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β-Peptido sulfonamides: for-Met-Leu-Phe-OMe analogues containing taurine and chiral β-amino-ethanesulfonic acid residues

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Abstract

A series of new β-peptido sulfonamides, related to the chemotactic tripeptide fMLF-OMe, has been synthesized. The examined **1a,b–7a,b** models contain the achiral –HN–$(CH_2)_2$–SO_2– taurine (Tau) residue or the chiral –HN–CH(*n*Bu)–CH_2–SO_2– and –HN–CH(*i*Bu)–CH_2–SO_2– residues, corresponding to the β-aminocarboxylic acid counterparts β^3-HNle and β^3-HLeu, respectively. The biological activity of the new analogues has been determined on human neutrophils and compared with that of the reference ligand as well as that of the previously studied related models. The results are analyzed in terms of structure–activity relationships. The conformational preferences of the new tripeptides **1b** and **2b**, containing a central chiral β-amino-ethanesulfonic acid residue, have also been discussed.

Keywords: Chemotactic peptides; Human neutrophils; β-Peptido sulfonamides; Pseudopeptides; Taurine

1. Introduction

During our research on the structure–activity relationships in the field of chemotactic peptides related to For-Met-Leu-Phe-OMe (fMLF-OMe) we started recently a program centered on the examination of the biochemical consequences of incorporating residues of β-amino-carboxylic and β-amino-ethanesulfonic acids into the molecule of the reference ligand fMLF-OMe. At present, two types of these new analogues have been examined. A first type is characterized by the presence of the achiral residue of β-alanine (H_2N–CH_2–CH_2–CO_2H) or taurine (H_2N–CH_2–CH_2–SO_3H) replacing the central native L-leucine (Leu) [1]. A second type includes the mixed α-peptide/β-peptide analogues of fMLF-OMe [2] incorporating the chiral residues of β^3-HMet, β^3-HLeu and β^3-HPhe [3,4] in place of the native L-methionine (Met), L-leucine (Leu) and L-phenylalanine (Phe) residues, respectively.

The analysis of the results obtained by examining the above described models showed that the incorporation of a β-amino-carboxylic acid residue at the central position of the parent sequence is the least tolerated structural modification. Thus, For-Met-βAla-Phe-OMe was found to be completely inactive and For-Met-β^3-HLeu-Phe-OMe, notwithstanding the presence in the β^3-HLeu residue of the central proteinogenic isobutylic side chain, resulted to be a weak agonist and the least active among the three mixed α/β^3 fMLF-OMe analogues incorporating a β^3-HMet, β^3-HLeu or β^3-HPhe residue, respectively.

At variance with the above reported findings concerning β-aminocarboxylic acids, all the pseudopeptidic fMLF-OMe analogues which incorporate the achiral residue of the β-amino-ethanesulfonic acid taurine (Tau) at the central position (namely: For-Met-Tau-Phe-OMe; Boc-Met-Tau-Phe-OMe and Met-Tau-Phe-OMe) exhibit a significant chemotactic activity, comparable to that shown by the reference ligand [1]. This despite the absence in the Tau residue of the isobutylic side chain and the different nature of the amino

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Table 1
Activity at the optimal peptide concentration of fMLF-OMe derivatives containing a β-aminoacid residue at the central position

Compounds	Chemotactic index [a]	O_2^- (nmoles) [a]	% Lysozyme release [a]
Boc-Met-Tau-Phe-OMe	0.93 (10^{-9})	2.2 (10^{-5})	10 (10^{-5}
HCO-Met-Tau-Phe-OMe	1.06 (10^{-9})	10.2 (10^{-5})	14 (10^{-5})
HCl.Met-Tau-Phe-OMe	1 (10^{-9})	5.5 (10^{-7})	17 (10^{-7})
Boc-Met-β^3-HLeu-Phe-OMe	0.23 (10^{-10})	4 (10^{-5})	16 (10^{-5})
HCO-Met-β^3-HLeu-Phe-OMe	0.55 (10^{-8})	33 (5×10^{-5})	38 (8×10^{-5})
Boc-Met-Leu-ψ[CH_2SO_2]-Phe-OMe (**2a**)	0.47 (10^{-8})	4 (10^{-7})	3 (10^{-7})
HCO-Met-Leu-ψ[CH_2SO_2)-Phe-OMe (**2b**)	0.58 (10^{-8})	41 (10^{-5})	56 (10^{-5})
Boc-Met-Tau-Phe-Phe-OMe (**6a**)	0.84 (10^{-10})	7 (10^{-7})	17 (10^{-7})
HCO-Met-Tau-Phe-Phe-OMe (**6b**)	0.45 (10^{-10})	27 (10^{-5})	27 (10^{-5})

[a] The number in parentheses indicates the peptide molarity.

terminal group in the three models. Particularly unexpected is the activity shown by the pseudopeptide Boc-Met-Tau-Phe-OMe (see Table 1) whose bulky protecting group at the Met amino group is generally associated with antagonistic activity [5,6].

As a continuation of our early studies in the field of linear and cyclic β-sulfonamido peptides [7–13] and in order to acquire more information on the role and potentiality of fMLF-OMe analogues incorporating residues of β-amino ethanesulfonic acids into the molecule of this potent chemotactic tripeptide, we report here synthesis and activity of a series of new models.

