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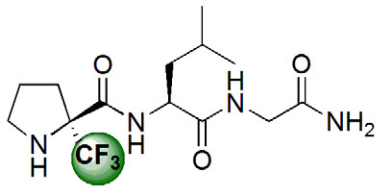
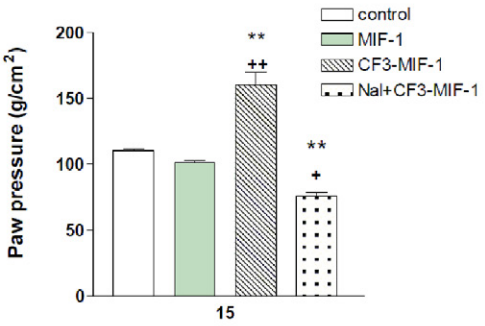


CF_3-(MIF-1)



Synthesis of a MIF-1 analogue containing Enantiopure (*S*)-α-Trifluoromethyl Proline and Biological Evaluation on Nociception

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Abstract[1]

The synthesis and the effect of a novel MIF-1 analogue on nociception during acute pain in rat model are reported. The synthesis of this enantiopure trifluoromethyl group containing tripeptide was performed through a peptide coupling reaction beetween the HCl. Leu-Gly-NH_2 and the (*S*)-α-Tfm-proline. The analgesic effect of the CF_3-MIF-1 **2** has been evaluated *in vivo* on rat model by paw pressure (PP) and hot plate (HP) tests and compared to the native peptide MIF-1. Highest analgesic effect was observed with CF_3-(MIF-1) **2** only in PP test. In order to study the mechanisms of nociception induced by the studied peptides, the involvement of the opioid and the nitric oxideergic systems was investigated. The results are in favor of a participation of both system since pretreatment, 20 min before injection of the CF_3-(MIF-1) **2**, with the

Abbreviations: S.E.M., Standard error of the mean; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, Hydroxybenzotriazole; BOP-Cl, Bis(2-oxo-3-oxazolidinyl)phosphonic chloride; TEA, Triethylamine; Boc, *t*-Butoxycarbonyle; DCM, Dichloromethane.

non-competitive antagonist of opiate receptors naloxone, the nitric oxide synthase (NOS) inhibitor L-N^G-nitroarginine ester (L-NAME) or the nitric oxide (NO) donor L-arginine (L-Arg) significantly decreased the pain perception in PP and HP tests.

Keywords

Fluorinated amino acids

Fluorinated peptides

MIF-1

Nociception

Acute pain

Analgesia

Highlights

- A MIF-1 analogue containing enantiopure (*S*)-α-trifluoromethyl proline was synthesized

- The nociception during acute pain of the CF_3-MIF-1 was evaluated in rat model

- Significant analgesic effect in paw pressure test compared to the native peptide

1. Introduction

The tripeptide PLG **1** (Pro-Leu-Gly-NH_2), known as melanocyte–stimulating hormone release inhibition factor (MIF-1), is an endogenous brain peptide that belongs to the Tyr-MIF-1 family of peptides isolated from bovine hypothalamus and human parietal cortex. It has been shown to be involved in a wide spectrum of physiological processes, including the development of stress [1-10]. MIF-1 demonstrates its ability to modulate dopaminergic neurotransmission *in vitro* and *in vivo*, by probably an allosteric effect increasing the binding affinity of agonists of dopamine receptor [10-13]. Therefore, it is considered as a promising starting point for the development of new pharmaceutical agents for various brain disorders including Parkinson's disease [11,14-18], tardive dyskinesia [19,20] and depression [21,22]. MIF-1 represents also a class of naturally occurring opiate antagonists. It does not bind to opiate receptors and it is the first peptide known to exert anti-opioid effects [3]. In particular, MIF-1 binds to its own non-opiate sites and is able to block respectively the analgesic effect of morphine in paw-pressure and tail-flick tests [23] and of

enkephalin in a radiant heat tail-flick assay [24]. This peptide antagonizes also the effects of morphine in a double-blind study in humans [25]. Because of its structural simplicity, and its relevance to activate CNS pathways related to dopamine-, serotonin-, opioid- and noradrenergic systems, several conformationally constrained analogues of MIF-1 have been reported in literature [26-32].

