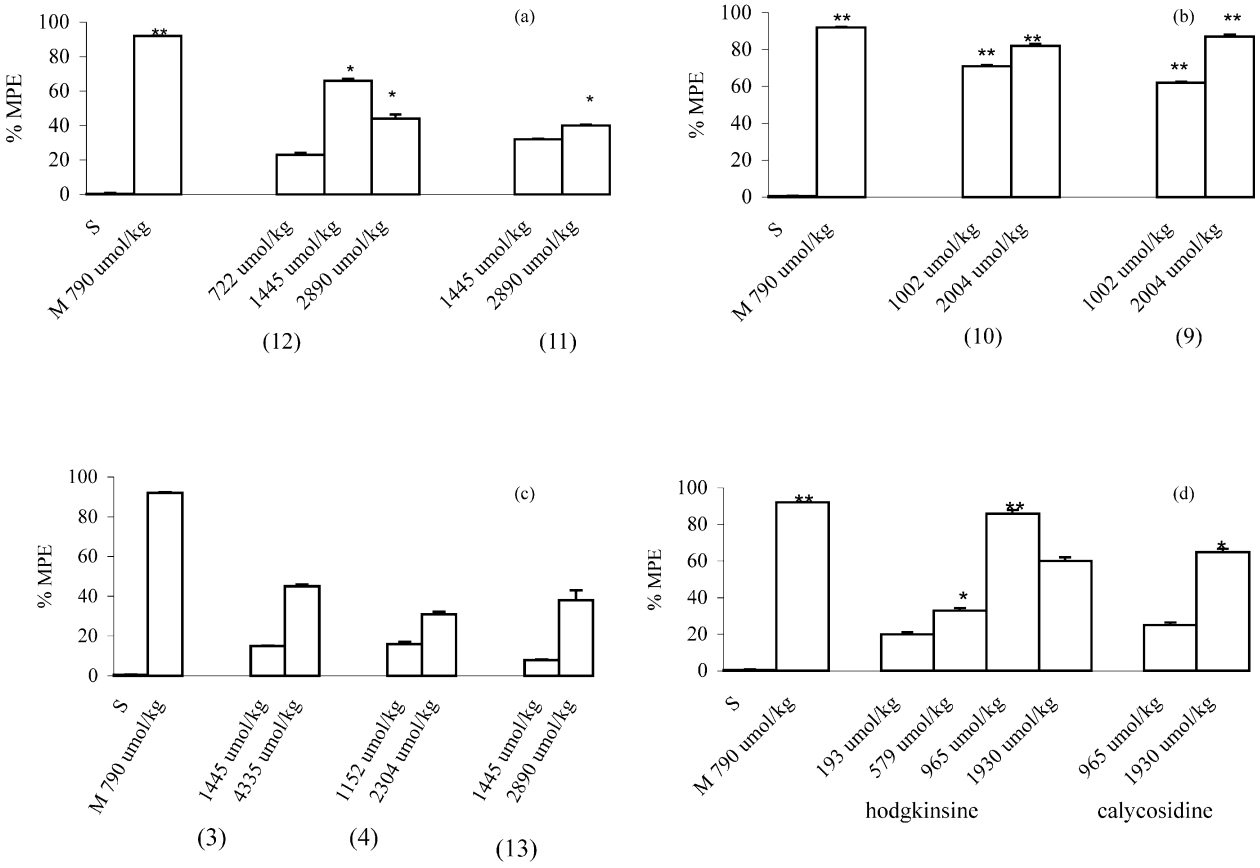


Figure 1. Effects of morphine (M), chimonanthines, hodgkinsine and calycosidine on the tail flick test. **1a** dose–effect analysis of the (+)- and (−)-chimonanthine (**12**) and (**11**); **1b** effects of (+)- and (−)-chimonantine monourethanes (**10**) and (**9**); **1c** effects of rac-chimonanthine, (meso)-carbomethoxychimonanthine (**4**) and meso-chimonanthine (**13**); **1d** effects of hodgkinsine and calycosidine. $N = 6$–8. S = saline. Columns represent % of maximum possible effect (%MPE) and vertical bars SEM. * = $p < 0.05$; ** = $p < 0.01$, ANOVA/Student–Newman–Keuls.

Tail-flick

Figure 1 shows results with the tail-flick model. (+)-chimonanthine (**12**) presents 66% of MPE at 1445 μmol/kg (5 mg/kg) [compared to morphine 790 μmol/kg (6 mg/kg)]) (Fig. 1a); nevertheless, it presents a bell shaped dose–effect relationship with 44% of MPE at 2890 μmol/kg (10 mg/kg). Its enantiomer (−)-chimonanthine (**11**) presents 40% of MPE at 2890 μmol/kg (10 mg/kg). Figure 1b shows that monourethanes of (−)- and (+)-chimonanthine (**9** and **10**) are equally active at 1002 μmol/kg (5 mg/kg), 71 and 62% of MPE, respectively, at the same dose. Figure 1c shows that (rac)-(chimonanthine), (meso)-chimonanthine (**13**) and (meso)-carbomethoxychimonanthine (**4**) do not bring about significant effect in any of the doses tested. Figure 1d shows that hodgkinsine 965 μmol/kg (5 mg/kg) results in 86% of MPE, comparable to morphine, and calycosidine 1930 μmol/kg (10 mg/kg) results in 70% of MPE.

Table 1. Receptor binding affinities of chimonanthines and pyrrolidinoindoline-type alkaloid analogues to the human μ- and κ-opioid receptors

Compound	(μ) K_i (nM)	(κ) K_i (nM)
4	>2500	>2500
9	5.7±1.4	996±263
10	4.8±0.6	≥2500
11	271±85	>2500
12	652±159	>2500
13	341±29	1447±45
Hodgkinsine	277±49	>1000
Morphine	0.76±0.04	63.9±10.5

Data are expressed as mean±SEM derived from at least three independent determinations.

Capsaicin-induced pain

Figure 2 shows data from capsaicin-induced pain. Figure 2a shows that (+)-chimonanthine (**12**) results in 38% inhibition at 72.2 μmol/kg (0.25 mg/kg) and lacks a clear dose–effect relationship. (−)-Chimonanthine (**11**) 72.2 μmol/kg (0.25 mg/kg) results in 47% inhibition of licking time, and looses activity at 144.5 μmol/kg (0.5 mg/kg). Figure 2b shows that monourethanes of (+)- and (−)-chimonanthine are inactive in the same mg/kg dose range. Figure 2c shows that (rac)-chimonanthine at 144.5 μmol/kg (0.5 mg/kg) results in 29% inhibition