R-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe (1)

R-Met-Leu-ψ[CH_2SO_2]-Phe-OMe (2)

R-Tau-Phe-OMe (3)

R-Tau-Leu-Phe-OMe (4)

R-Tau-Leu-Phe-Phe-OMe (5)

R-Met-Tau-Phe-Phe-OMe (6)

R-Met-Leu-Tau-Phe-OMe (7)

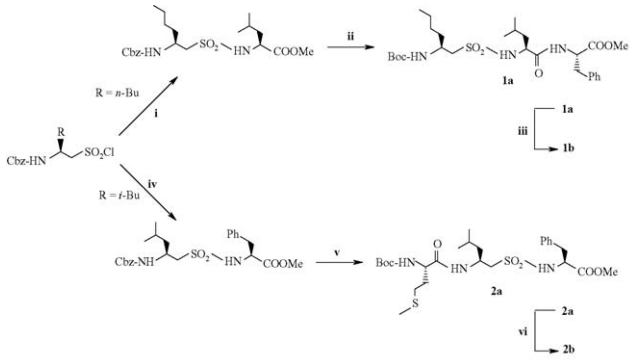
(a) *R* = *tert*-Bu-OCO- (Boc-); (b) *R* = HCO- (For-).

A first group includes the *N*-Boc- and the *N*-For-derivatives **1a,b** and **2a,b**. These peptidosulfonamide/peptide hybrids have been synthesized with the aim of comparing the consequences of the replacement of the β-aminocarboxylic acid residues, incorporated into the previously studied α/β^3 hybrid tripeptides [2], with the corresponding chiral β-amino-ethanesulfonic acid residues. It should be noted here that in compounds **1a,b** (which correspond to the analogues of the previously studied Boc- and For-β^3-HMet-Leu-Phe-OMe peptides) the –HN–CH(*n*Bu)–CH_2–SO_2– residue related to the β^3-norleucine (β^3-HNle), instead of the expected –NH–CH(CH_2–CH_2–S–CH_3)–CH_2–SO_2– residue related to β^3-methionine (β^3-HMet), has been incorporated. This strategy is based on the observation that methionine (Met) and norleucine (Nle) show bioisosterism [5,14,15] and the utilization of this latter residue avoids the laborious synthesis of the chiral β-amino-ethanesulfonyl derivative possessing the methylthio–ethyl side chain of the native methionine.

The second group of here reported peptides is directly related to the above mentioned remarkable chemotactic activity previously observed in the case of the pseudotripeptides containing a residue of taurine at the central position [1]. Thus, in order to get information on this point, the synthesis of the new series of β-sulfonamido peptides (**3–7**) containing the achiral Tau residue in place of a natural amino acid, has been performed. In particular, in addition to the dipeptides **3a,b** and tripeptides **4a,b**, characterized by a N-terminal Tau residue, a positional scan of the sequence -Met-Leu-Phe-, typical of the native ligand fMLF-OMe, has been performed by synthesizing the three β-sulfonamido-tetrapeptides **5, 6, 7** in which the Tau residue replaces systematically one of the first three α-aminoacid residues of the highly active tetrapeptide For-Met-Leu-Phe-Phe-OMe (fMLFF-OMe) [14,16].

2. Chemistry

The synthesis of Boc-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe (**1a**), Boc-Met-Leu-ψ[CH_2SO_2]-Phe-OMe (**2a**) and of the corresponding *N*-formyl analogues HCO-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe (**1b**) and HCO-Met-Leu-ψ[CH_2SO_2]-Phe-



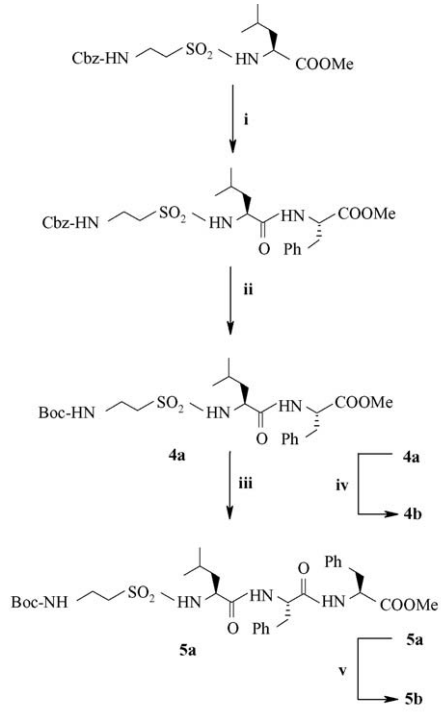
Reagents: **i**) HCl·Leu-OMe, TEA; **ii**) a: NaOH, MeOH; b: HCl·Phe-OMe, *i*-BuOCOCl, NMM; c: HBr/AcOH; d: $(Boc)_2O$; **iii, vi**) HCOOH, EEDQ; **iv**) HCl·Phe-OMe, TEA; **v**) a: HBr/AcOH; b: Boc-Met-OH, *i*-BuOCOCl, NMM.

Scheme 1.

OMe (**2b**) is reported in Scheme 1. The key synthons along this route were Cbz-Nle-ψ[CH_2SO_2]-Cl and Cbz-Leu-ψ[CH_2SO_2]-Cl, which were prepared by using the mesylate–thioacetate intermediates starting from Cbz-Nle-OH and Cbz-Leu-OH, respectively, according to the literature [17]. Coupling of Cbz-Nle-ψ[CH_2SO_2]-Cl with HCl. Leu-OMe in the presence of triethylamine afforded the Cbz-Nle-ψ[CH_2SO_2]-Leu-OMe which, after alkaline hydrolysis of the methyl ester, gave the corresponding free acid. This latter was coupled with HCl.Phe-OMe by mixed anhydride method giving Cbz-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe. Removal of the Cbz group by a solution of HBr in acetic acid and N-protection of the amino group with di-*tert*-butyl-dicarbonate afforded **1a**. Coupling of Cbz-Leu-ψ[CH_2SO_2]-Cl with HCl.Phe-OMe by analogous procedure afforded Cbz-Leu-ψ[CH_2SO_2]-Phe-OMe which, after cleavage of the Cbz group by HBr in acetic acid solution, was coupled with Boc-Met-OH by the mixed anhydride method giving **2a**.

