Synthesis and Biological Effects of c(Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu) (JMV2012), a New Analogue of Neurotensin that Crosses the Blood−Brain Barrier

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The central administration of neurotensin (NT) or of its C-terminal hexapeptide fragment NT(8-13), produces strong analgesic effects in tests evaluating acute pain. The use of NT-derived peptides as pharmaceutical agents to relief severe pain in patients could be of great interest. Unfortunately, peptides do not readily penetrate the blood−brain barrier. We have observed that the cyclic NT(8-13) analogue, c(Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu) (JMV2012, compound **1**), when peripherally administered to mice produced analgesic and hypothermic effects, suggesting the peptide penetrates the blood−brain barrier and functions effectively like a drug. Moreover, dimeric compounds show increased potency compared to their corresponding monomer. We present the synthesis of the cyclic dimer compound **1** (JMV2012). In mice, compound **1** induced a profound hypothermia and a potent analgesia, even when peripherally administered. Compound **1** appears to be an ideal lead compound for the development of bioactive NT analogues as novel analgesics drugs.

Introduction

Neurotensin (NT,[a] pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) is a tridecapeptide first isolated by Carraway and Leeman (1973)[1] from bovine hypothalami. This peptide, which fulfills the major criteria attributed to a neuromodulator/neurotransmitter, exerts a wide range of biological actions when injected in the central nervous system, including modulation of dopamine transmissions in the nigro-striatal and mesocorticolimbic systems,[2] hypothermia,[3] and analgesia.[4] This last effect is of particular interest because this analgesia was shown to be more potent than morphine[5] and principally naloxone-insensitive[4,6,7] on a molar basis. For this reason, NT agonists might be useful as nonopioid analgesics from a therapeutic point of view.

Several receptors are involved in NT biological activity. So far, three NT receptors have been cloned and designated NTS1, NTS2, and NTS3. NTS1[8] and NTS2[9,10] belong to the family of G protein coupled receptors, while NTS3,[11] constituted by a single transmembrane domain, corresponds to the gp95/sortilin.[12] Although it was recently shown that stimulation of the NTS1 receptor was required for the full expression of NT analgesia when a thermal stimulus was used to induce nociception,[13–15] several studies demonstrate that NTS2 receptors are the main receptors involved in NT analgesia, independently of the nature of the nociceptive stimulus (thermal, mechanical, or chemical) used to evaluate nociception.[15–22] The development of NT agonists able to cross the blood−brain barrier and selective for the NTS2 receptors is of significant interest as potential novel analgesic agents. In this way, the C-terminal hexapeptide fragment of NT [NT(8-13)], which corresponds to the shorter fragment of NT that maintains full biological activities[23] is an obvious lead compound for development.

Thus, peptide NT1 or "Eisai peptide",[24–26] a modified analogue of NT(8-13), was the first agonist able to induce biological activity, such as analgesia, hypothermia, and hypolocomotion, after systemic administration in animals.[27,28] More recently, the group of Richelson was interested in peptidase-resistant NT agonists able to cross the blood−brain barrier and presenting high binding affinity for the NTS1 receptor. Among the different peptides they developed, NT69L was the most studied. This peptide exhibits high affinity for the neurotensin receptor (0.82 nM for the rat NTS1; 1.55 nM for the human NTS1; 2.1 nM for the human NTS2 receptor) and induces potent analgesia and hypothermia after intraperitoneal injection in rats.[29]

A different strategy to obtain agonist that cross the blood−brain barrier is the development of cyclic analogues. A few years ago, a structure−activity relationship study revealed that a cyclic analogue (JMV1193) comprising the minimal effective NT fragment was able to cross the blood−brain barrier.[30] This cyclic compound has the sequence c(Lys-Lys-Pro-Tyr-Ile-Leu) and displays an affinity in the 0.3 μM range for mouse brain NT receptors. The i.v. administration of this compound induces the same hypothermic and analgesic effects as icv injection. However, like NT and NT(8-13), compound JMV1193 was inactivated by the endopeptidase neprilysin. It has been shown that dimeric compounds were usually able to show increased potency compared to their corresponding monomer.[31] We then decided to synthesize the dimeric compound of Lys-Lys-Pro-

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[a] Abbreviations: Boc, N^{α}-*tert*-butyloxycarbonyl; DCM, dichloromethane; DE_{35}; dose that brings the mean body temperature to 35 °C over 4 h; DIEA, diisopropylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; ED_{50}, effective dose 50; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; icv., intracerebroventricular; i.v., intravenous; NT, neurotensin; NTS1, neurotensin receptor type 1; NTS2 neurotensin receptor type 2; NTS3, neurotensin receptor type 3; per os, oral route of administration; s.c., subcutaneous; tBu, *tert*-butyl; TEA, triethylamine; TFA, trifluoroacetic acid. The symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem J.* **1984**, *219*, 345–373.