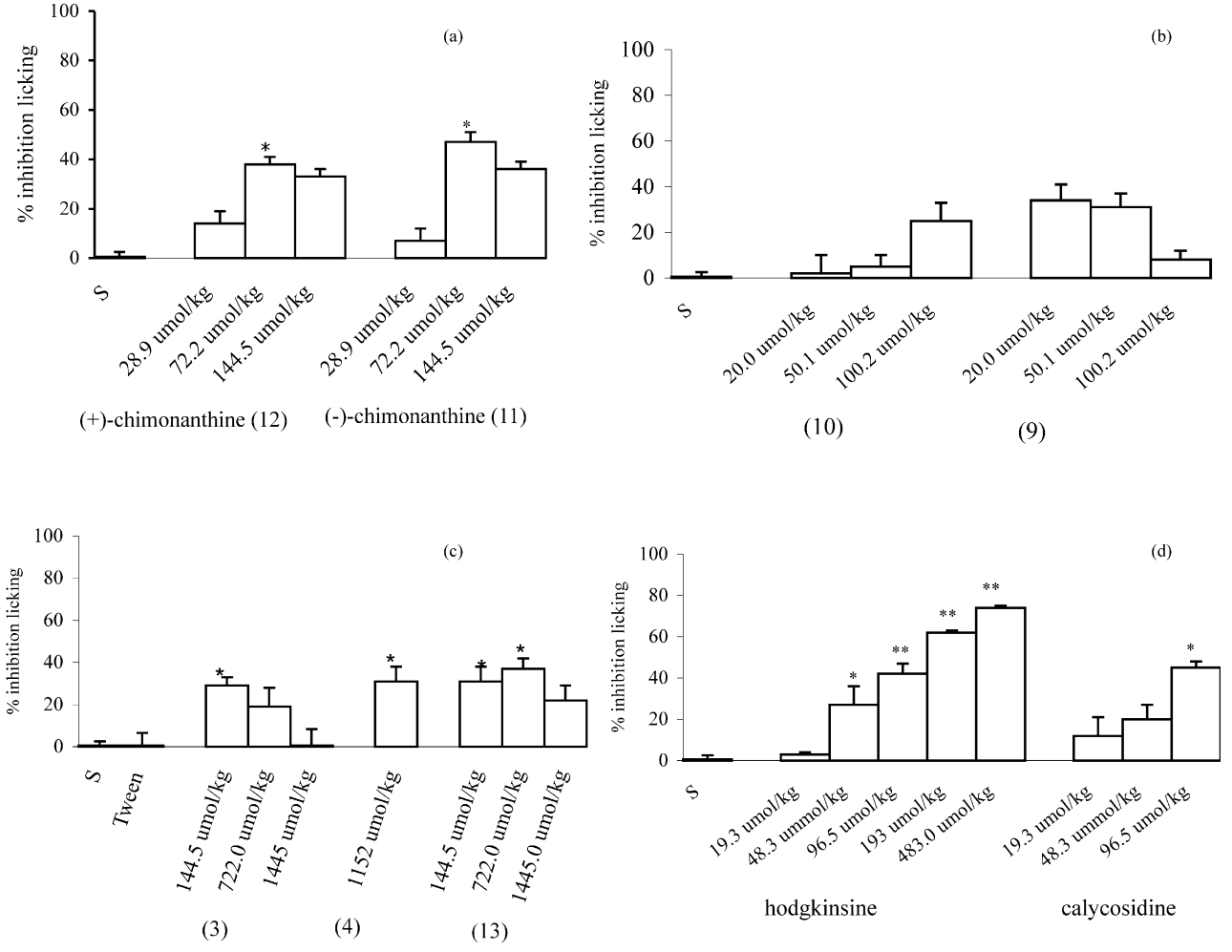
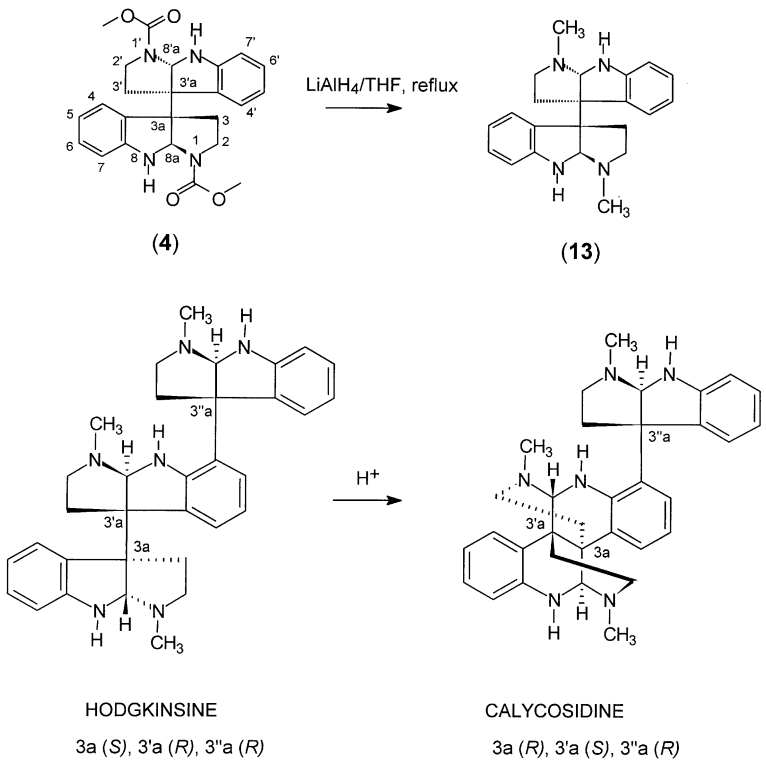


Figure 2. Effects of the chimonanthines, hodgkinsine and calycosidine in capsaicin-induced pain. **2a** Dose–effect analysis of (+)-chimonanthine (**12**) and (−)-chimonanthine (**11**); **2b** effects of (+)- and (−)-chimonantine monourethanes (**10**) and (**9**); **2c** effect of rac-chimonanthine, (meso)-carbomethoxychimonanthine (**4**) and meso-chimonanthine (**13**); **2d** effects of hodgkinsine and calycosidine. $N=6$–10. S = saline. Columns represent percentage of inhibition and the vertical bars SEM. * = $p<0.05$, ANOVA/Student–Newman–Keuls.



[being inactive at 722.0 and 1445 μmol/kg (2.5 and 5.0 mg/kg)] that (meso)-carbomethoxychimonanthine (**4**) at 1125 μmol/kg (5 mg/kg) results in 45% inhibition of licking time, and that (meso)-chimonanthine (**13**) at 144.5 and 722.0 μmol/kg (0.5 and 2.5 mg/kg) results in 30–35% inhibition, losing activity at 1445 μmol/kg (5.0 mg/kg). Hodgkinsine and calycosidine decreased dose-dependently the licking time in the same dose range (Fig. 2d).

It is important to note that the purpose of this pharmacological analysis was to differentiate active from inactive compounds, comparing activities when possible.