Our research group is interested in the development of convenient and scalable experimental procedures for the stereoselective synthesis of various trifluoromethyl group containing α-amino acids (α-Tfm-AAs) with linear [33,34], cyclic [35-37] or functionalized side chain [38]. It is well documented that proline [39-43] and its derivatives such as pyroglutamic acid [44,45] or pseudoprolines [46,47] play a unique and important role in the conformation of peptides and proteins resulting in the modulation of their biological activities. Therefore, enantiopure (*S*)- and (*R*)-α-Tfm-prolines ((*S*)-**3** and (*R*)-**3**) are very attractive target molecules for the design of biologically active compounds. However, the use of α-Tfm-AAs remains very limited since their incorporation into a peptide chain is still a challenge due to the low nucleophilicity of the α-Tfm-AAs amino group. We previously reported an efficient protocol allowing the coupling at the *C*-terminal position of the non-protected α-Tfm-proline or α-Tfm-alanine leading to original α-Tfm-AAs containing dipeptides [48]. The challenging *N*-terminal coupling of an α-Tfm-alanine dipeptide using a Fmoc-amino acyl chloride was also described.

As the chemical barriers for the coupling reaction were unlocked, we decided to prepare trifluoromethylated analogues of native peptides in order to investigate the effect of the α-Tfm-AAs on the biological activity. Indeed, the incorporation of α-Tfm-AAs into peptides increases their chemical and thermal stability and their resistance to degradation by proteases and enhances hydrophobicity resulting in better affinities for lipid membranes [49-54]. Moreover, their incorporation into peptides may induce stabilization of particular conformations and better auto-assembly [55-60]. Additionally, the trifluoromethyl group is a label for ^{19}F NMR studies allowing an access to pharmacokinetic and dynamic data [61-63].

The objectives of the present study were the synthesis of the enantiopure (*S*)-α-Tfm-proline containing MIF-1 analogue **2** (Figure 1) and the investigation of its effect on nociception during acute pain in rat model. An enhancement of the lipophilicity is expected since the amino group of the Tfm-proline at the *N*-terminal position should not be protonated at physiological pH.

[Fig 1]

2. Results and discussion

2.1. Chemistry

Due to the stereoelectronic effects impart by the CF_3 group, chemical and physical properties of the neighboring functions are modified. The nucleophilicity and the basicity of the vicinal amino group is

decreased while the reactivity of the acid function is increased. These properties allow efficient coupling reactions at the *C*-terminal position under classical protocols without any protection of the amino group of the α-Tfm-proline residue [48]. It should be reminded that such protection is required for native amino acids.

The retrosynthetic pathway to CF_3-(MIF-1) **2** involved a coupling reaction, at the final stage, between the enantiopure α-Tfm-proline residue (*S*)-**3** and the HCl.Leu-Gly-OEt **4a** or the preformed HCl.Leu-Gly-NH_2 dipeptide **4b** (Figure 2).

[Fig 2]

Both enantiomers of the α-Tfm-proline **3** ((*S*)-**3** and (*R*)-**3**) were prepared according to our convenient gram-scale procedure [37,48]. The CF_3-oxazolidine **5**, prepared by condensation of (*R*)-*N*-Boc-phenylglycinol with the commercially available ethyl trifluoropyruvate [36], was treated with allyltrimethylsilane under Lewis acid activation to give, after acidic treatment, a 75:25 diastereomeric mixture of allylmorpholinones (*R*,*S*)-**6** and (*R*,*R*)-**6** (Scheme 1) [64]. In the presence of iodine, **6** afforded the pyrrolidinic bicyclic compound **7**. The iodocompound **7** was hydrogenolyzed to give the corresponding bicyclic morpholinone **8**. After separation of the two diastereomers **8** by column chromatography and deprotection with Pearlman's catalyst, the (*S*)- and (*R*)-α-Tfm-proline [(*S*)-**3** and (*R*)-**3**] were obtained in enantiopure form.

[Scheme 1]

The initial strategy adopted for the synthesis of the expected CF_3-(MIF-1) **2** tripeptide involved an amidification step of the tripeptide **12** bearing an ester function at the *C*-terminal position.

The coupling reaction between Boc-Leu-OH **9** and HCl.Gly-OEt **10a** using standard coupling reagents such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBt) in DMF gave the corresponding dipeptide **11a** (Scheme 2). The deprotection of the *N*-terminal amino group under acidic conditions led to the corresponding hydrochloride salt of the Leu-Gly-OEt dipeptide **4a** in good yield (84%). We previously reported that the coupling reaction at the *C*-terminal position of a non-protected α-Tfm-proline **3** with an amino ester or a peptide chain needed a reverse addition protocol to avoid the formation of the diketopiperazine byproduct [48]. Accordingly, by addition of one equivalent of α-Tfm-proline (*S*)-**3** to two equivalents of the non-fluorinated dipeptide **4a** in the presence of HOBt and EDCI, the tripeptide **12** was obtained in a reasonable good yield (62%). Surprisingly, an amidification step using dissolved NH_3 in EtOH already reported for the non-fluorinated Pro-Leu-Gly-OEt tripeptide gave a messy mixture and an incomplete conversion when applied to the fluorinated tripeptide analogue **12**.