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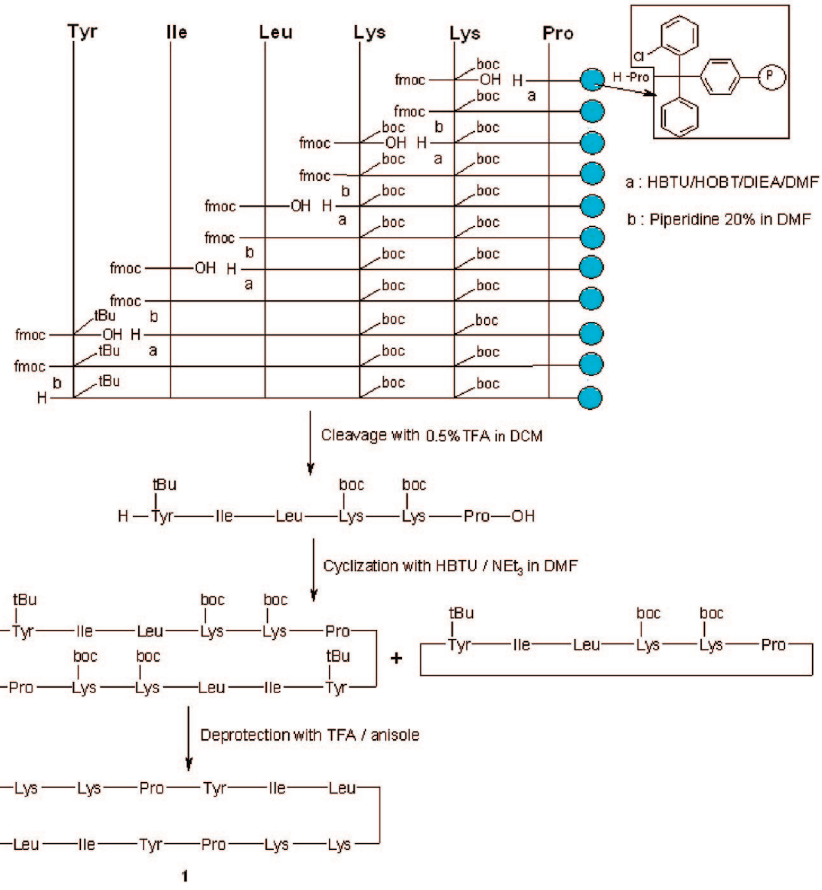
Scheme 1. Synthetic Procedure for Cyclic Peptide Compound **1**



Tyr-Ile-Leu, maintaining the cyclic character of this molecule. We synthesized compound JMV2012 (compound **1**), c(Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu). The cyclization conditions were modulated to favor dimerization starting from the hexapeptide Lys-Lys-Pro-Tyr-Ile-Leu.

Initial studies performed in vitro showed that this peptide was able to bind to both human NTS1 and NTS2 receptors with a similar affinity close to 150 ± 100 nM. Under the same experimental conditions, NT was found to bind to the human NTS1 receptor with an affinity of 0.16 nM and to the human NTS2 receptor with an affinity of 1.10 nM. Furthermore, it has been shown that NT(8-13) binds to the rat and human NTS1 receptor with an affinity of 0.16 and 0.14 nM, respectively.[32] Nevertheless, we decided to evaluate the in vivo activity (hypothermia and analgesia) of compound **1** after various routes of administration in mice.

In mice, compound **1** was able to induce a profound hypothermia and an NTS2-dependent analgesia in the acetic acid-induced writhing and hot plate test after icv injection. Consequently, compound **1** was further characterized with respect to its ability to cross the blood−brain barrier. For this purpose, we studied its ability to trigger hypothermia and analgesia observed with the help of acetic acid-induced writhing test after various routes of administration such as the intravenous (i.v.), subcutaneous (s.c.), and oral routes (per os) in mice.

Results

Chemistry. The linear hexapeptide precursor was synthesized by solid-phase using the 2-chlorotrityl chloride resin preloaded with proline residue (Scheme 1). The 9-fluorenylmethyloxycarbonyl (Fmoc) protection was used as temporary protection of the *N*-terminal amino groups, and N^{α}-*tert*-butyloxycarbonyl (Boc) and *tert*-butyl (tBu) were used for the side-chain protections. Couplings of protected amino acids were carried out with a solution of HBTU/HOBt reagents.

Table 1. Optimization of the Cyclization Reaction

coupling reagent	base	concentration (linear peptide)	reaction time	yields[a] (mono/dimer)
HBTU (1.5 equiv)	TEA (10 equiv)	1 mmol/L	1 h 30 min	87/0%
HBTU (1.5 equiv)	TEA (10 equiv)	100 mmol/L	1 h 30 min	0/85%

[a] Yields after purification on preparative HPLC.