A surprisingly high binding affinity to the μ-opioid receptor, was found with both (+)- and (−)-chimonanthine monourethanes (**10**) and (**9**). (−)-Chimonanthine (**11**), meso-chimonanthine (**13**) and hodgkinsine displayed lower affinities, while, as expected, carbomethoxychimonanthine (**4**) did not show affinity for this receptor. Compared to the trimer hodgkinsine, all the products showed a fairly minor activity when tested in the tail-flick model (see Fig. 1); carbomethoxychimonanthine (**4**), (rac)-chimonanthine and (meso)-chimonanthine (**13**) were inactive in this model in the doses studied, while (−)-chimonanthine (**11**) was moderately active. The compound which better resembles the behaviour of hodgkinsine is (+)-chimonanthine (**12**).[17]

Among the different chemical structures with affinity for the opioids receptors, two common features are found: a basic amino group and an aromatic ring. Effective receptor interaction is further dependent on the conformation assumed by the drug, in which the aromatic ring and the hydrogen acceptor exhibit a spatial relationship similar to that of morphine. Of the two basic centers N1 and N8 present in the molecules here studied, the weak hydrogen acceptor N8 does not appear to be particularly critic with respect to the activity, a fact demonstrated by the strong activity of (−)- and (+)-chimonanthine urethanes (**9**–**10**). On the contrary, N1 is required to maintain the basic character, such as in morphine, as shown by the lack of activity of carbomethoxychimonanthine (**4**).

A 3a(*R*) configuration of the pyrrolidinoindoline unit seems to be favorable for maintaining activity, since calycosidine, the product obtained by acid treatment of hodgkinsine (see Experimental), is as active as hodgkinsine in the tail-flick model (see Fig. 1d). Since the bisquinoline part of the molecule is devoid of activity in this model, as demonstrated by the inactivity of (−)-calycanthine (data not shown), the presence of a pyrrolidinoindoline unit possessing a 3a(*R*) configuration can be considered preferential.

The behaviour of the monourethanes (**9**–**10**) is particularly intriguing. The derivatives of both (−)- and (+)-chimonanthine show the same activity, again supporting the poor selectivity of the optically active bis-pyrrolidinoindoline moiety. Since chimonanthines possess fairly low binding affinities for the opioid μ receptor, the strong activities shown by **9** and **10** are to be attributed to the benzylcarbamate moiety. A similar behaviour is shown by some well known benzamide and benzeneacetamide amines, which are morphine-like analgesics with high affinity for the μ and κ opioid receptors. In these benzamide and benzeneacetamide amines a pyrrolidine type nitrogen is linked through two cyclic carbon atoms to a tertiary arylamide.[20–22]

Concerning capsaicin-induced pain, only hodgkinsine and calycosidine presented clear dose-dependent responses (Fig. 2), hodgkinsine being clearly more active. The other compounds did not show significant activity or clear dose–effect relationship.

While it is accepted that the NMDA glutamate receptor play a prominent role in capsaicin-induced pain, a definitive conclusion on the presence of a NMDA antagonistic property would require NMDA binding data, still lacking for most of these compounds. Nevertheless, given that hodgkinsine and psychotridine (a pentameric pyrrolidinoindoline alkaloid also isolated from *P. colorata*[12,13]) dose-dependently inhibit capsaicin-induced pain[14] and the binding of the NMDA antagonist MK801,[23] the results obtained with the capsaicin model can be regarded as an indication of a potential NMDA antagonism. In any case, only the highest (more than two units) members of our oligomeric series seem to be active in this model.

A better correlation between in vivo and opioid in vitro data is seen with results obtained with tail flick rather than with capsaicin. This is to be expected, since thermal pain models are known to be more directly associated with the opioid system, whereas the glutamate NMDA receptors are particularly important in capsaicin-induced pain. The opioid κ subtype receptor does not seem to be involved in the activity (very low affinity for all compounds), while the opioid δ subtype (not evaluated) has been reported to be less associated to both tail flick and capsaicin-induced pain models.[24]

Because NMDA antagonism is of relevance for slowing opioid tolerance, compounds presenting both opioid activation and NMDA inhibition properties are of interest. The biological data so far obtained with these oligomeric pyrrolinoindoline alkaloids point to the validity of identifying a synthetic approach for obtaining a compound series in enough quantities for the necessary comprehensive pharmacological analysis. A definitive SAR pattern could only be attained if all possible pyrrolidinoindoline-type oligomers compatible with the existing stereocenters could be prepared.

Experimental

Chemistry

Mps have been measured on a Buchi 510 mp apparatus and are reported uncorrected. Optical rotations have been measured on a Perkin-Elmer 241 polarimeter. UV spectra have been recorded on a Hewlett-Packard 8452A spectrophotometer (average concentrations 10^{-4} M); CD spectra have been measured on a Jasco J-500 dicrograph (*c* 10^{-4} M). TLC (silica gel 60 F_{254}) were eluted with $CHCl_3$–MeOH–NH_4OH 9:1:0.15. Spots were revealed by spraying with modified Ehrlich reagent (*p*-dimethylaminocinnamaldehyde in 25% methanolic HCl) or 10% methanolic H_2SO_4, followed by heating, or by absorption of the UV light (254 nm). Column chromatography was performed with silica gel 60 (63–200 or 40–63 μm) or with neutral Alumina (activity I) (art 1.0177, Merck). HPLC analyses were carried out on a Waters 600-MS liquid-liquid chromatograph, connected with a Waters PDA 991 detector and a NEC 386/25 personal computer. A Symmetry C18 column (5 μm, 4.6×250 mm, Waters) was used, eluted with MeOH/H_2O/Et_2NH 80:20:0.1 at a flow rate of 1 mL/min. Samples were filtered prior to each injection through Millex FH_{13} filters (5 μm, Millipore). EI (70 eV) and CI (isobutane) mass spectra were recorded on a VG 7070 EQ mass spectrometer. 200 and 300 MHz NMR spectra were recorded on a Bruker AC 200, AC 300 and Varian Unity Inova 300 spectrometers. Samples were dissolved in $CDCl_3$ or DMSO-d_6 and TMS was used as reference. DQF-COSY, E-COSY, NOESY, ROESY, GHSQC, GHMBC experiments were performed by using the available Varian software (VNMR). Medium pressure liquid chromatography was carried out with a Büchi 681 Chromatography Pump; a glass column was packed with Lichroprep RP8 (25–40 μm; art. 9324 Merck).