[Scheme 2]

In order to achieve the synthesis of the CF_3-(MIF-1) **2**, we attempted the direct condensation of Boc-Leu-OH **9** with HCl.Gly-NH_2 **10b** using classical (HOBt/EDCI) coupling reagents (Scheme 2). The corresponding Boc-Leu-Gly-NH_2 dipeptide **11b** was obtained in moderate yield (62%) due to a tedious chromatographic purification to remove the HOBt byproduct and DMF traces. Another protocol using BOPCl activation in DCM was found to be more efficient giving **11b** in higher yields (81%). The Boc amino group deprotection by acidic treatment (HCl_g in 1,4-dioxane) followed by a coupling reaction of the α-Tfm-proline (*S*)-**3** under reverse addition conditions gave the tripeptide CF_3-(MIF-1) **2** in a good yield (89%).

2.2. Biology

As pain is a frequently observed symptom of various diseases and as the development of analgesic drugs is one of the greatest achievements in medicine, we focused our investigation on the CF_3-(MIF-1) **2** effects on acute pain.

Involvement of opioidergic system in analgesic effects of CF_3-MIF-1

In order to evaluate the nociceptive activity of the CF_3-(MIF-1) **2** and to compare it with the native (MIF-1) **1** peptide, we first administrated the peptide alone by intraperitoneal (i.p.) injection on male Wistar rats and measured the analgesic effect in PP and HP tests. The first measurements were recorded 15 min after the injection of the peptides. Alone, MIF-1 (**1**, 1 mg/kg, i.p.) had no analgesic effect in PP test and significantly increased HP latency ($P < 0.05$) only after 15 min from the beginning of the investigated period. (Fig. 3 and 4). The results are in accordance with literature data reporting that MIF-1 had a similar action to naloxone (Nal), a known opiate antagonist. (*S*)-α-Tfm-proline containing MIF-1 analogue (**2**, 1 mg/kg, i.p.), developed a significant analgesic effect after 15 and 30 min in PP test ($P < 0.01$), and had no effect on HP latency (Fig. 3 and 4). These results suggest that the incorporation of the CF_3 group is responsible for the analgesic effect of MIF-1 in PP test only.

We may speculate that the opposite effects are related to different receptors (mechano- and thermoreceptors) and different pain pathways.

[Fig.3].

[Fig.4]

In order to investigate the involvement of the opioid system in the CF_3-MIF-1 peptide induced analgesia, the non-competitive antagonist of opiate receptors naloxone (Nal, 1 mg/kg, i.p.) was tested. Pretreatment with Nal 20 min before injection of the CF_3-(MIF-1) **2** peptide significantly decreased the pain threshold ($P < 0.01$) of about 50% and 40% after 15 min and 30 min respectively (Fig. 3 and 4). These results suggest the involvement of opioid receptors in the observed CF_3-MIF-1 effects.

Involvement of nitric oxideergic system in analgesic effects of CF_3-MIF-1

Nitric oxide (NO) is a unique neurotransmitter, which participates in many physiological and pathological processes including pain transmission in the organism. It is biosynthesized from L-arginine, by the nitric oxide synthase enzyme (NOS) [65,66]. The morphological studies present evidence for the existence of a signaling pathway between the opioidergic and the nitric oxideergic systems in the periaqueductal gray (PAG) of the rat brain, the critical site involved for coping with different types of stress and pain [67]. It is also known that NO is an important and many-sided regulator of a number of physiological functions in animals. It also acts as a neurotransmitter itself and/or as a neuromodulator and influences plastic properties of the neurons such as the phenomenon of long-lasting potentiation [68].

In a second experimental series, NOS inhibitor L-N^G-nitroarginine ester (L-NAME, 10 mg/kg, i.p.) or NO donor L-arginine (L-Arg, 1 mg/kg, i.p.) were injected 20 min before the studied analogues. Each compound used alone showed analgesic effects in both tests. The effect of L-NAME was more pronounced and long lasting compared to that of L-Arg (Fig. 5-8).

The results of our investigations showed that both non fluorinated and fluorinated peptides significantly decreased the analgesic effects of L-NAME and L-Arg in PP and HP tests during the whole investigated period (Fig.5-8) and demonstrate that the nitric oxideergic system is also involved in the mechanisms of nociception of the investigated peptides.