The use of the 2-chlorotrityle resin, as well as of mild conditions (0.5% trifluoroacetic acid (TFA) in DCM) for cleaving the peptide from the resin, allowed the release of the protected peptide from the resin. Cyclization of the resulting protected linear hexapeptide was achieved using *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and triethylamine (TEA) in dimethylformamide. We modulated the cyclization conditions to favor dimerization. The linear precursor concentration was used at a high concentration to obtain the desired cyclic peptide **1** (Table 1).

Finally, the protected cyclic analogue was deprotected with TFA in the presence of anisole as scavenger. The free cyclic analogue **1** was purified by preparative reverse-phase HPLC on a C_{18} column, and its structure was confirmed by ESI mass spectrometry. To confirm the structure of this compound, we prepared the corresponding linear dodecapeptide stepwise on the same solid support, followed by cyclization, deprotection, and purification. The cyclic compound obtained by this way

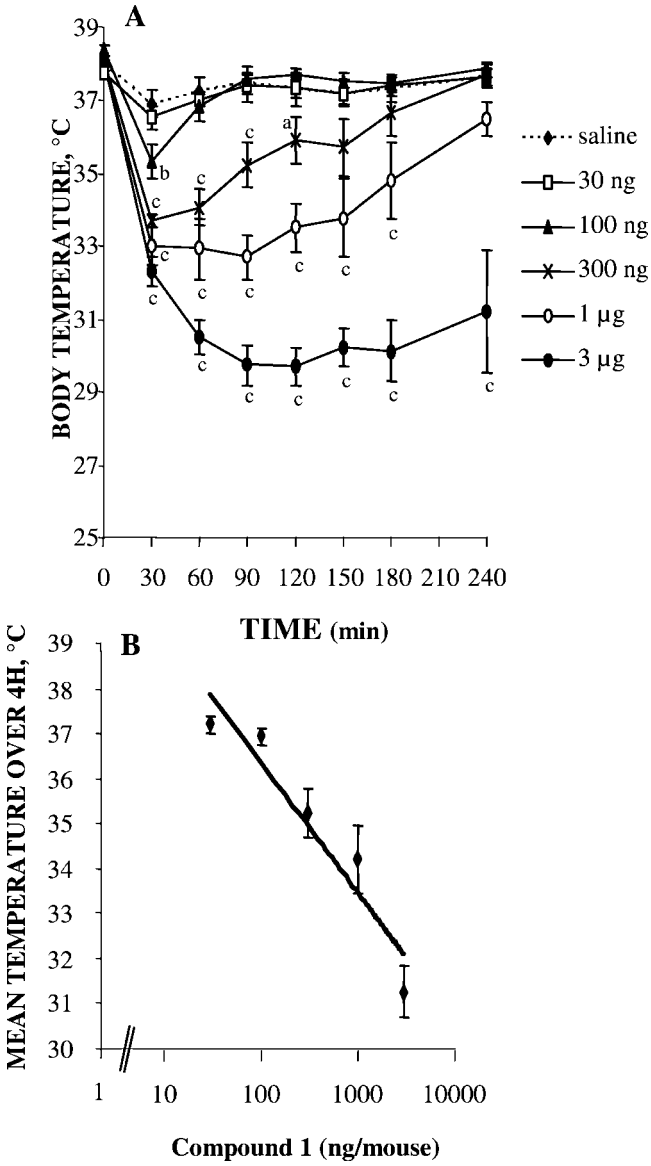


Figure 1. Time course and dose–response relationships for the hypothermic effect of icv injected compound **1** in the mouse. (A) Body temperature as a function of time after icv injections of saline or compound **1** at the indicated doses. (B) Mean body temperature over 4 h after icv injection of compound **1** at various doses. M ± SEM from eight mice per group; (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ vs saline controls.

has the same chemical and physical properties than compound **1** prepared previously.

The biological profile of the cyclic peptide was then evaluated by its ability to induce analgesic and hypothermic activities after various systemic routes of administration.

Biological Results. Effects of Compound 1 on Body Temperature and Nociception in Mice after icv Administration. In a first series of experiments, compound **1** was injected icv for studying its ability to trigger hypothermia and analgesia in mice.

Hypothermia. The two-way repeated measures analysis of variance performed on the results of this experience indicates a statistically significant interaction between the different doses of compound **1** icv administered and the different time of the measure ($F(35238) = 16.699$; $p < 0.001$). Thus, compound **1** icv administered between the dose of 30 ng and 3 μg reduced dose and time-dependent mice body temperature (Figure 1A). This hypothermia was significant from the dose of 100 ng at 30 min postinjection ($p = 0.006$ vs saline). At the highest tested dose (3 μg), a profound hypothermia occurred with a maximal effect at 90 min postinjection ($p < 0.001$ vs saline). This hypothermia was prolonged because at 4 h postinjection the body temperature of mice treated with compound **1** (3 μg) remained significantly lower than that of the saline controls ($p < 0.001$). To integrate the intensity of the hypothermia and its duration into one parameter, the mean body temperature over 4 h was calculated as the area under the time−response curve for each mouse over a period of 240 min (4 h) divided by 240 min (Figure 1B). From this dose–response curve, we determined that the ED_{35} for compound **1** (dose of compound **1** that brings the mean body temperature to 35 °C over 4 h) was 300 ng. Moreover, this hypothermia was not potentiated by the mixed multipeptidase inhibitor JMV390-1 (data not shown), indicating that compound **1** is resistant to the major NN/NT degrading enzymes, such as aminopeptidase N and endopeptidases 24.11, 24.15, and 24.16.[33,34]