3-[2′(Methoxycarbonyl)amino]ethyl]indole (2). Tryptamine (100.3 g, 0.626 mol) was dissolved in dry CH_2Cl_2 (1.6 L), under nitrogen atmosphere, and dry Et_3N (124 mL) was added. The mixture was cooled to 0 °C, and with stirring, methylchloroformate (55 mL, 0.718 mol) in dry CH_2Cl_2 (300 mL) was added dropwise over 30 min. After warming to room temperature, stirring was continued until the disappearance of tryptamine (2 h 30 min). The mixture was washed with 2% HCl (410 mL), then with brine (850 mL). The aqueous phase was extracted twice with CH_2Cl_2. The pooled organic phases were dried (Na_2SO_4) and the solvent evaporated in vacuum. The residue was crystallized (iPr_2O) to give 2 (74.3 g, 69%). Mp 79 °C. ^{1}H NMR ($CDCl_3$) δ 8.01 (bs, 1H, N1-H), 7.60 (d, J=7.5 Hz, 1H, C7-H), 7.36 (d, J=7.5 Hz, 1H, C4-H), 7.20 (t, J=7.5 Hz, 1H, C6-H), 7.12 (t, J=7.5 Hz, 1H, C5-H), 7.03 (s, 1H, C2-H), 4.73 (bs, 1H, N3′-H), 3.65 (s, 3H,-OCH_3), 2.96 (t, J=8 Hz, 2H, C2′-H_2), 2.52 (m, 2H, C1′-H_2); EI–MS *m*/*z* 218 $[M]^+$ (17), 143 (27), 130 (100). Anal. ($C_{12}H_{14}N_2O_2$) C: calcd 66.04, found 66.10, H calcd 6.47, found 6.40, N calcd 12.84, found 12.90.

(±)-Carbomethoxychimonanthine (3) and (meso)-carbomethoxychimonanthine (4). The reaction was performed on compound **2** (45 g), according to ref 16 for 40 h at room temperature. The crude material was purified on silica gel eluted with $CHCl_3$/EtOAc 9:1 to give 17% of starting material and 30% of the mixture 3+4. 13.5 g. of 3+4 were repeatedly purified on silica gel eluted with $CHCl_3$/EtOAc 85.15, to give 7.2 g of (±)-carbomethoxychimonanthine (**3**) and 4.4 g of (meso)-carbomethoxychimonanthine (**4**).

3. Mp 191 °C (lit 207), R_f 0.31 ($CHCl_3$/EtOAc 8:2). ^{1}H NMR ($CDCl_3$) δ 7.27 (d, J=7.4 Hz, 1H, C4-H), 7.04 (t, J=7.4 Hz, 1H, C6-H), 6.65 (t, J=7.4 Hz, 1H, C5-H), 6.59 (dd, J=7.4, 3.3 Hz, 1H, C7-H), 6.43 (bs, 1H, N8-H), 4.73 (s, 1H, C8a-H), 3.53 (s, 3H, OCH_3), 3.52 (m, 1H, C2-H_B), 2.64 (ddd, J=16.0, 11.0, 5.7 Hz, 1H, C2-H_A), 2.51 (m, 1H, C3-H_B), 2.51 (m, 1H, C3-H_A); CI MS *m*/*z* 435 $[M+H]^+$ (33); 217 $[C_{12}H_{13}N_2O_2]^+$ (100), 187

$[C_{11}H_{11}N_2O]^+$ (30), 144 (39), 130 $[C_9H_9N]^+$ (50), 81 (40), 69 (91). Anal. ($C_{12}H_{14}N_2O_2$) C: calcd 66.34, found 66.60, H calcd 6.08, found 5.90, N calcd 12.89, found 13.00.

4. Mp 276 °C (lit 295) R_f 0.22 ($CHCl_3$/EtOAc 8:2). ^1H NMR ($CDCl_3$) δ 6.97 (t, $J=7.9$ Hz, 1H, C6-H), 6.57 (d, $J=7.9$ Hz, 1H, C4-H), 6.48 (t, $J=7.9$ Hz, 1H, C5-H), 6.45 (d, $J=7.9$ Hz, 1H, C7-H), 6.22 (bs, 1H, N8-H), 3.64 (s, 3H, OCH_3), 3.64 (m, 1H, C2-H_B), 2.80 (m, 1H, C2-H_A), 2.30 (m, 1H, C3-H_B), 2.20 (dd, $J=12.3$, 6.1 Hz, C3-H_A); CI MS m/z 435 $[M+H]^+$ (20), 219 (100), 217 $[C_{12}H_{13}N_2O_2]^+$ (99), 187 $[C_{11}H_{11}N_2O]^+$ (30), 185 (34), 144 (37), 130 $[C_9H_9N]^+$ (34), 85 (38), 73 (81). Anal. ($C_{12}H_{14}N_2O_2$) C: calcd 66.34, found 66.60, H calcd 6.08, found 5.90, N calcd 12.89, found 13.10.

(+)-Carbomethoxychimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (6) and (−)-carbomethoxychimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (5). To a stirred solution of (−)-carbomethoxychimonanthine (3) (6.8 g, 15.65 mmol) in dry $CHCl_3$ (125 mL), kept under nitrogen atmosphere, 30 mL of Et_3N were added, followed by a solution of 5 mL of (−)-(*S*)-(α-methyl-benzyl) isocyanate in dry $CHCl_3$ (20 mL) added dropwise. The mixture was refluxed for 24 h. The solution was allowed to cool to room temperature, H_2O–EtOH 1:1 (20 mL), then 5% HCl were added to reach pH 6 and extracted with $CHCl_3$ (2×600 mL). The organic phases were washed with H_2O, dried (Na_2SO_4) and evaporated to dryness under vacuum. The crude material (9.1 g) was purified on silica gel (flash chromatography, 8 cm) eluted with $CHCl_3$/EtOAc 6:4, to give four fractions: fr. A (1.0 g), fr. B (1.3 g, starting material, 38%), fr. C (2.8 g) and fr. D (1.5 g).

Fr. A was flash chromatographed on silica gel (petrol/EtOAc 6:4) obtaining a mixture of the di-carbamates (**7**) and (**8**) (0.64 g) which was crystallized ($iPrO_2$), to give 248 mg of (+)-carbomethoxychimonanthine N8, N8′-(−)-(*S*)-(α-methyl-benzyl)dicarbamate (**8**). The mother liquors were repeatedly purified on silica gel, eluted with toluene/EtOAc 85:15, obtaining a fraction (69 mg) which was crystallized ($iPrO_2$) to give 27 mg of (−)-carbomethoxychimonanthine N8, N8′-(−)-(*S*)-(α-methyl-benzyl)dicarbamate (**7**).

8. Mp 262 °C (iPr_2O); $[\alpha]_D^{20}=+160.2°$ (*c* 0.9, $CHCl_3$); R_f 0.33 (toluene/EtOAc 8:2); ^1H NMR ($CDCl_3$) δ 8.37 (d, $J=7.6$ Hz, 1H, ArN*H*CO), 7.81 (d, $J=8.2$ Hz, 1H, C7-H), 7.46 (d, $J=7.7$ Hz, 2H, Ar2+6-H_2), 7.36 (t, $J=7.3$ Hz, 2H, Ar3+5-H_2), 7.27 (m, 1H, C6-H), 7.26 (m, 1H, Ar4-H), 7.15 (d, $J=7.7$ Hz, 1H, C4-H), 7.02 (t, $J=7.3$ Hz, C5-H), 5.69 (bs, 1H, C8a-H), 5.07 (dq, $J=7.2$ Hz, Ar–C*H*–N), 3.74 (s, 3H, OCH_3), 3.87 (dd, $J=10.4$, 6.5 Hz, 1H, C2-H_A), 2.87 (m, 1H, C2-H_B), 2.16 (m, 2H, C3-H_{A+B}), 1.61 (d, $J=7.0$ Hz, Ar–CH–CH_3); CI MS m/z: 729 $[M+H]^+$ (100), 625 (12), 582 $[C_{33}H_{35}N_5O_5]^+$ (57), 435 $[C_{24}H_{27}N_4O_4]^+$ (12), 366 (39), 279 (21), 262 (20), 257 (19), 219 (75), 217 $[C_{12}H_{13}N_2O_2]^+$ (97), 187 (29), 174 (15), 159 (23). Anal. ($C_{42}H_{44}N_6O_6$) C: calcd 69.21, found 69.40, H calcd 6.08, found 5.90, N calcd 11.53, found 11.70.