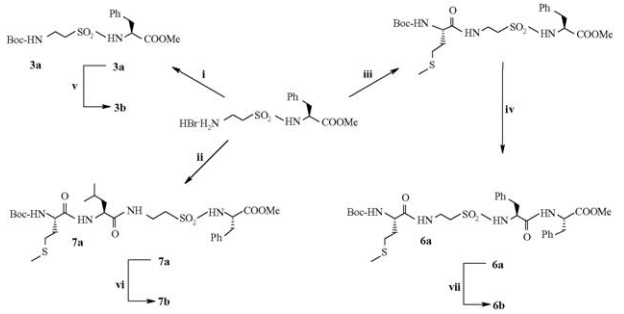
The synthesis of the pseudopeptides Boc-Tau-Leu-Phe-OMe (**4a**) and Boc-Tau-Leu-Phe-Phe-OMe (**5a**) and of the corresponding *N*-formyl analogues HCO-Tau-Leu-Phe-OMe (**4b**) and HCO-Tau-Leu-Phe-Phe-OMe (**5b**) was performed according to Scheme 2: alkaline hydrolysis of Cbz-Tau-Leu-OMe [13] gave the free acid which was coupled with HCl.Phe-OMe by EDC/HOBT to give Cbz-Tau-Leu-Phe-OMe. Removal of Cbz group by catalytic hydrogenation in the presence of trifluoroacetic acid (TFA) furnished TFA.H_2N-Tau-Leu-Phe-OMe which was N-protected with di-*tert*-butyldicarbonate to give **4a**. Cbz- instead of Boc-group, for the N-protection of the starting material, is requested to avoid the instability of the latter in the amino-ethanesulphonyl chloride preparation step. Alkaline hydrolysis of **4a** gave the free acid which was coupled with HCl.Phe-OMe to give **5a**.

In Scheme 3 the synthesis of the pseudopeptides Boc-Tau-Phe-OMe (**3a**), Boc-Met-Tau-Phe-Phe-OMe (**6a**) and Boc-Met-Leu-Tau-Phe-OMe (**7a**) and of the corresponding *N*-formyl analogues HCO-Tau-Phe-OMe (**3b**), HCO-Met-Tau-Phe-Phe-OMe (**6b**) and HCO-Met-Leu-Tau-Phe-OMe (**7b**) is reported. The starting HBr.Tau-Phe-OMe was prepa-



Reagents: **i, iii**) a: NaOH, MeOH; b: HCl·Phe-OMe, EDC, HBT, NMM; **ii**) a: H_2, Pd/C; b: $(Boc)_2O$; **iv, v**) HCOOH, EEDQ.

Scheme 2.



Reagents: **i**) $(Boc)_2O$; **ii**) Boc-Met-Leu-OH, *i*-BuOCOCl, NMM; **iii**) Boc-Met-OH, *i*-BuOCOCl, NMM; **iv**) a: NaOH, MeOH; b: HCl·Phe-OMe, EDC, HBT, NMM; **v, vi, vii**) HCOOH, EEDQ.

Scheme 3.

red according to reference [18]. A direct transformation of the *N*-Boc derivatives **1a–7a** into the corresponding *N*-formyl analogues was performed by following the procedure of Lajoie and Kraus [19]. Thus, treatment of the *N*-Boc derivatives with formic acid, followed by ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate (EEDQ), gave the respective formyl derivatives **1b–7b**.

3. Biological activity

The biological activity of the new analogues **1–7** has been determined on human neutrophils and compared with that of the reference ligand fMLF-OMe. Directed migration (chemotaxis), superoxide anion production and lysozyme release have been measured. In Fig. 1 the agonistic activity of the

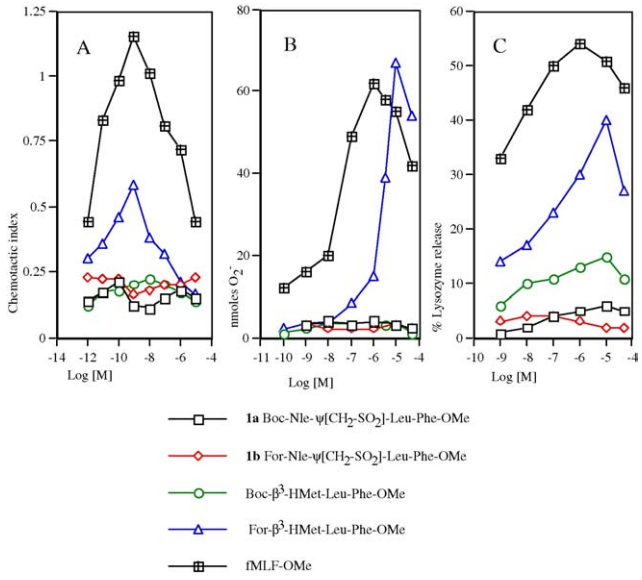


Fig. 1. Biological activity of the two β-sulfonamido tripeptides **1a,b** and of the corresponding analogues containing a N-terminal chiral β^3-aminocarboxylic acid residue, compared with the reference ligand fMLF-OMe. (A) Chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining lysozyme activity.