Nociception. In the hot plate test, compound **1** increased dose-dependently, between 300 ng and 3 μg, latencies of both paw licking and jumping ($F(3,27) = 9.976$; $p < 0.001$ for the paw licking latency, and $F(3,24) = 7.297$; $p = 0.001$ for the jump latencies; Figure 2A,B). This result indicates that compound **1** triggers analgesia by icv administration in mice. Furthermore, the NTS2 receptor antagonist levocabastine (1350 pmol), which prevents the increase in jump latency produced by neurotensin after icv administration,[18] also prevented the increase in jump latency induced by compound **1** (300 ng; interaction [levocabastine × compound **1**]: $F(1,22) = 5.201$; $p = 0.035$). As it was already demonstrated for neurotensin,[18] these data clearly established that NTS2 receptors are involved in the analgesic effect developed by compound **1** (Figure 2C).

Compound **1** was also tested in the writhing test. This test is more sensitive than the hot plate test. In the writhing test, compound **1**, injected 20 min before testing, reduced dose-dependently the number of writhes produced by acetic acid injection in mice ($F(3,37) = 9.740$; $p < 0.001$) from the dose of 30 ng ($p = 0.001$ vs saline; Figure 3). In this experiment, the ED_{50} was below this dose of 30 ng.

This test was then used for the evaluation of the analgesic activity of compound **1** by the other route of administration.

Effects of Compound 1 on Body Temperature and Nociception in Mice after i.v. Administration. Considering that compound **1** induced hypothermia and analgesia after icv administration, we have evaluated its ability to produce hypothermia and analgesia after i.v. administration.

With regard to hypothermia, the two-way repeated measures analysis of variance performed on the results of this experience indicates a significant interaction between the different doses of i.v. administered compound **1** and the different times of the measure ($F(12,114) = 19.236$; $p < 0.001$), thus, compound **1**, i.v. administered at the dose of 1 and 3 mg/kg, induced a dose and time-dependent hypothermia that lasted at least 60 min, with a maximal effect at 30 min postinjection (Figure 4).

Compound **1** produced a strong analgesic effect by i.v. administration ($F(3,42) = 7.914$; $p < 0.001$) in the writhing test from the dose of 0.3 mg/kg ($p = 0.042$ vs saline). Furthermore, at the dose of 1 mg/kg, compound **1** was close to abolish writhing in mice ($p = 0.001$ vs saline; Figure 5A).

Finally, at the dose of 0.3 mg/kg, the two-way analysis of variance indicates that compound **1**, by i.v. administration, induced a dose- and time-dependent analgesia ($F(3,78) = 4.529$; $p = 0.006$). This analgesia was still evident for 60 min (Figure