7. Mp 152–154 °C (iPr_2O); $[\alpha]_D^{20}=-10.6°$ (*c* 0.34, $CHCl_3$); R_f 0.29 (toluene/EtOAc 8:2); ^1H NMR ($CDCl_3$) δ 8.66 (d, $J=5.2$ Hz, 1H, ArN*H*CO), 7.61 (d, $J=8.2$ Hz, 1H, C7-H), 7.46 (d, $J=7.1$ Hz, 2H, Ar2+6-H_2), 7.35 (t, $J=7.1$ Hz, 2H, Ar3+5-H_2), 7.26 (t, $J=7.1$ Hz, 1H, Ar4-H), 7.18 (m, 1H, C6-H), 6.79 (m, 2H, C4+5-H_2), 5.95 (s, 1H, C8a-H), 5.09 (dq, $J=6.9$, 5.5 Hz, Ar–C*H*–N), 3.78 (s, 3H, OCH_3), 3.70 (m, 1H, C2-H_A), 2.83 (dd, $J=10.1$, 9.4 Hz, 1H, C2-H_B), 1.87 (m, 2H, C3-H_{A+B}), 1.65 (d, $J=7.1$ Hz, Ar–CH–CH_3); CI MS m/z: 729 $[M+H]^+$ (97); 625 (10); 582 $[C_{33}H_{35}N_5O_5]^+$ (57); 435 $[C_{24}H_{27}N_4O_4]^+$ (12); 366 (38); 279 (24); 262 (18); 219 (78); 217 $[C_{12}H_{13}N_2O_2]^+$ (100); 187 (35); 174 (16); 159 (24). Anal. ($C_{42}H_{44}N_6O_6$) C: calcd 69.21, found 69.40, H calcd 6.08, found 5.90, N calcd 11.53, found 11.70.

Fr. C was purified on silica gel eluted with $CHCl_3$/EtOAc 85:15, to give (−)-carbomethoxychimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (**5**) (1.68 g, 16.5%). Mp 128–130 °C (iPr_2O); $[\alpha]_D^{20}=-261°$ (*c* 0.3, $CHCl_3$), R_f 0.13 (*n*-hexane/EtOAc 6:4); 0.167 (CH_3OH/H_2O 74:26); $t_R=8.36$ min (CH_3OH/H_2O/Et_2NH 80:20:0.1); ^1H NMR (DMSO-d_6) δ 8.29 (d, $J=6.5$ Hz, 1H, ArN*H*CO), 7.56 (d, $J=8.3$ Hz, 1H, C7-H), 7.44 (d, $J=8.3$ Hz, 1H, C4-H), 7.41 (d, $J=8.0$ Hz, 2H, Ar2+6-H_2), 7.38 (m, 2H, Ar3+5-H_2), 7.27 (m, 1H, Ar4-H), 7.24 (t, $J=7.5$ Hz, 1H, C6-H), 7.17 (d, $J=7.5$ Hz, 1H, C4′-H), 7.06 (m, 1H, C6′-H), 7.02 (t, $J=7.5$ Hz, 1H, C6-H), 6.60 (m, 1H, C5′-H), 6.58 (d, $J=7.0$ Hz, 1H, C7′-H), 5.49 (bs, 1H, C8a-H), 4.74 (s, 1H, C8′a-H), 4.93 (m, Ar–C*H*--N), 3.63 (s, 3H, OCH_3), 3.56 (s, 3H, OCH_3), 3.70 (m, 1H, C2-H_A), 3.51 (m, 2H, C2-H_{A+B}), 2.70 (m, 1H, C2-H_B), 2.49 (m, 1H, C3-H_A), 2.38 (m, 1H, C3-H$_A$), 2.22 (m, 1H, C3-H_B), 2.16 (m, 1H, C3-H_B),1.41 (d, $J=6.5$ Hz, Ar–CH–CH_3); CI MS m/z: 582 $[M+H]^+$ (58), 478 $[M-106]^+$ (3), 435 $[C_{24}H_{27}N_4O_4]^+$ (10), 366 (14), 257 (13), 217 $[C_{12}H_{13}N_2O_2]^+$ (69), 199 (20), 187 $[C_{11}H_{11}N_2O]^+$ (26), 144 (44), 130 $[C_9H_9N]^+$ (57), 105 (100), 91 $[C_7H_7]^+$ (84), 81 (50), 71 (92). Anal. ($C_{33}H_{35}N_5O_5$) C: calcd 68.14, found 68.50, H calcd 6.07, found 5.80, N calcd 12.04, found 12.20.

Fr. D was purified on silica gel eluted with petrol/EtOAc 85:15, to give (+)-carbomethoxychimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (**6**) (1.33 g, 14.6%). Mp 131–133 °C (iPr_2O); $[\alpha]_D^{20}=+274.4°$ (*c* 0.6, $CHCl_3$); R_f 0.093 (*n*-hexane/EtOAc 6:4); 0.167 (CH_3OH/H_2O 74:26); $t_R=11.04$ min (CH_3OH/H_2O/Et_2NH 80:20:0.1); ^1H NMR (DMSO-d_6) δ 8.24 (d, $J=7.9$ Hz, 1H, ArN*H*CO), 7.51 (d, $J=7.4$ Hz, 1H, C4-H), 7.41 (d, $J=7.4$ Hz, 1H, C7-H), 7.33 (m, 2H, Ar2+6-H_2), 7.33 (m, 2H, Ar3+5-H_2), 7.32 (d, $J=7.0$ Hz, 1H, C4′-H), 7.24 (m, 1H, Ar4-H), 7.23 (m, 1H, C6-H), 7.11 (t, $J=7.6$ Hz, 1H, C6′-H), 7.04 (t, $J=7.3$ Hz, 1H, C6-H), 6.70 (t, $J=7.0$ Hz, 1H, C5′-H), 6.66 (d, $J=7.0$ Hz, 1H, C7′-H), 5.27 (bs, 1H, C8a-H), 4.54 (s, 1H, C8′a-H), 4.90 (m, Ar–C*H*–N), 3.61 (s, 3H, OCH_3), 3.55 (s, 3H, OCH_3), 3.69 (m, 1H, C2-H_A), 3.60 (m, 1H, C2′-H_A), 2.70 (m, 2H, C2-H_B+C2′-H_B), 2.54 (m, 1H, C3-H_A), 2.49 (m, 1H, C3′-H_A), 2.34 (m, 1H, C3-H_B), 2.31 (m, 1H, C3′-H_B), 1.41 (d, $J=7.0$ Hz, Ar–CH–CH_3); CI MS m/z: 582 $[M+H]^+$ (58), 522 $[M-59]^+$ (2), 478