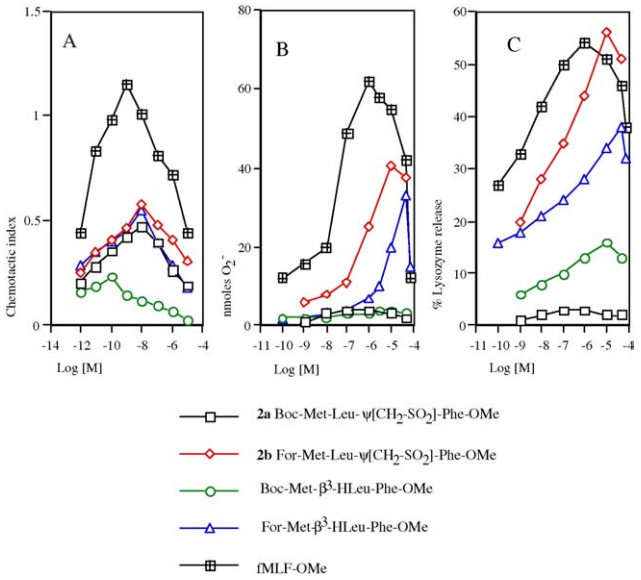


Fig. 2. Biological activity of the two β-sulfonamido tripeptides **2a,b** and of the corresponding analogues containing a central chiral β^3-aminocarboxylic acid residue, compared with the reference ligand fMLF-OMe. (A) Chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining lysozyme activity.

pseudotripeptides Boc-Nle-ψ[CH_2-SO_2]-Leu-Phe-OMe (**1a**) and HCO-Nle-ψ[CH_2-SO_2]-Leu-Phe-OMe (**1b**), together with the biological responses elicited by the previously studied [2] α/β^3 mixed tripeptides Boc-β^3-HMet-Leu-Phe-OMe and HCO-β^3-HMet-Leu-Phe-OMe, are reported. It can be seen that the replacement of the N-terminal β^3-HMet with the -Nle-ψ[CH_2-SO_2]- residue to give the *N*-formyl derivative **1b**, causes the loss of the activity shown by the corresponding β^3-HMet containing analogue and leaves unaltered the inactivity of the *N*-Boc β^3-HMet containing α/β^3 mixed tripeptide.

In Fig. 2 the agonistic activity of the pseudotripeptides Boc-Met-Leu-ψ[CH_2-SO_2]-Phe-OMe (**2a**) and HCO-Met-Leu-ψ[CH_2-SO_2]-Phe-OMe (**2b**) is compared with that shown by the previously studied α/β^3 mixed tripeptides Boc-Met-β^3-HLeu-Phe-OMe and HCO-Met-β^3-HLeu-Phe-OMe [2]. Concerning the chemotactic activity (Fig. 2A and Table 1) it can be seen that the replacement of the central β^3-HLeu with the corresponding β-sulfonyl residue leaves unaltered the activity of the formylated model while renders chemoattractant the otherwise inactive Boc-Met-β^3-HLeu-Phe-OMe. This latter result parallels the unexpected agonistic activity previously observed in the case of the tripeptide Boc-Met-Tau-Phe-OMe as compared with the inactive Boc-Met-βAla-Phe-OMe. As for the superoxide anion production (Fig. 2B and Table 1) the tripeptide HCO-Met-Leu-ψ[CH_2SO_2)-Phe-OMe (**2b**) was found more potent and efficient than the corresponding α/β^3 mixed formyl derivative. A similar trend was also observed in the case of lysozyme release (Fig. 2C and Table 1). Here, it is worth noting the considerable activity exhibited by the formyl β-peptidosulfonamide **2b** which superates both the analogue For-Met-β^3-HLeu-Phe-OMe containing the central β-aminocarboxylic acid residue and, at slightly higher concentration, the parent fMLF-OMe.

The biological assays were then extended to the pseudopeptides Boc-Tau-Phe-OMe (**3a**), HCO-Tau-Phe-OMe (**3b**), Boc-Tau-Leu-Phe-OMe (**4a**), and HCO-Tau-Leu-Phe-OMe (**4b**) containing a N-terminal Tau residue. All these derivatives were found practically inactive in each of the performed assays (not shown).

Finally, Fig. 3 shows the biological activity of the fMLFF-OMe pseudotetrapeptide analogues containing the Tau residue in the first (**5a,b**), second (**6a,b**), and third (**7a,b**) position, respectively. Inspection of Fig. 3A shows that *N*-Boc derivative **6a** exhibits the highest chemotactic activity and this is in agreement with the remarkable activity found for the previously studied tripeptide Boc-Met-Tau-Phe-OMe [1] (Table 1). On other hand, the low chemotactic response elicited by the corresponding formyl derivative HCO-Met-Tau-Phe-Phe-OMe (**6b**) is not expected in view of both the remarkable activity exhibited by the correlated β-peptidosulfonamide HCO-Met-Tau-Phe-OMe (Table 1) and of the expected beneficial effect on the activity induced by the presence of an additional *C*-terminal Phe residue [5,14,15]. Concerning the superoxide anion production and the lysozyme release, the formyl derivative **7b**, containing the Tau residue in the third position, was found to be the most active agonist in both the biological assays. It should be also noted that the pseudopeptides containing the Tau residue in the first position (**5a,b**) were essentially inactive in the three tested functions. These data, together with the biological inactivity shown by the derivatives **3a,b** and **4a,b**, confirm that the occurrence of N-terminal Tau is not tolerated.