[Fig.5]

[Fig.6]

[Fig.7]

[Fig.8]

3. Conclusions

In summary, we reported a convenient synthesis of a novel trifluoromethylated analogue of MIF-1 from the commercially available ethyl trifluoropyruvate. Our choice was to incorporate a 2-trifluoromethylproline in place of the native proline in order to investigate the biological activity improvement imparted by the presence of the CF_3 group. The PLG analogue (CF_3-MIF-1) was synthesized in a good yield. Its involvement on opiate and in nitric oxideergic systems was shown by *in vivo* acute pain experiments on rat model. The results from this study demonstrated that the CF_3-MIF-1 has significant analgesic effect in PP test ($P < 0.01$) compared to the native MIF-1, but has no effect on HP latency. This study illustrates that the introduction of an α-Tfm-amino acid into a peptide can modulate its biological profile compared to the native peptide. Moreover it could be anticipated that the incorporation of a trifluoromethylated amino acid at the *N*-terminal position of a peptide will increase its lipophilicity.

4. Experimental Section

4.1. Chemistry

Unless otherwise mentioned, all the reagents were purchased from commercial source. All glassware was dried in an oven at 150 °C prior to use. Ether and THF were distilled under nitrogen from sodium/benzophenone prior to use. CH_2Cl_2 was distilled under nitrogen from CaH_2 prior to use. ^{1}H NMR (400.00 MHz), ^{13}C NMR (100.50 MHz) and ^{19}F NMR (376.20 MHz) were measured on a JEOL ECX400 spectrometer. Chemical shifts of ^{1}H NMR are expressed in parts per million downfield from tetramethylsilane ($\delta = 0$) in $CDCl_3$. Chemical shifts of ^{13}C NMR are expressed in parts per million downfield from $CDCl_3$ as internal standard ($\delta = 77.0$). Chemical shifts of ^{19}F NMR are expressed in parts per million downfield from C_6F_6 as an internal standard ($\delta = -164.9$). Coupling constants are reported in Hertz. Column chromatography was performed on SDS 60Å, (40–63 μm) silica gel, employing a mixture of the specified solvent as eluent. Thin-layer chromatography (TLC) was performed on Merck silica gel (Merck 60 PF254) plates. Silica TLC plates were visualized under UV light, by a 10% solution of phosphomolybdic acid in ethanol followed by heating. Mass spectra (MS) were obtained on a GC/MS apparatus HP 5973 MSD with an HP 6890 Series GC. Ionization was obtained by electronic impact (EI 70 eV). Infrared spectra (IR) were obtained by Fourier-transformation on BRÜCKER TENSOR 27, wavenumbers are given in cm^{-1}. Liquid Chromatography-Mass Spectrometry (LC-MS) analyses were done on a Shimadzu LCSM-2010 A on a HPLC, column Alltima HP C8 3μ (Alltech), reversed phase (L= 53 mm; ID = 7 mm), PDA diodes detector SPD-M10 A (D_2, lamp from 190 to 400 nm) and light scattering detector ELSD-LT. The LC were run using a 1 ml/min flow using a gradient between acetonitrile and water containing formic acid (0,1%): 0 to 1 min : 30% CH_3CN, 1 to 5 min : from 30% to 100% CH_3CN, 5 to 12 min : 100% CH_3CN, 12 to 14,99 min : from

100% to 30% CH_3CN, 14,99 to 20 min : 30% CH_3CN. MS spectrum was recorded between *m/z* = 100 to 500 at the exit of the column using an ESI ionization and positive ion mode (detector = 1.5 kV, quadripole = 5 V). Elemental analyses were performed on Perkin–Elmer CHN 2400. Optical rotations were determined using a JASCO P1010 polarimeter. HRMS analyses were performed on a Jeol JMS-GC Mate II. Melting points were obtained on a Büchi apparatus and are uncorrected.

*4.1.1. General procedure for the synthesis of dipeptides (**11a-11b**).*

Boc-Leu-OH **9** and HOBt were dissolved in DMF. The reaction mixture was cooled to 0 °C, EDCI was added and the solution was stirred for 1 h. HCl.Gly-OEt **10a** or HCl.Gly-NH_2 **10b** and TEA were added. The reaction mixture was stirred at r.t. for 16 h, then cooled to 0°C and the urea precipitate was removed by filtration. The solvent was evaporated under vacuum and the crude residue was dissolved in ethyl acetate. The organic phase was extracted with 5% $NaHCO_3$ solution, brine, dried over $MgSO_4$ and concentrated under reduced pressure to afford the dipeptides **11a** and **11b** in 65-84%.