$[M-106]^+$ (5.4), 435 $[C_{24}H_{27}N_4O_4]^+$ (9.2), 366 (17), 317 $[C_{10}H_{21}N_4]^+$ (3), 217 $[C_{12}H_{13}N_2O_2]^+$ (92), 187 $[C_{11}H_{11}N_2O]^+$ (24), 144 (31), 130 $[C_9H_9N]^+$ (55), 105 (100). Anal. ($C_{33}H_{35}N_5O_5$) C: calcd 68.14, found 68.40, H calcd 6.07, found 5.80, N calcd 12.04, found 12.20.

(−)-chimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (9). To a suspension of $LiAlH_4$ (890 mg, 24.1 mmol) in dry THF (50 mL), 5 (1.3 g, 2.24 mmol, in 70 mL of dry THF), was added dropwise at 0 °C. The reaction is warmed to reflux for 3 h 30 min. The mixture was left to reach room temperature, then was carefully basified to pH 9 with 10% NaOH and filtered on Celite which was repeatedly washed with THF. The solution was concentrated to water and extracted with $CHCl_3$ (2×250 mL). The organic phases were washed with H_2O, dried (Na_2SO_4) and evaporated to dryness under vacuum.

The crude material was purified on silica gel, eluted with EtOAc/Et_2NH 98:2 (800 mL) and 96:4 (1 L), to give (−)-chimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (**9**) (750 mg, 68%) and 270 mg of a fraction which was further purified on silica (EtOAc/Et_2NH 97:3) to give (−)-chimonanthine (**11**) (145 mg, 18%).

9. Mp 112–113 °C (*n*-hexane/iPr_2O 1:1); $[\alpha]_D^{20} = -83.4°$ (*c* 0.7, $CHCl_3$); R_f 0.24 (EtOAc/Et_2NH 96:4) ^1H NMR (DMSO-d_6) δ 8.32 (s, 1H, ArN*H*CO), 7.52 (d, $J = 8.0$ Hz, 1H, C7-H), 7.46 (d, $J = 7.4$ Hz, 2H, Ar2 + 6-H_2), 7.36 (t, $J = 7.1$ Hz, 2H, Ar3 + 5-H_2), 7.25 (t, $J = 7.1$ Hz, 1H, Ar4-H), 7.16 (d, $J = 7.5$ Hz, 1H, C4-H), 6.86 (m, 1H, C6-H), 6.84 (m, 1H, C4′-H), 6.68 (1H, C5-H), 6.66 (m, 1H, C6′-H), 6.24 (d, $J = 7.2$ Hz, 1H, C7′-H), 6.06 (bs, 1H, N8′-H), 5.62 (m, 2H, C8a + 8′a,-H_2), 5.02 (q, $J = 7.2$ Hz, 1H, Ar–C*H*–N), 2.70 (m, 1H, C2-H_A), 2.60 (m, 1H, C2′-H_A), 2.50 (m, 1H, C3-H_B), 2.44 (s, 3H, N1′-CH_3), 2.41 (m, 1H, C3′-H_A), 2.32 (s, 3H, N1-CH_3), 2.30 (m, 1H, C2′-H_B), 2.25 (m, 1H, C2-H_B), 2.01 (m, 1H, C3-H_A)1.95 (m, 1H, C3′-H_B), 1.52 (s, 3H, Ar–CH–*C*H_3); CI MS *m*/*z*: 494 $[M+H]^+$ (32), 347 $[M+H\text{-}147]^+$ (6), 319 $[C_{20}H_{21}N_3O]^+$ (37), 174 $[C_{11}H_{14}N_2]^+$ (75), 172 $[C_{11}H_{12}N_2]^+$ (71), 131 (37), 130 $[C_9H_9N]^+$ (42), 108 (55), 105 (62), 91 $[C_7H_7]^+$ (100). Anal. ($C_{31}H_{34}N_5O$) C: calcd 75.43, found 75.60, H calcd 7.15, found 6.80, N calcd 14.19, found 14.00.

11. Mp 171–172 °C (iPr_2O/$CHCl_3$); $[\alpha]_D^{20} = -310°$ (*c* 0.8, EtOH); R_f 0.32 ($CHCl_3$/CH_3OH/NH_3 9:1:0.15) $t_R = 6.16$ min (CH_3OH/H_2O/Et_2NH 80:20:0.1); ^1H NMR ($CDCl_3$) δ 7.23 (d, $J = 7.5$ Hz, 1H, C4-H), 7.04 (t, $J = 7.5$ Hz, 1H, C6-H), 6.71 (t, $J = 7.5$ Hz, 1H, C5-H), 6.59 (d, $J = 7.5$ Hz, 1H, C7-H), 4.48 (bs, 1H, C8a-H), 2.59 (m, 2H, C2-H_{A+B}), 2.58 (m, 1H, C3-H_A), 2.37 (s, 3H, N–CH_3), 2.12 (dt, $J = 12.0$, 6.4 Hz, 1H, C3-H_B), ^1H NMR (DMSO-d_6) δ 7.04 (bs, 1H, C4-H), 6.81 (bs, 1H, C6-H), 6.40 (bs, 1H, C5-H), 6.37 (bs, 1H, C7-H), 6.11 (bs, 1H, NH), 4.11 (bs, 1H, C8a-H), 2.59 (m, 1H, C2-H_A), 2.46 (m, 1H, C3-H_A), 2.31 (m, 1H, C2-H_B), 2.26 (s, 3H, N–CH_3), 1.85 (m, 1H, C3-H_B); ^{13}C NMR ($CDCl_3$) δ 150.8 (s, C7a), 133.2 (s, C4a), 128.5 (d, C6), 124.9 (d, C4), 119.1 (d, C5), 109.8 (d, C7), 85.6 (s, C8a), 63.7 (s, C3a), 53.0 (t, C2), 37.4 (q, N–CH_3), 35.9 (t, C3); EI MS *m*/*z*: 346 M^+(2), 174 (43), 173 (94), 172 (100), 171 (51), 157 (14), 143 (16), 131 (68), 130 $[C_9H_9]^+$ (100), 117 (16), 103 (19), 77 (25). Anal. ($C_{22}H_{26}N_4$) C: calcd 76.27, found 76.30, H calcd 7.56, found 7.40, N calcd 16.17, found 16.30.