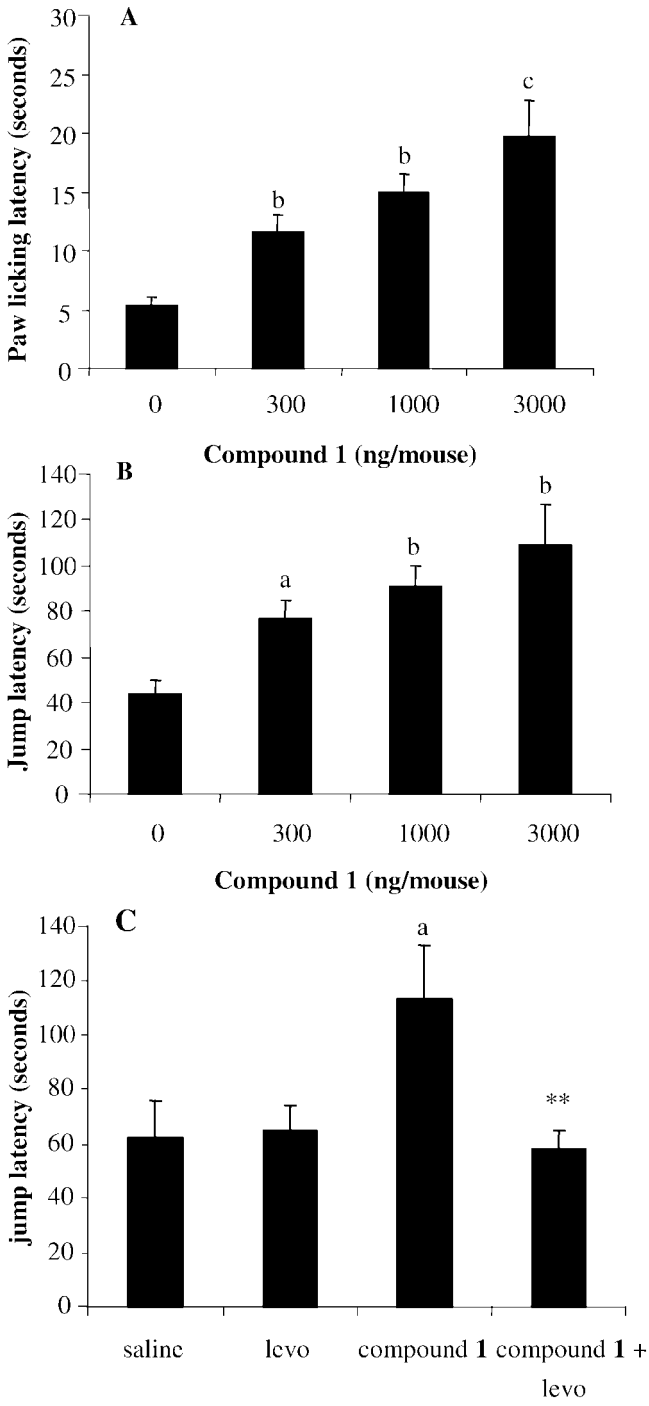


Figure 2. Dose–response relationships for the analgesic effect of icv injected compound **1** in the hot plate test. Mice were icv injected with compound **1** at various indicated doses 20 min before being placed on the hot plate at 55 °C. (A) Dose–response relationship for the paw licking latency. (B) Dose–response relationship for the jump latency. M ± SEM from 6 to 9 mice per group; (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ vs saline controls. (C) Effect of icv injected levocabastine on the increase in jump latency produced by icv injected compound **1**. Mice were icv injected with saline, compound **1** (300 ng), levocabastine (1350 pmol), or with a coadministration of compound **1** (300 ng) and levocabastine (1350 pmol) 20 min before being placed on the hot plate at 55 °C. M ± SEM from 6 to 8 mice per group; (a) $p < 0.05$ vs saline controls; **$p < 0.01$ vs compound **1** alone.

5B). These data indicate that compound **1** was able to cross the blood−brain barrier.

Effects of Compound 1 on Body Temperature and Nociception after s.c. Administration in Mice. The ability of

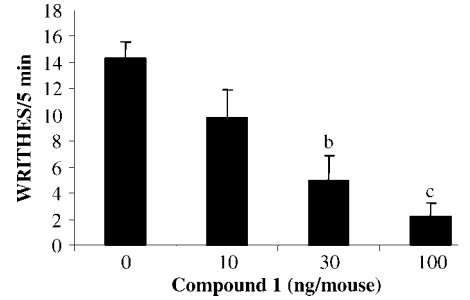


Figure 3. Dose–response relationships for the analgesic effect of icv injected compound **1** in the writhing test. Mice were icv injected with compound **1** at various indicated doses 15 min before receiving acetic acid solution 0.5% (i.p.). Mice were then placed individually in large beakers and the stretches were counted over a 5 min period 5 min after the acetic acid solution injection. M ± SEM from 8 to 14 mice per group; (b) $p < 0.01$ and (c) $p < 0.001$ vs saline controls.

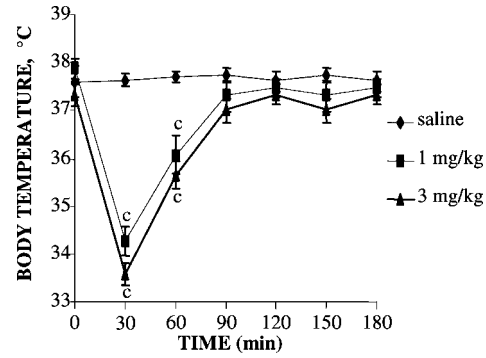


Figure 4. Time course for the hypothermic effect of i.v. injected compound **1** at various doses in the mouse. M ± SEM from 6 to 8 mice per group; (c) $p < 0.001$ vs saline controls.

compound **1** to induce hypothermia and analgesia in mice was also tested after s.c. administration.

Compound **1**, s.c. administered, produced a time- and dose-dependent hypothermia (interaction [dose × time]: $F(24,272) = 7.647$; $p < 0.001$) with a maximal effect reached for each tested dose at 30 min postinjection (Figure 6A). This hypothermic effect lasted between 30 and 45 min for the doses of 1 and 3 mg/kg, whereas at the highest tested dose of 10 mg/kg, this hypothermia was more pronounced and still significant for more than 3 h (Figure 6A).

In the writhing test, compound **1**, between the doses of 1 and 10 mg/kg, reduces dose-dependently the number of writhes ($F(3,22) = 16.465$; $p < 0.001$) at the dose of 3 mg/kg ($p < 0.001$ vs saline; Figure 6B).