*4.1.1.1. Boc-Leu-Gly-OEt (**11a**)*

The dipeptide **11a** was prepared following the general procedure starting from Boc-Leu-OH **9** (1.0 g, 4.3 mmol, 1.0 equiv), HOBt (0.6 g, 4.7 mmol, 1.1 equiv) in DMF (4 mL), then EDCI (0.8 g, 4.3 mmol, 1.0 equiv), HCl.Gly-OEt **10a** (0.6 g, 4.3 mmol, 1.0 equiv) and TEA (1.2 mL, 8.6 mmol, 2.0 equiv). Purification by recrystallization in ethyl acetate/petroleum ether (40:60) gave 1.14 g (84%) of pure dipeptide **11a** as a white solid. Spectroscopic data of **11a** are in accordance with the literature reported data [69]. White solid; mp 72-74 °C; IR (neat) 3290, 2954, 2868, 1754, 1661, 1246, 1166 cm^{-1}; $[\alpha]^{26}_D$ –16 (*c* 0.5, $CHCl_3$); ^1H RMN (400 MHz, $CDCl_3$) δ 0.94 (d, *J* = 6.3 Hz, 3 H), 0.99 (d, *J* = 6.3 Hz, 3 H), 1.29 (t, *J* = 7.1 Hz, 3 H), 1.45 (s, 9 H), 1.68-1.71 (m, 3 H), 4.04 (d, *J* =5.0 Hz, 2 H), 4.16 (bs, 1 H), 4.18 (q, *J* = 7.1 Hz, 2 H), 4.85 (m, 1 H), 6.64 (s, 1 H); ^{13}C RMN (100 MHz, $CDCl_3$) δ 14.0, 21.8, 22.8, 24.5, 28.2, 41.1, 41.3, 52.8, 61.2, 79.8, 155.7, 169.6, 173.1; LCMS (ESI+) *rt* = 4.98 min, *m/z* = 339 $[M + Na]^+$ (100), 261; ELSD pur. 99%, UV pur. 100%.

*4.1.1.2. Boc-Leu-Gly-NH_2 (**11b**)*

HOBt activation protocol: The product was prepared following the corresponding general procedure starting from Boc-Leu-OH **9** (2.0 g, 8.6 mmol, 1.0 equiv), HOBt (1.7 g, 12.0 mmol, 1.5 equiv) in DMF (8 mL), then EDCI (2.5 g, 13.0 mmol, 1.5 equiv), HCl.Gly-NH_2 **10b** (2.0 g, 18.1 mmol, 2.1 equiv) and TEA (4.2 mL, 30 mmol, 3.5 equiv) to give 1.46 g (62%) of dipeptide **11b** as a white solid. Dipeptide **11b** was engaged in the next step without further purification. Spectroscopic data of **11b** are in accordance with the literature reported data [69].

BOP-Cl activation protocol: Boc-Leu-OH **9** (9.5 g, 41.1 mmol), HCl.GlyNH_2 **10b** (5.0 g, 45.2 mmol, 1.1 equiv) and TEA (17.6 mL, 127 mmol, 3.1 equiv) were mixed in DCM (1.7 L) and the resulting solution was

stirred for 10 min. BOP-Cl (13 g, 51 mmol, 1.25 equiv) was then added and the reaction mixture was stirred at r.t. overnight, quenched with HCl aqueous solution (1 M, 500 mL). The layers were separated and the organic phase was washed with a saturated $NaHCO_3$ aqueous solution (2 x 500 mL), brine (500 mL), dried over $MgSO_4$ and concentrated under reduced pressure to afford 10.1 g (81%) of the crude dipeptide **11b** which was engaged in the next step without further purification. White solid; mp 70-72 °C; IR (neat) 3282, 2956, 1657, 1246, 1162 cm^{-1}; $[\alpha]^{27}_D$ –15 (*c* 0.5, $CHCl_3$); ^{1}H RMN (400 MHz, $CDCl_3$, 323 K) δ 0.93 (d, *J* = 6.7 Hz, 3 H), 0.96 (d, *J* = 6.7 Hz, 3 H), 1.41 (s, 9 H), 1.50 (m, 1 H), 1.56-1.76 (m, 2 H), 3.80 (dd, *J* = 17.0, 5.0, 1 H), 4.00 (dd, *J* = 17.0, 5.0 Hz), 5.31 (bs, 1 H), 6.09 (bs, 1 H), 6.75 (bs, 1 H), 7.26-7.36 (m, 2 H); ^{13}C RMN (100 MHz, $CDCl_3$) δ 21.8, 22.8, 24.7, 28.3, 40.8, 42.7, 53.6, 80.3, 156.3, 172.4, 173.7; LCMS (ESI+) *rt* = 3.74 min, *m*/*z* = 310 $[M + Na]^+$ (100), 232, 188; ELSD pur. 99%, UV pur. 100%.

4.1.2. General procedure for the Boc deprotection.

Boc-Leu-Gly-OEt **11a** or Boc-Leu-Gly-NH_2 **11b** was dissolved in 1,4-dioxane and was treated at r.t. for 40 min with dry HCl gas. The reaction mixture was stirred at r.t. until disappearance of the starting material (monitoring by TLC), then the solvent was removed under vacuum to afford quantitatively the crude dipeptides **4a** and **4b** which were engaged in the next step without further purification.