(+)-Chimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (10). According to the procedure used for making compound **9**, from compound **6**, (+)-chimonanthine N8-(−)-(*S*)-(α-methyl-benzyl)carbamate (**10**) (605 mg, 59%) and (+)-chimonanthine (**12**) (120 mg, 16%) were obtained.

10. Mp = 106–108 °C (*n*-hexane/iPr_2O 1:1); $[\alpha]_D^{20} = +119.7°$ (*c* 0.8, $CHCl_3$); R_f 0.37 (EtOAc/Et_2NH 96:4) ^1H NMR (DMSO-d_6) δ 7.06 (m, 1H, ArNHCO), 7.50 (d, $J = 7.8$ Hz, 1H, C7-H), 7.44 (d, $J = 7.4$ Hz, 2H, Ar2 + 6-H_2), 7.35 (t, $J = 7.2$ Hz, 2H, Ar3 + 5-H_2), 7.26 (t, $J = 7.2$ Hz, 1H, Ar4-H), 7.18 (d, $J = 7.6$ Hz, 1H, C4-H), 6.96 (t, $J = 7.9$ Hz, 1H, C6-H), 6.87 (d, $J = 6.5$ Hz, 1H, C4′-H), 6.75 (t, $J = 7.4$ Hz, 1H, C5-H), 6.73 (t, $J = 7.4$ Hz, 1H, C6′-H), 6.27 (d, $J = 7.4$ Hz, 1H, C7′-H), 6.07 (bs, 1H, N8′-H), 5.45 (m, 2H, C8a + 8′a,-H_2), 5.06 (dq, $J = 7.2$ Hz, 1H, Ar–C*H*–N), 2.62 (m, 1H, C2-H_A), 2.53 (m, 1H, C2′-H_A), 2.41 (m, 1H, C3-H_A); 2.33 (s, 3H, N1′-CH_3), 2.23 (m, 1H, C2-H_B), 2.28 (m, 1H, C2′-H_B), 2.28 (s, 3H, N1-CH_3), 1.98 (m, 1H, C2′-H_A), 1.92 (1H, m, C3-H_B), 1.52 (s, 3H, Ar–CH–*C*H_3); CI MS *m*/*z*: 494 $[M+H]^+$ (77), 376 $[M+H\text{-}2\times CH_3OCO]^+$ (13), 349 (17), 347 $[M+H\text{-}147]^+$ (16), 215 (14), 186 (16), 174 $[C_{11}H_{14}N_2]^+$ (99), 172 $[C_{11}H_{12}N_2]^+$ (100), 158 (17), 144 (30), 132 (43), 130 $[C_9H_9N]^+$ (47), 105 (48), 91 $[C_7H_7]^+$ (15). Anal. ($C_{31}H_{34}N_5O$) C: calcd 75.43, found 75.60, H calcd 7.15, found 6.90, N calcd 14.19, found 14.00.

12. Mp 168–170 °C (iPr_2O/$CHCl_3$ 3:1); $[\alpha]_D^{20} = +270°$ (*c* 0.9, EtOH); R_f 0.32 ($CHCl_3$/CH_3OH/NH_3 9:1:0.15) $t_R = 6.16$ min (CH_3OH/H_2O/Et_2NH 80:20:0.1); ^1H NMR, ^{13}C NMR and MS identical to **11**. Anal. ($C_{22}H_{26}N_4$) C: calcd 76.27, found 76.40, H calcd 7.56, found 7.40, N calcd 16.17, found 16.20.

(−)-Chimonanthine (11). Compound **9** (27.5 mg, 55.7 mmol) was treated with 1 N sodium pentoxide (1.8 mL) and the mixture was refluxed for 3 h. At room temperature, 0.5 N HCl was added to reach pH 8; the mixture was diluted with H_2O and extracted with $CHCl_3$ (2×15 mL). The organic phases were washed with water, dried (Na_2SO_4) and evaporated to dryness under vacuum. The crude material was purified on silica gel, eluted with EtOAc/Et_2NH 96:4, to give (−)-**11** (14.1 mg, 73%). The same procedure was followed to obtain **12** from **10** (yield 75%).

(meso)-Chimonanthine (13). According to the procedure used for making **9** and **10**, compound 4 was treated with $LiAlH_4$ in THF to give **13** (yield 45%). Mp 194–196 °C (lit 198, 201 °C) (EtOH); $R_f = 0.25$ ($CHCl_3$/CH_3OH/NH_3 9:1:0.15) $t_R = 10.20$ min (CH_3OH/H_2O/Et_2NH 80:20:0.1); ^1H NMR ($CDCl_3$) δ 7.06 (m, 2H, C6-H + C5-H), 6.60 (d, $J = 8.0$ Hz, 2H, C4-H + C7-H), 2.86 (m, 1H, C2-H_A), 2.52 (m, 1H, C2-H_B), 2.50 (m, 1H,

C3-H_A), 2.45 (s, 3H, N-CH_3), 2.11 (m, 1H, C3-H_B), ^{1}H NMR (DMSO-d_6) δ 6.88 (m, 2H, C6-H + C5-H), 6.30 (d, J = 8.0 Hz, 2H, C4-H + C7-H), 2.73 (m, 1H, C2-H_A), 2.48 (m, 1H, C3-H_A), 2.30 (s, 3H, N–CH_3), 2.23 (m, 1H, C2-H_B), 1.88 (m, 1H, C3-H_B); ^{13}C NMR ($CDCl_3$) δ 152.5 (s, C7a), 133.7 (s, C4a), 128.9 (d, C6), 125.2 (d, C4), 119.1 (d, C5), 109.5 (d, C7), 84.1 (s, C8a), 64.7 (s, C3a), 53.1 (t, C2), 37.5 (t, C3), 36.3 (q, N–CH_3); ^{13}C NMR (DMSO-d_6) δ 153.5 (s, C7a), 133.6 (s, C4a), 128.4 (d, C6), 124.7 (d, C4), 117.0 (d, C5), 108.0 (d, C7), 83.6 (s, C8a), 63.8 (s, C3a), 52.3 (t, C2), 36.3 (q, N–CH_3), 31.6 (t, C3); EI MS m/z: 346 M^+ (13), 245 (12), 174 (88), 173 (89), 172 (100), 171 (75), 157 (16), 143 (17), 131 (35), 130 $[C_9H_9]^+$ (82), 117 (15), 103 (11). Anal. ($C_{22}H_{26}N_4$) C: calcd 76.27, found 76.30, H calcd 7.56, found 7.50, N calcd 16.17, found 16.15.