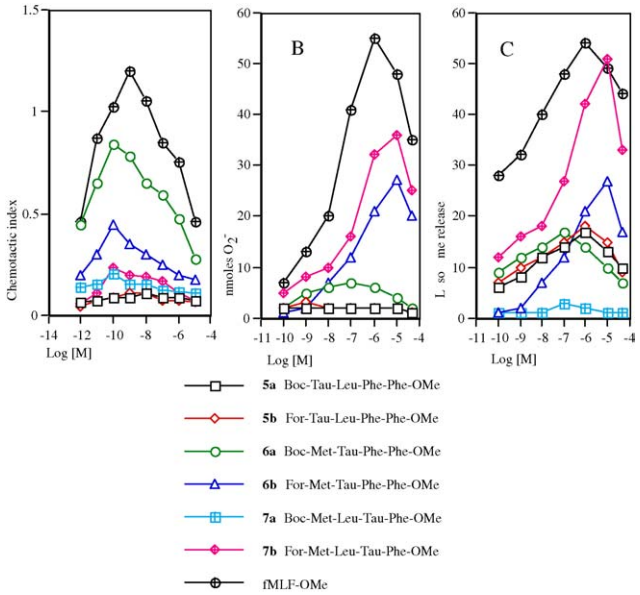


Fig. 3. Biological activity of β-sulfonamido tetrapeptides **5a,b–7a,b** containing a taurine residue, compared with the reference ligand fMLF-OMe. (A) Chemotactic activity; (B) superoxide anion production; compounds **5a** and **7a** exhibit almost identical values. (C) Release of neutrophil granule enzymes evaluated by determining lysozyme activity.

4. Solution conformation

Information on the conformational preferences of pseudotripeptides **1** and **2** containing a chiral β-sulphonamido residue have been obtained by examining the involvement of the NH groups of the formyl derivatives HCO-Nle-ψ[CH_2-SO_2]-Leu-Phe-OMe (**1b**) and HCO-Met-Leu-ψ[CH_2-SO_2]-Phe-OMe (**2b**) in intramolecular H-bonds using ^{1}H NMR. In Fig. 4 the chemical shift dependence of the NH proton resonances as a function of increasing DMSO-d_6 concentrations in $CDCl_3$ solution (10 mM) is reported. In Table 2 the solvent exposure of the NH groups of **1b** and **2b**, expressed as the difference ($\Delta\delta$, ppm) between the NH chemical shift values observed in a $CDCl_3$ solution containing 10% DMSO and in neat $CDCl_3$, is compared with the accessibility of the corresponding groups of the previously studied [2] α/β^3 mixed tripeptides HCO-β^3-HMet-Leu-Phe-OMe and HCO-Met-β^3-HLeu-Phe-OMe. It appears that in the two models under study, all the NH groups interact efficiently with the solvent, thus indicating the absence of intramolecularly H-bonded conformations. Thus, the pseudopeptides **1b** and **2b** do not adopt folded conformations stabilized by intramolecular H-bonds and the chiral β-sulphonamido residue does not induce the locally folded structures, centred at the β-residues through a six membered ring (C_6 conformation) observed for the related α/β^3 mixed tripeptides HCO-β^3-HMet-Leu-Phe-OMe and HCO-Met-β^3-HLeu-Phe-OMe [2].

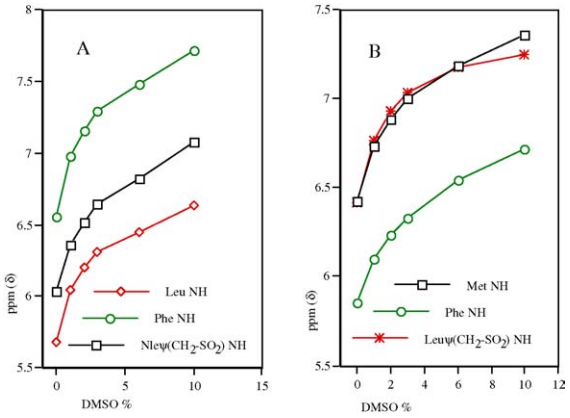


Fig. 4. Plots of NH proton chemical shifts in the ^{1}H NMR spectra of the β-sulfonamido tripeptides **1b** (A) and **2b** (B) as a function of increasing amounts of DMSO-d_6 (v/v) added to the $CDCl_3$ solution (peptide concentration: 10 mM).

5. Conclusions

Some significant points of the present study are the followings:

- a β-sulphonamido residue, carrying or not the proteinogenic side chain, when located at the N-terminal position of fMLF-OMe analogues, leads to derivatives devoid of biological activity in all the examined models. It should be noted that the related *N*-For analogue, containing a β^3-HMet residue in place of the native N-terminal Met, shows significant chemotactic activity (see Fig. 1A).
- The incorporation of the proteinogenic isobutylic side chain into the Tau residue of Boc- or For-Met-Tau-Phe-OMe, leading to compounds **2a,b**, causes a reduction of their remarkable chemotactic activity and the loss of the selectivity shown by the *N*-For derivative (see Fig. 2 and Table 1).
- An unexpected decrease of the remarkable chemotactic activity exhibited by the previously studied For-Met-Tau-Phe-OMe [1] is observed on passing from this tripeptide to the related tetrapeptide For-Met-Tau-Phe-Phe-OMe (**6b**). The corresponding *N*-Boc pseudotetrapeptide **6a** maintains, on other hand, the remarkable chemotactic activity exhibited by the tripeptide Boc-Met-Tau-Phe-

Table 2
Solvent accessibility of peptide NH groups: Differences ($\Delta\delta$, ppm) between NH proton chemical shift values observed in a $CDCl_3$ solution containing 10% DMSO and in neat $CDCl_3$

Compound	*N*-terminal NH	Central NH	*C*-terminal NH
HCO-Nle-ψ[CH_2-SO_2]-Leu-Phe-OMe (**1b**)	1.05	0.95	1.16
HCO-β^3-HMet- Leu-Phe-OMe	0.48	1.25	0.67
HCO-Met-Leu-ψ[CH_2-SO_2]-Phe-OMe (**2b**)	0.93	0.84	0.86
HCO-Met-β^3-HLeu-Phe-OMe	0.80	0.36	1.04

OMe. Furthermore, it should be noted that these two *N*-Boc derivatives are selective ligands since show only chemotactic activity (Fig. 3 and Table 1). An opposite selectivity is observed in the case of the *N*-For tetrapeptide **7b** which is not chemoattractant but induces superoxide anion production and lysozyme release (Fig. 3).