*4.1.2.1. HCl.Leu-Gly-OEt (**4a**)*

The product was prepared following the corresponding general procedure starting from Boc-Leu-Gly-OEt **11a** (315 mg, 1 mmol) in 1,4-dioxane (5 mL) to give 260 mg (quantitative) of dipeptide **4a**. Pale yellow oil; IR (neat) 3240, 3080, 2960, 2850, 1735, 1671, 1205, 870 cm^{-1}; $[\alpha]^{29}_D$ +12.7 (*c* 0.75, MeOH); ^{1}H RMN (400 MHz, DMSO-*d6*) δ 0.89 (d, *J* = 7.1 Hz, 3 H), 0.91 (d, *J* = 7.1 Hz, 3 H), 1.19 (t, *J* = 7.1 Hz, 3 H), 1.46-1.64 (m, 2 H), 1.72 (m, 1 H), 3.80 (m, 1 H), 3.85 (dd, *J* = 17.0, 5.7 Hz, 1 H), 3.96 (dd, *J* = 17.0, 6.0 Hz, 1 H), 4.10 (q, *J* = 7.1 Hz, 2 H), 8.29 (bs, 3 H), 9.04 (t, *J* = 5.2 Hz, 1 H); ^{13}C RMN (100 MHz, DMSO-*d6*): δ 13.9, 22.1, 22.3, 22.4, 23.2, 50.5, 60.5, 66.3, 169.0, 169.4; LCMS (ESI+) *rt* = 1.40 min, *m*/*z* = 217 $[M + H – HCl]^+$ (100); ELSD pur. 100%, UV pur. 100%.

*4.1.2.2. HCl.Leu-Gly-NH_2 (**4b**)*

The product was prepared following the corresponding general procedure starting from Boc-Leu-Gly-NH_2 **11b** (5 g, 17.4 mmol) in 1,4-dioxane (95 mL) to give 3.89 g (quantitative) of dipeptide **4b**. Colorless hygroscopic oil; IR (neat) 3380, 3220, 3130, 2955, 1726, 1670, 1647, 1507, 1264 cm^{-1}; $[\alpha]^{29}_D$ +29.0 (*c* 1.3, MeOH); ^{1}H RMN (400 MHz, D_2O) δ 0.76 (d, *J* = 5.5 Hz, 3 H), 0.77 (d, *J* = 5.5 Hz, 3 H), 1.45-1.63 (m, 3 H), 3.72 (d, *J* = 16.9 Hz, 1 H), 3.83 (d, *J* = 16.9 Hz, 1 H), 3.88 (m, 1 H); ^{13}C RMN (100 MHz, D_2O**)** δ 21.5, 22.0, 24.1, 40.0, 42.3, 52.3, 171.2, 173.6; LCMS (ESI+) *rt* = 4.98 min, *m*/*z* = 188 $[M + H – HCl]^+$ (100); ELSD pur. 99%, UV pur. 100%.

*4.1.3. General procedure for the synthesis of the tripeptides (**12**) and (**2**).*

HCl.Leu-Gly-OEt **4a** or HCl.Leu-Gly-NH_2 **4b** was dissolved in DMF. TEA was added and the solution was stirred at 0 °C for 1 h, followed by the addition of HOBt and EDCI to the reaction mixture. After stirring for 20 min at 0 °C, (*S*)-(αTfm)-Proline was added. The resulting mixture was warmed to r.t. for 72 h, then cooled to 0 °C and the urea precipitate was removed by filtration. The DMF was evaporated under reduced pressure to dryness and the corresponding crude mixture was diluted with ethyl acetate and water. The layers were separated and the aqueous phase was extracted with ethyl acetate. The combined organic phases were evaporated and the crude product was purified by reverse phase chromatography (gradient H_2O/ CH_3CN). The residue was dissolved in 5% $NaHCO_3$ aqueous solution, extracted with ethyl acetate (3 x), dried over $MgSO_4$ and concentrated to give the tripeptides **12** and **2** in respectively 62% and 89% yield.