Effects of Compound 1 on Nociception after Administration per os in Mice. After oral administration, compound **1** administered at the dose of 30 mg/kg reduced significantly the number of writhes induced by the i.p. injection of an acetic acid solution (Figure 7).

Conclusion

This paper presents the synthesis and the pharmacological evaluation of a new cyclic dimeric NT derivative. This compound, c(Tyr-Ile-Leu-Lys-Lys-pro-Tyr-Ile-Leu-Lys-Lys-Pro) (**1**), exhibited analgesic and hypothermic activities after icv., i.v., s.c., and per os administration. These data suggest that this peptide, owing to its cyclic structure, resists to the NT degrading enzymes and crosses the blood−brain barrier. Moreover, the ability of levocabastine to inhibit the analgesic activity

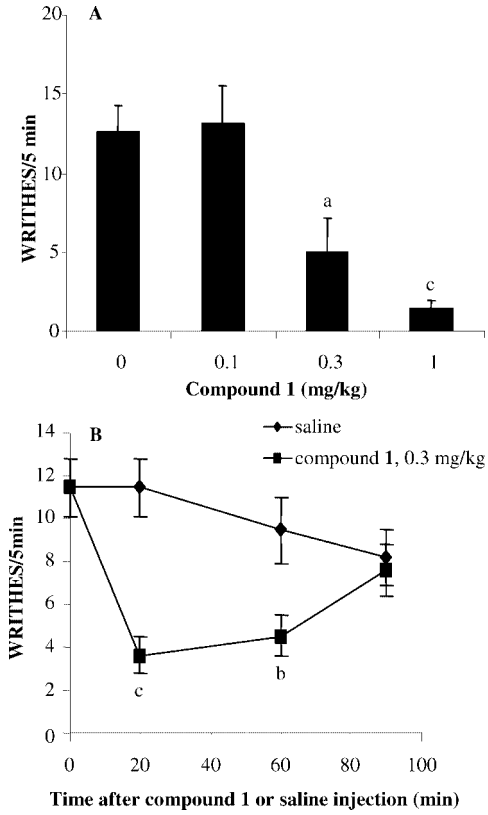


Figure 5. Dose–response and time-course relationships for the analgesic effect of i.v. injected compound **1** in the writhing test. (A) Dose–response relationships for the analgesic effect of i.v. injected compound **1** in the writhing test. Mice were i.v. injected with compound **1** at various indicated doses 15 min before receiving acetic acid solution 0.5% (i.p.). Mice were then placed individually in large beakers and the stretches were counted over a 5 min period 5 min after the acetic acid solution injection. (B) Time-course for the analgesic effect of i.v. injected compound **1** (0.3 mg/kg) in the writhing test. M ± SEM from 8 to 18 mice per group; (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ vs saline controls.

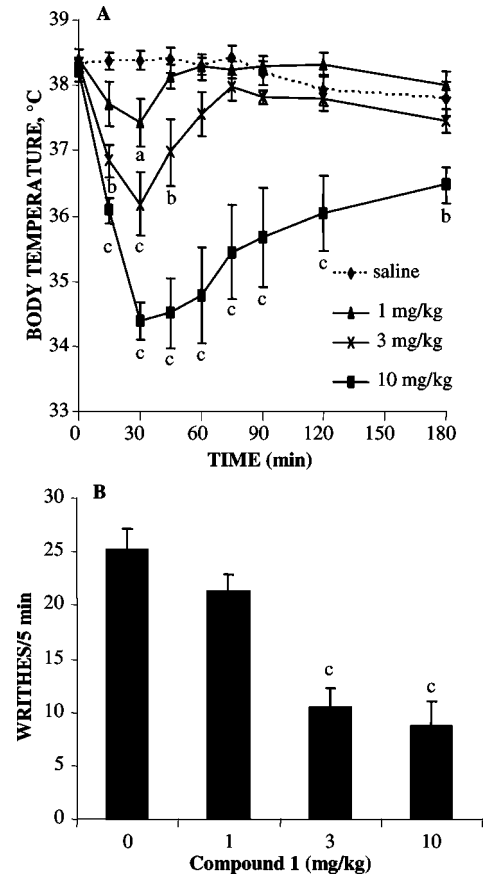


Figure 6. Time-course for the hypothermic effect of s.c. injected compound **1** and dose–response relationship for the analgesic effect of s.c. injected compound **1** in the writhing test. (A) Body temperature as a function of time after s.c. injections of saline or compound **1** at the indicated doses. M ± SEM from 8 to 10 mice per group. (B) Dose–response relationships for the analgesic effect of s.c. injected compound **1** in the writhing test. Mice were s.c. injected with compound **1** at various indicated doses 15 min before receiving acetic acid solution 0.5% (i.p.). Mice were then placed individually in large beakers and the stretches were counted over a 5 min period 5 min after the acetic acid solution injection. M ± SEM from 6 to 8 mice per group; (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ vs saline controls.

of compound **1** in the hot plate test also suggests that compound **1** behaves like an agonist of the NTS2 receptors. However, compound **1** probably triggers other NT receptors because it produces hypothermia in mice, a NT effect independent of the NTS2 receptors stimulation. In conclusion, compound **1** appears at this stage to be an ideal lead compound for the development of bioactive cyclic dimer more selective for the NTS2 receptors as novel analgesic drugs.