- The replacement of the β-amino-carboxylic acid residue with the corresponding β-amino-sulfonic acid leads to the models **1b** and **2b** (see Fig. 4 and Table 2) which do not maintain the locally folded conformations stabilized by intramolecular H-bonds found in the case of the corresponding previously studied models [2] containing the β^3-HMet and β^3-HLeu residues.

A main indication which emerges from the above reported results concerns the role of the sequence -Met-Tau-Phe- which appears associated, both in the tripeptide and in the tetrapeptide models, with the unusual chemotactic activity shown by the *N*-Boc derivatives. Although more data are necessary to better define this point, the available data suggest that the sulphonamide junction, due to its high polar character and hydrogen bonding capabilities, can significantly alter the usual mode of ligand–receptor interaction and when centrally located into fMLF-OMe analogues determines a profound change of the established requirements for an efficient binding [14,20]. Consistent with this hypothesis is the observed decrease of activity on passing from the tripeptides containing a central Tau to the corresponding models **2a,b** possessing the isobutylic proteinogenic Leu side chain as well as the higher activity shown by the *N*-Boc derivative **6a** as compared with the corresponding *N*-For analogue **6b**.

Further studies on the properties of fMLF-OMe analogues containing β-sulfonic acid residues are in progress in our laboratories.

6. Experimental section

6.1. Chemistry

Melting points were determined with a Büchi B 540 apparatus and are uncorrected. Optical rotations were taken at 20 °C with a Schmidt-Haensch Polartronic D polarimeter (1 dm cell, *c* 1.0 in $CHCl_3$, unless otherwise specified). IR spectra were recorded in 1% $CHCl_3$ (unless otherwise specified) solution employing a Perkin-Elmer FT-IR Spectrum 1000 spectrometer. ^1H NMR spectra were determined in $CDCl_3$ solution with a Bruker AM 400 spectrometer and chemical shifts were indirectly referred to TMS. Thin-layer and preparative layer chromatographies were performed on silica gel Merck 60 F_{254} plates. The drying agent was sodium sulphate. Elemental analyses were performed in the laboratories of the Servizio Microanalisi del CNR, Area della Ricerca di Roma, Montelibretti, Italy, and were within ±0.4% theoretical values. The abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; EEDQ, ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole; KRPG, Krebs–Ringer phosphate containing 0.1% w/v D-glucose; NMM, *N*-methylmorpholine; DMF, dimethylformamide; TEA, triethylamine.

6.1.1. Coupling of Cbz-β-aminoethane sulphonylchlorides with aminoacid methylester hydrochlorides. General procedure A

To an ice-cooled mixture containing the Cbz-β-aminoethane sulphonylchloride (1.0 mmol) and the aminoacid methylester hydrochloride (2.0 mmol) in dry CH_2Cl_2 (8 ml), TEA (2.0 mmol) in dry CH_2Cl_2 (8 ml) was added under nitrogen. The resulting suspension was stirred overnight allowing to warm to room temperature. After dilution with CH_2Cl_2 (25 ml), the mixture was washed with 1 M HCl, saturated $NaHCO_3$ and brine. The organic phase was dried and evaporated under reduced pressure.

6.1.2. Cbz-group removal. General procedure B

General procedure B-1 by HBr/AcOH: the *N*-protected aminoacid or peptide (1 mmol) was dissolved in a solution of 36% HBr in acetic acid (3.0 ml). After 15 min at room temperature the reaction mixture was evaporated to dryness without heating. The residue was repeatedly coevaporated with anhydrous diethyl ether then dried under high vacuum overnight.

General procedure B-2 by catalytic hydrogenation: a solution of the *N*-protected aminoacid or peptide (1 mmol) and trifluoroacetic acid (1.2 mmol) in MeOH (10 ml) was flushed with H_2 stream in the presence of 10% palladium on charcoal at room temperature until the reaction was complete (TLC). The mixture was filtered on celite and the filtrate evaporated to dryness under reduced pressure. The oily residue was repeatedly coevaporated with anhydrous diethyl ether to remove the TFA excess and then kept under high vacuum overnight.

6.1.3. Hydrolysis of methylesters. General procedure C

The aminoacid or peptide methylester (1 mmol) was mixed with 2 N NaOH (1.5 ml) and MeOH (5 ml) and left at room temperature overnight. After removal of MeOH at reduced pressure, water (10 ml) was added and the solution extracted with diethyl ether (2 × 25 ml). The aqueous phase was then acidified with 1 N HCl and the product extracted with ethyl acetate (3 × 20 ml). The organic phases were washed with brine, pooled, dried and evaporated.

6.1.4. Preparation of the N-Boc derivatives. General procedure D

To a solution of the aminoacid or peptide (as hydrobromide or trifluoroacetate 1 mmol) and TEA (1.5 mmol) in dioxane (10 ml), di-*tert*-butyldicarbonate (1.2 mmol) in dioxane (3 ml) was added and the mixture was allowed at room temperature overnight. After evaporation at reduced

pressure, the residue was dissolved in ethyl acetate (25 ml) and washed with 1 M $KHSO_4$, $NaHCO_3$ saturated solution and brine. The organic phase was dried and evaporated under reduced pressure.