*4.1.3.1. (S)-(α–Tfm)-Pro-Leu-Gly-OEt (**12**)*

The product was prepared following the corresponding general procedure starting from HCl.Leu-Gly-OEt **4a** (278 mg, 1.1 mmol, 2 equiv) in DMF (3 mL), TEA (260 µL, 1.9 mmol, 3.4 equiv), HOBt (110 mg, 0.8 mmol, 1.5 equiv), EDCI (157 mg, 0.8 mmol, 1.5 equiv), (*S*)-(α–Tfm)-Proline (100 mg, 1 equiv). Purification by reverse phase chromatography (100% H_2O then 5% gradient H_2O/CH_3CN) gave 127 mg (62%) of tripeptide **12**. Colorless hygroscopic oil; IR (neat) 3309, 2957, 2871, 1747, 1655, 1284, 1158 cm^{-1}; $[\alpha]^{25}_D$ –58.0 (*c* 1.0, MeOH); ^{1}H RMN (400 MHz, CD_3OD) δ 0.93 (d, *J* = 5.3 Hz, 3 H), 0.96 (d, *J* = 4.6 Hz, 3 H), 1.26 (t, *J* = 7.1 Hz, 3 H), 1.57-1.67 (m, 3 H), 1.73 (m, 1 H), 1.89 (m, 1 H), 2.21-2.27 (m, 2 H), 3.01-3.09 (m, 2 H), 3.85 (d, *J* = 17.4 Hz, 1 H), 4.00 (d, *J* = 17.4 Hz, 1 H), 4.17 (q, *J* = 7.1 Hz, 2 H), 4.50 (m, 1 H); ^{13}C RMN (100 MHz, CD_3OD) δ 14.4, 21.9, 23.4, 25.9, 26.4, 33.3, 42.0, 42.3, 53.1, 62.2, 72.1 (q, *J* = 26.8 Hz), 127.4 (q, *J* = 283.7 Hz), 170.9, 171.3, 174.6; ^{19}F RMN (376 MHz, CD_3OD) δ –79.14 (s, CF_3); LCMS (ESI+) *rt* = 4.43 min, *m*/*z* = 382 $[M + H]^+$ (100); ELSD pur. 99%, UV pur. 100%.

*4.1.3.2. (S)-(α–Tfm)-Pro-Leu-Gly-NH_2 (**2**)*

The product was prepared following the corresponding general procedure starting from HCl.Leu-Gly-NH_2 **4b** (200 mg, 0.9 mmol, 1.6 equiv) in DMF (6 mL), TEA (260 µL, 1.9 mmol, 3.4 equiv), HOBt (110 mg, 0.8 mmol, 1.5 equiv), EDCI (157 mg, 0.8 mmol, 1.5 equiv), (*S*)-(αTfm)-Proline (100 mg, 1 equiv). Purification by reverse phase chromatography (100% H_2O then 5% gradient H_2O/CH_3CN) gave 170 mg (89%) of tripeptide **2**. Colorless hygroscopic oil; IR (neat) 3305, 3080, 3200, 2958, 2928, 2872, 1654, 1514, 1283, 1158 cm^{-1}; $[\alpha]^{24}_D$ –30.0 (*c* 0.5, MeOH); ^{1}H RMN (400 MHz, CD_3OD) δ 0.93 (d, *J* = 6.0 Hz, 3 H), 0.97 (d, *J* = 5.7 Hz, 3 H), 1.58-1.80 (m, 4 H), 1.89 (m, 1 H), 2.21-2.29 (m, 2 H), 2.99-3.09 (m, 2 H), 3.77 (d, *J* = 17.0 Hz, 1 H), 3.90 (d, *J* = 17.0 Hz, 1 H), 4.40 (m, 1 H); ^{13}C RMN (100 MHz, CD_3OD) δ 21.9, 23.4, 26.0, 26.4, 33.3, 41.6, 43.1, 48.2, 53.9, 72.1 (q, *J* = 26.2 Hz), 127.4 (q, *J* = 289.0 Hz), 171.9, 174.0, 174.8; ^{19}F RMN (376 MHz, CD_3OD) δ –79.1 (s, CF_3); LCMS (ESI+) *rt* = 2.41 min, *m*/*z* = 353 $[M + H]^+$ (100); ELSD pur. 99%, UV pur. 100%.

4.2. Biology

4.2.1. Animals

The experiments were carried out on male Wistar rats (180–200 g), housed at 12 h light/dark cycle. Food and water were available ad libitum. All experiments were carried out between 09.00 a.m. and 12.00 p.m. Each group included five rats for nociceptive tests.

4.2.2. Nociceptive tests

Paw-pressure test (Randall-Selitto test): The changes in the mechanical nociceptive threshold of the rats were measured using an analgesimeter (Ugo Basile). The pressure was applied to the hind-paw and the pressure (g) required eliciting nociceptive responses, such as squeak, and struggle was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw.

Hot plate test: The latency of response to pain was measured from the moment of placing an animal on a metal plate (heated to 55 ± 0.5^{o}C) to the first signs of pain (paw licking, jump). The cut-off time was 30 s.

4.2.3. Drugs and treatment

MIF-1, a non-specific opioid receptor antagonist naloxone (Nal), L-Arginine (L-Arg, the natural precursor of NO) (all at a dose of 1 mg/kg) and NO synthase inhibitor L-N^{G}-nitroarginine ester (L-NAME) (10 mg/kg), were obtained from Tocris. All drugs were dissolved in a sterile saline (0.9% NaCl) solution and injected intraperitoneally (i.p.).