Experimental Section

The starting 2-chlorotrityl chloride resin preloaded with proline residue was purchased from Novabiochem; Fmoc-amino acids were obtained from Bachem.

HBTU, HOBt, DIEA, TEA, and piperidine were purchased from Aldrich. Water was obtained from Milli-Q plus system (Millipore), and acetonitrile and trifluoroacetic acid (TFA) were obtained from Merck. Mass spectra were obtained by electron spray ionization (ESI-MS) on a Micromass Platform II quadrupole mass spectrometer (Micromass) fitted with an electrospray source coupled with an HPLC Waters. HPLC runs were performed on Waters equipment, using columns packed with Nucleosil 100 Å, 5 μm, C18 particles, unless otherwise stated. The analytical column (250 × 4.6 mm) operated at 1 mL/min, with a photodiode array detector 996, wavelength 214 nm. Solvent A consisted of 0.1% TFA in H_2O, and solvent B consisted of 0.1% TFA in acetonitrile. After lyophilisation, the product was stored at −20 °C.

Preparative HPLC was performed on a Waters Delta-Prep 4000 chromatography equipped with a Waters 486 UV detector with detection at 214 nm, using a Delta-Pak C18 column (40 × 100 mm, 15 μm, 100 Å) at a flow rate of 50 mL/min of a binary eluent system of A/B (A: H_2O, TFA 0.1%; B: CH_3CN, TFA 0.1%).

Synthesis of Protected Linear Peptide. The linear hexapeptide was synthezised by the solid-phase method on a Perkin-Elmer ABI433A automatic synthesizer on a 0.25 mmol scale with Pro-preloaded 2-chlorotrityl chloride resin (loading 0.56 mmol/g, 446 mg). The coupling reagent was a 0.45 M solution of HBTU/HOBt (2 mL). Four times excess of amino acid was used in coupling (1 mmol). Deprotection cycles were carried out in piperidine/DMF (20/80, 5 mL) and monitored by conductimetry. Elongation was performed by single 30 min couplings in DMF (15 mL) with DIEA (2 M, 1 mL) as base. Washings were carried out with DMF (3 × 15 mL) and DCM (1 × 15 mL). Final cleavage was carried out with 0.5% TFA in DCM for 15 min (10 mL). The resin was washed extensively with DCM, then dried in vacuo, dissolved in an acetonitrile−water mixture (5/5, 10 mL), and freeze-dried. The side chain protected intermediate peptide was obtained in 89% yield (247 mg of TFA salt). t_R = 18.4 min (20–50% B, 30 min, C18). ES-MS $[M + H]^+$ 1017.4.

Cyclization of Protected Peptide. The resulting protected linear hexapeptide (220 mg, 0.22 mmol)) was allowed to cyclize for 90 min at high concentration in DMF (2.2 mL), with HBTU (125 mg,

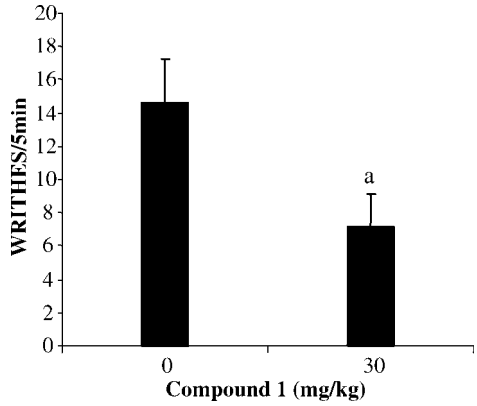


Figure 7. Analgesic effect of compound **1** administered at the dose of 30 mg/kg per os in the writhing test. Mice were treated with compound **1** 15 min before receiving acetic acid solution 0.5% (i.p.). Mice were then placed individually in large beakers and the stretches were counted over a 5 min period 5 min after the acetic acid solution injection. M ± SEM from 10 mice per group; (a) $p < 0.05$ vs saline controls.

0.33 mmol) as coupling reagent and TEA (300 μl, 2.20 mmol) as base (Table 1). After concentration under reduced pressure, the residue was dissolved in ethyl acetate (6 mL) and then successively washed (3 × 1.5 mL) with 1 M aqueous solution of KHO_4S, saturated NaCl aqueous solution, saturated $NaHCO_3$ aqueous solution, and water. The ethyl acetate phase was dried over $MgSO_4$, filtered off, then concentrated under reduced pressure to afford a colorless oil, which precipitated from ether/hexane (1/5) to afford a white solid (92% yield, 195 mg).