6.1.5. Mixed anhydride coupling. General procedure E

Isobutyl chloroformate (1 mmol) was added at –15 °C to a stirred solution of the N-protected aminoacid or peptide (1.0 mmol) and NMM (0.153 g, 1.0 mmol) in dry THF (8.0 ml). The temperature was maintained at –15 °C for 10 min and a freshly prepared mixture of the *C*-protected aminoacid hydrochloride (1.0 mmol) and NMM (1.0 mmol) in dry THF (5.0 ml) was then added. The reaction mixture was stirred at –15 °C for 30 min and left at 0 °C overnight. Ethyl acetate was then added and the solution washed with 1 M $KHSO_4$, brine, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried and evaporated under reduced pressure.

6.1.6. Carbodiimide coupling. General procedure F

A mixture containing the N-protected aminoacid or peptide (1.0 mmol), the *C*-protected aminoacid or peptide salt (1.0 mmol), HOBt (1.2 mmol) and TEA (1.2 mmol) in anhydrous ethyl acetate (6 ml) was prepared at room temperature. After cooling to 0 °C, EDC (1.0 mmol) was added and the reaction mixture allowed to warm slowly to room temperature. After 12 h ethyl acetate was added and the organic layer washed with 1 M $KHSO_4$, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried and evaporated under reduced pressure.

6.1.7. Preparation of the N*-formyl-derivatives. General procedure G*

The *N*-Boc derivative (1.0 mmol) was dissolved in formic acid (3 ml) and the mixture allowed to stand at room temperature overnight. After remotion of the excess of formic acid under vacuum, the residue was dissolved in dry DMF (2 ml). EEDQ 97% (1.2 mmol) was added and the solution stirred at room temperature for 24 h. Evaporation under reduced pressure afforded the crude product.

6.1.8. Cbz-Nle-ψ[CH_2SO_2]-Leu-OMe

From Cbz-Nle-ψ[CH_2SO_2]-Cl (0.920 g, 2.76 mmol) and HCl.Leu-OMe (0.501 g, 2.76 mmol) according to general procedure A. White solid (0.760 g, 78%). R_f = 0.40 (CH_2Cl_2). M.p. 82–83 °C (Et_2O/hexane). $[\alpha]_D$ = +2°. IR: *ν* 3434, 2960, 1742, 1709, 1517, 1338, 1139 cm^{-1}. ^1H NMR *δ*: 0.83–1.03 [9H, m, $CH_2CH(CH_3)_2$ and CH_2CH_3], 1.25–1.87 [9H, m, $CH_2CH(CH_3)_2$ and $CH_2CH_2CH_2CH_3$], 3.07–3.17 (2H, m, CH_2SO_2), 3.77 (3H, s, $COOCH_3$), 4.20 (1H, m, Leu α-CH), 4.43 (1H, m, $CHCH_2SO_2$), 4.97 (1H, d, *J* = 9.4 Hz, Nle-ψ[CH_2SO_2] NH), 5.12 (2H, s, $PhCH_2O$), 5.77 (1H, d, *J* = 9.0 Hz, Leu NH), 7.28–7.40 (5H, m, aromatics).

6.1.9. Cbz-Leu-ψ[CH_2SO_2]-Phe-OMe

From Cbz-Leu-ψ[CH_2SO_2]-Cl (0.534 g, 1.6 mmol) and HCl.Phe-OMe (0.690 g, 3.2 mmol) according to general procedure A. White solid (0.780 g, 79%). R_f = 0.25 ($CHCl_3$/MeOH 98:2). M.p. 107–108 °C (EtOAc/hexane). $[\alpha]_D$ = +7°. IR: *ν* 3431, 2951, 1743, 1711, 1510, 1331, 1144 cm^{-1}. ^1H NMR *δ*: 0.92 [6H, two superimposed d, $CH_2CH(CH_3)_2$], 1.25–1.77 [3H, three m, $CH_2CH(CH_3)_2$], 2.98 (2H, sharp m, CH_2SO_2), 3.08 and 3.16 (2H, A and B of an ABX, *J* = 4.2, 5.2 and 10.5 Hz, Phe β-CH_2), 3.75 (s, 3H, $COOCH_3$), 4.28 (1H, m, $CHCH_2SO_2$), 4.45 (1H, m, Phe α-CH), 4.90 (1H, d, *J* = 6.9 Hz, Leu-ψ[CH_2SO_2] NH), 5.13 (2H, m, $PhCH_2O$), 5.61 (1H, d, *J* = 6.3 Hz, Phe NH), 7.18–7.40 (5H, m, aromatics).

6.1.10. Cbz-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe

The above described Cbz-Nle-ψ[CH_2SO_2]-Leu-OMe (0.76 g, 1.71 mmol) was C-deprotected according to procedure C. The residue was coupled with HCl.Phe-OMe (0.37 g, 1.71 mmol) according to general procedure E. White solid (0.818 g, 81%). R_f = 0.55 ($CHCl_3$/MeOH 95:5). M.p. 146–148 °C (EtOAc/hexane). $[\alpha]_D$ = +17°. IR: *ν* 3429, 2959, 1740, 1707, 1516, 1330, 1234, 1140 cm^{-1}. ^1H NMR *δ*: 0.82–0.98 [9H, m, $CH_2CH(CH_3)_2$ and CH_2CH_3], 1.23–1.80 [9H, m, $CH_2CH(CH_3)_2$ and $CH_2CH_2CH_2CH_3$], 2.86–3.21 (4H, m, Phe β-CH_2 and CH_2SO_2), 3.74 (3H, s, $COOCH_3$), 3.97 (1H, m, Leu α-CH), 4.27 (1H, m, $CHCH_2SO_2$), 4.87 (1H, m, Phe α-CH), 5.11 (2H, s, $PhCH_2O$), 5.69 (1H, d, *J* = 6.3 Hz, Nle-ψ(CH_2SO_2) NH], 6.65 (1H, d, *J* = 6.1 Hz, Leu NH), 7.14–7.38 (6H, m, aromatics and Phe NH).