Calycosidine. Hodgkinsine ($[\alpha]_D^{20} = -33.6$) (94 mg) was treated with 1 N CH_3COOH (2 mL). After 2 h at rt, the solution was refluxed for 2 h. At the formation of by-products, the reaction was poured in ice, diluted to pH 9 with 15% NH_4OH, then extracted with CH_2Cl_2. After drying, the mixture was taken to dryness under vacuum and purified by flash chromatography (Ø 1.7 cm, EtOAc–Et_2NH 98:2, 60 mL). Combined fractions give 52 mg of calycosidine. $[\alpha]_D^{20} = -22°$ (c 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.12 (dd, J = 7.4, 1.0 Hz, 1H, C4″-H), 7.01 (dd, J = 7.8, 1.4 Hz, 1H, C4-H), 6.90 (d, J = 6.9 Hz, 1H, C6′-H), 6.86 (t, J = 7.6 Hz, 1H, C6-H), 6.82 (t, J = 7.7 Hz, 1H, C6″-H), 6.69 (d, J = 7.0 Hz, 1H, C4′-H), 6.56 (t, J = 7.0 Hz, 1H, C5-H), 6.55 (t, J = 6.9 Hz, 1H, C5″-H), 6.49 (t, J = 6.9 Hz, 1H, C5′-H), 6.45 (d, J = 7.6 Hz, 1H, C7-H), 6.42 (d, J = 7.7 Hz, 1H, C7″-H), 5.84 (s, 1H, C8″a-H), 5.02 (d, J = 3.6 Hz, 2H, NH), 4.96 (bs, 1H, NH), 4.50 (s, 1H, C8′a-H), 4.09 (d, J = 3.9 Hz, 1H, C8a-H), 2.57 (m, 2H, C2″-H_{A+B}), 2.31 (s, 3H, CH_3′), 2.30 (m, 1H, C3″-H_A), 2.28 (s, 3H, CH_3'), 2.27 (m, 1H, C2′-H_A), 2.18 (m, 1H, C3″-H_B), 2.09 (s, 3H, CH_3), 2.09 (m, 1H, C2′-H_B), 2.08 (m, 1H, C3′-H_B), 2.01 (m, 2H, C2-H_{A+B}), 1.75 (m, 1H, C3-H_B), 1.12 (m, 1H, C3′-H_A), 0.86 (m, 1H, C3-H_A).

Opioid binding assay

Binding experiments have been performed in membranes prepared from Chinese hamster ovary (CHO) or human embrionic kidney (HEK) cells stably expressing human cloned μ- and κ-opioid receptors, respectively.

A stable expression of μ receptors (h-MOR) in CHO cell line has been performed in house, using pCDN vectors.[25] Membranes were prepared by lysis in hypotonic phosphate-buffer according to the method described by Scheideler and R. S. Zukin.[26] The highly potent and selective radioligands [^{3}H]-[D-Ala,^{2} Mephe,^{4} Gly-ol^5]enkephalin ([^{3}H]-DAMGO, 50 Ci/mmol, New England Nuclear, Bruxelles, Belgium), and [^{3}H]-U-69593 (63 Ci/mmol, Amersham, Italy) were used to label μ- and κ-opioid receptors, respectively.[25]

Non-specific binding was determined in the presence of 10 μM Naloxone.

Binding experiments were performed in 25 mM monobasic potassium phosphate buffer, containing 3 mM $MgCl_2$, pH 7.4 in polypropylene 96-deep-well plates. Incubation was carried on for 60 min at 25 °C at the final volume of 0.5 mL (κ assay) or 0.7 mL (μ assay). The reaction was terminated by filtration using a Packard Filtermate harvester containing a GF/B Unifilter plate. After filtration, Unifilter plates were dried, each well filled with 50 μL of Packard Microscint 30 and radioactivity counted by a Packard Topcount NXT.

IC_{50} values were determined using the non linear least-squares fitting program GraFit (Erithacus Software Ltd, Horley, UK)[27] and were then trasformed into K_i values by applying the Cheng and Prusoff equation.[28]

Pharmacology

Animals. Male albino mice, CF1, 30–35 g, maintained at 20 ± 2 °C, 12 h light/dark cycle, with food and water ad libitum were used in all experiments.

Drugs. Morphine sulfate, naloxone, capsaicin, MK-801 (dizolcipine) were acquired from Sigma (USA). With the exception of mesocarboxychimonanthine diluted in Tween (80), all other compounds were transformed into salts by adding stechiometric amounts of HCl and diluted in distilled water.

Tail-flick test. Analgesia was assessed with a Tail flick apparatus (Albarsch Electronic Equipment) following the method detailed elsewhere.[9] Forty-five minutes before testing, animals were placed individually in acrylic chambers (20×20×20 cm) which also served for observation. Pre-drug latency (reaction time for removing the tail from heat source) was obtained as the mean of three measures (after each measure animals were returned to the observation chambers for 2 min). At this point, animals presenting two measures of 6 (or more) seconds were discarded. A cut-off time of 10 s was used to prevent tissue damage. Treatments were administered (ip) immediately after the third pre-drug measure. Thirty min later, another set of three measures was taken and the mean considered as post-drug reaction time. Reversibility by naloxone (ip, 10.0 mg/kg) was tested by administering naloxone 10 min before treatments. Statistical analysis used Kruskal–Wallis/Mann–Whitney. Differences in pre- and post-drug latencies were analyzed by the Wilcoxon test. Data are presented as% of Maximum Possible Effect (% MPE), obtained through the following formula:% MPE = T1−T0/T2−T0×100 where, T1 = time post-drug, T0 = time pre-drug and T2 = cut-off time (10 or 20 s, respectively). Statistical analysis used ANOVA/Student–Newmann–Keuls. Differences in pre- and post-drug latencies were analyzed by the Wilcoxon test.

Capsaicin-induced pain. Experiments were performed according to a previously described method:[29,30] 20 min before the experiment, animals were placed individually in acrylic boxes, which also serve as observation cham-

bers. After this adaptation period, 20 μL of capsaicin (1.6 μg/paw) was injected under the dorsal skin of the right hindpaw using a Hamilton microsyringe with a 26-gauge needle. Treatments were administered sc (neck) or ip 30 min before capsaicin and animals were individually observed for 5 min after capsaicin administration. The time spent in licking the injected paw was recorded and taken as the pain index. Data were analyzed through ANOVA/Student–Newmann–Keuls.

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17. It has been reported that both the two enantiomers of eseroline show comparable affinity with the opiate receptors, with the (−)-enantiomer being twice as active as the (+)-enantiomer.[18] In vivo assays have demonstrated that only (−)-eseroline has potency comparable to morphine.[18] In our case, (−)-chimonanthine shows stronger affinity to μ receptors than the (+)-enantiomer, while opposite results are obtained in the tail-flick test. Nevertheless, a direct comparison with eseroline is risky, since the phenol OH in eseroline can play a crucial role, acting as hydrogen donor in the formation of hydrogen bondings with the receptor. Moreover, eseroline closely resembles the morphinane structures. Very recently, a paper has appeared[19] describing the strong affinity and selectivity of 3-deoxy, 14 β-cinnamoylamino derivatives of morphinone (deoxyclocinnamox). Accordingly, in this paper, the authors report that the cinnamoylamino group appears to have a grater influence on opioid binding than the presence of a phenolic hydroxyl group.
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