Preparation of Free Cyclic Peptide. The dried residue from the cyclization reaction (150 mg, 0.15 mmol) was dissolved in a mixture TFA/anisole (8/2, 2 mL) and stirred for 30 min. The reaction mixture was evaporated under reduced pressure and then coevaporated several times with hexane to remove residual TFA. Addition of a solution ether/hexane (1/5) allowed the peptide to precipitate as a TFA salt. The afforded solid was dried under vacuum and then purified on preparative HPLC.

These conditions afforded the expected cyclic dimer (**1**) in 85% yield, after purification. $t_R = 18.1$ min (20–50% B, 30 min, C18). ES-MS $[M + H]^+$ 1485.9.

Animals. Male Swiss albino mice (CD1, Charles River, L'Arbresle, France) weighing 20–22 g were obtained at least one week before the beginning of the experiments. The animals were housed in a room maintained at a constant temperature (21 ± 1 °C), with a regular light cycle (light on between 7 a.m. and 7 p.m.). Food and water were freely available, except at the time of testing. This study was performed in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health No. 85-23, revised 1985), and the principles were presented in the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs and Solutions. c(Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro) **1** was dissolved in saline. Levocabastine (a generous gift from Janssen-Cilag laboratory, Beerse, Belgium) was dissolved in dimethylsulfoxide (DMSO) and cremophor EL and then diluted with saline to a final concentration of 5% DMSO and 5% cremophor.

Compound **1** was injected in mice intravenously (i.v.), subcutaneously (s.c.), and by a gastric canula (per os) in a volume of 10 mL/kg. The intracerebroventricular injections (icv.) were performed according to the method of Haley and Mc Cormick (1957)[35] in a volume of 10 μL/mouse. These protocols were approved by the Regional Ethical Committee for Animal Research (Normandy) with the following numbers: N/10-04-04-12 (intracerebroventricular injection in mouse) and N/04-05-04-23 (per os injection).

Binding Experiments. Binding potencies of compound **1** were determined in competition experiments, as described previously[9,19] performed with homogenates freshly prepared from COS 7 cells (0.01 mg protein per tube) expressing either the human NTS1 or NTS2. Competition experiments were carried out in 250 μL of 50 mM Tris/HCl buffer, pH 7.5, containing 0.2% BSA and 1 mM o-phenanthroline. Homogenates, 10 μg of protein from cells, were incubated for 30 min at 25 °C with increasing concentrations of compound **1** and ^{125}I-Tyr3-NT (100 Ci/mmol, 0.05 nM). Total, specific, and nonspecific binding were determined by using the filtration method, as described previously. It was checked that membranes from untransfected COS cells were devoid of specific ^{125}I-Tyr3-NT binding.

Analgesic Tests. Hot Plate Test. This test was derived from that of Eddy and Leimbach (1953).[36] A plastic cylinder (height = 20 cm, diameter = 14 cm) was used to confine the mouse to the heated surface of the plate. The plate was heated to a temperature of 55 ± 0.5 °C, using a thermoregulated water circulating pump. For each animal, the paw licking and the jump latencies were determined. To avoid injury, mice that did not respond within 240 s were removed from the hot plate. Each mouse was tested only once and sacrificed immediately thereafter. This protocol was approved by the Regional Ethical Committee for Animal Research (Normandy) with the following number: N/12-04-04-14 (hot plate test).

Writhing Test. This test was derived from that of Sigmund et al. (1957).[37] Mice received intraperitoneally (i.p.) a 0.5% acetic acid solution in a volume of 10 mL/kg. They were then placed individually in large beakers. The stretches were counted over a 5 min period from the fifth minute after the acetic acid solution injection. A stretch was characterized by an elongation of the body, the development of tension in the abdominal muscles, and the extension of the forelimbs. This protocol was approved by the Regional Ethical Committee for Animal Research (Normandy) with the following number: N/01-05-04-20 (writhing test).

Body Temperature. Colonic temperature was measured with a thermistor probe (Thermalert TH-5, Physitemp, NJ, U.S.A.) introduced to a depth of 2 cm into the rectum. This protocol was approved by the Regional Ethical Committee for Animal Research (Normandy) with the following number: N/16-04-04-18 (measurement of colonic temperature in rat and mouse).

Statistical Analysis. The data were expressed as means ± SEM. Results were analyzed using one-way analysis of variance or two-way analysis of variance, when appropriate, followed by Student Newman Keul's comparisons. Comparisons between two groups were analyzed by Student's *t*-test. For the study of the hypothermic effect of compound **1**, statistical analysis was performed using a two-way repeated measures followed by a Student Newman Keul's test. A *p*-value of <0.05 was considered significant, and statistical analyses were performed with SigmaStat (SPSS Inc., Chicago, IL, U.S.A.).

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Supporting Information Available: Mass spectrometry and HPLC tracing for compound **1**. This material is available free of charge via the Internet at the http://pubs.acs.org.

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