The experimental procedures were carried out in accordance with the institutional guidance and general recommendations on the use of animals for scientific purposes.

4.2.4. Statistical analysis

The results were statistically assessed by the analysis of variance ANOVA. Values are mean ± S.E.M. Values of $P < 0.05$ were considered to indicate statistical significance.

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List of captions

Fig 1. Chemical structure of (MIF-1) **1** and CF_3-(MIF-1) **2**

Fig 2. Retrosynthetic pathway of CF_3-(MIF-1) **2**

Scheme 1. Reagents and conditions : (a) AllylTMS, $BF_3.OEt_2$, CH_2Cl_2, 72 h, 0 °C → r.t.; (b) PTSA cat., Toluene, reflux, 24 h; (c) I_2, Toluene, 95°C, 48 h; (d) H_2 (5 bars), Pd/C, AcONa, EtOH, 24 h; (e) Chromatographic separation of both diastereomer; (f) H_2 (5 bars), $Pd(OH)_2$, EtOH, 48 h.

Scheme 2. Reagents and conditions : (a) HOBt, EDCI, Et_3N, DMF, 16 h, 0 °C → r.t.; (b) BOPCl, Et_3N, CH_2Cl_2, 12 h; (c) HCl_g, 1,4-dioxane; (d) (*S*)-**3**, HOBt, EDCI, Et_3N, DMF, 72 h, 0 °C → r.t.; (e) NH_3, EtOH, 2 h.

Fig.3. Effects of MIF-1, its analogue CF_3-MIF-1 and combination of CF_3-MIF-1 with NaI (all at a dose of 1 mg/kg, i.p.) estimated by PP test in male Wistar rats. Data are presented as mean ±S.E.M.; *P<0.05, ** P<0.01 vs. control; $^{+}$P<0.05, $^{++}$ P<0.01 vs. MIF-1.

Fig.4. Effects of MIF-1, its analogue CF_3-MIF-1 and combination of CF_3-MIF-1 with NaI (all at a dose of 1 mg/kg, i.p.) estimated by HP test in male Wistar rats. Data are presented as mean ±S.E.M.; *P<0.05, ** P<0.01 vs. control; $^{+}$P<0.05, $^{++}$ P<0.01 vs. MIF-1.

Fig.5. Effects of MIF-1, its analogue CF_3-MIF-1 (both at a dose of 1 mg/kg, i.p.) and their combination with L-NAME (10 mg/kg, i.p.) estimated by PP test in male Wistar rats. Data are presented as mean ±S.E.M.; ** P<0.01 vs. control; $^{++}$ P<0.01 vs. L-NAME.

Fig.6. Effects of MIF-1, its analogue CF_3-MIF-1 (both at a dose of 1 mg/kg, i.p.) and their combination with L-NAME (10 mg/kg, i.p.) estimated by HP test in male Wistar rats. Data are presented as mean ±S.E.M.; *P<0.05, ** P<0.01 vs. control; $^{++}$ P<0.01 vs. L-NAME.

Fig.7. Effects of MIF-1, its analogue CF_3-MIF-1 and their combination with L-Arg (all at a dose of 1 mg/kg, i.p.) estimated by PP test in male Wistar rats. Data are presented as mean ±S.E.M.; *P<0.05, ** P<0.01 vs. control; $^{++}$ P<0.01 vs. L-Arg.

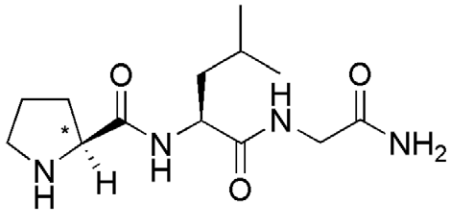
Fig.8. Effects of MIF-1, its analogue CF_3-MIF-1 and their combination with L-Arg (all at a dose of 1 mg/kg, i.p.) estimated by HP test in male Wistar rats. Data are presented as mean ±S.E.M.; *P<0.05, ** P<0.01 vs. control; $^{++}$ P<0.01 vs. L-Arg.

Highlights

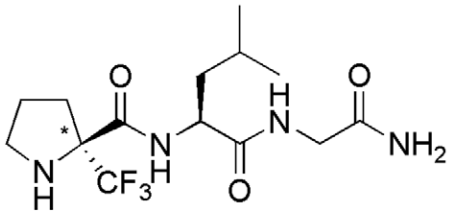
- A MIF-1 analogue containing enantiopure (*S*)-α-trifluoromethyl proline was synthesized

- The nociception during acute pain of the CF_3-MIF-1 was evaluated in rat model

- Significant analgesic effect in paw pressure test compared to the native peptide



(MIF-1) **1**



CF_3-(MIF-1) **2**