*6.1.11. Boc-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe (**1a**)*

Cbz-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe (0.575 g, 0.97 mmol) underwent to N-deprotection according to general procedure B-2. The trifluoroacetate salt (white solid) was *N*-Boc-protected according to general procedure D. Silica gel flash chromatography gave the pure product as a colourless oil (0.367 g, 68%). R_f = 0.78 ($CHCl_3$/MeOH 95:5). $[\alpha]_D$ = +14°. IR: *ν* 3435, 2960, 1741, 1686, 1509, 1368, 1234, 1166 cm^{-1}. ^1H NMR *δ*: 0.90–0.96 [9H, m, $CH_2CH(CH_3)_2$ and CH_2CH_3], 1.20–1.83 [9H, three m, $CH_2CH(CH_3)_2$ and $CH_2CH_2CH_2CH_3$], 1.44 [9H, s, $C(CH_3)_3$], 2.94 (2H, d, *J* = 5.8 Hz, CH_2SO_2), 3.08 and 3.19 (2H, A and B of an ABX, *J* = 5.1, 7.1 and 13.7 Hz, Phe β-CH_2) 3.75 (3H, s, $COOCH_3$), 3.99 (1H, m, Leu α-CH), 4.23 (1H, m, $CHCH_2SO_2$), 4.70 (1H, d, *J* = 9.5 Hz, Nle-ψ[CH_2SO_2] NH), 4.89 (1H, m, Phe α-CH), 5.80 (1H, d, *J* = 6.6 Hz, Leu NH), 6.57 (1H, d, *J* = 7.4 Hz, Phe NH), 7.16–7.35 (5H, m, aromatics).

*6.1.12. HCO-Nle-ψ[CH_2SO_2]-Leu-Phe-OMe (**1b**)*

From **1a** (0.25 g, 0.45 mmol) according to general procedure G. White solid (0.178 g, 82%). R_f = 0.48 ($CHCl_3$/MeOH 95:5). M.p. 112–115 °C (triturated with hexane). $[\alpha]_D$ = +17°. IR: *ν* 3423, 2959, 1742, 1682, 1513, 1362, 1143 cm^{-1}. ^1H NMR *δ*: 0.86–0.96 [9H, m, $CH_2CH(CH_3)_2$ and CH_2CH_3], 1.20–1.84 [9H, three m, $CH_2CH(CH_3)_2$ and $CH_2CH_2CH_2CH_3$], 2.97–3.25 (4H, m, Phe β-CH_2 and CH_2SO_2), 3.76 (3H, s, $COOCH_3$), 3.98 (1H, br, Leu α-CH),

4.61 (1H, m, C*H*CH_2SO_2), 4.89 (1H, m, Phe α-CH), 5.68 (1H, br, Leu NH), 6.03 (1H, d, *J* = 6.9 Hz, Nle-ψ[CH_2SO_2] NH), 6.56 (1H, d, *J* = 6.2 Hz, Phe NH), 7.16–7.36 (5H, m, aromatics), 8.18 (1H, s, HCO).

*6.1.13. Boc-Met-Leu-ψ[CH_2SO_2]-Phe-OMe (**2a**)*

Cbz-Leu-ψ[CH_2SO_2]-Phe-OMe (0.78 g, 1.63 mmol) was N-deprotected according to general procedure B-2. The trifluoroacetate salt (foam) was coupled with Boc-Met-OH (0.691 g, 1.63 mmol) according to general procedure E. Silica gel flash chromatography ($CHCl_3$/MeOH 95:5) gave the pure title compound. White foam (0.81 g, 86%). R_f = 0.16 ($CHCl_3$/MeOH 98:2). $[\alpha]_D$ = –9°. IR: *ν* 3430, 2918, 1743, 1692, 1533, 1332, 1155 cm^{-1}. ^1H NMR *δ*: 0.90 [6H, apparent t, $CH_2CH(CH_3)_2$], 1.27–1.62 [3H, two m, $CH_2CH(CH_3)_2$], 1.46 [9H, s, $C(CH_3)_3$], 1.85–2.22 (2H, two m, Met β-CH_2), 2.13 (3H, s, S–CH_3), 2.60 (2H, t, *J* = 5.4 Hz, Met γ-CH_2), 2.99 (2H, m, CH_2SO_2), 3.08 and 3.17 (2H, A and B of an ABX, *J* = 4.4, 5.4 and 10.4 Hz, Phe β-CH_2) 3.75 (3H, s, $COOCH_3$), 4.20 (1H, m, Met α-CH), 4.45 (1H, m, Phe α-CH), 4.53 (1H, m, C*H*CH_2SO_2), 5.10 (1H, d, *J* = 8.4 Hz, Met NH), 5.96 (1H, d, *J* = 9.3 Hz, Phe NH), 6.42 (1H, d, *J* = 8.8 Hz, Leu-ψ[CH_2SO_2] NH), 7.22–7.36 (5H, m, aromatics).

*6.1.14. HCO-Met-Leu-ψ[CH_2SO_2]-Phe-OMe (**2b**)*

From **2a** (0.438 g, 0.76 mmol) according to general procedure G. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure product. Pale yellow oil (0.322 g, 84%). R_f = 0.52 ($CHCl_3$/MeOH 98:2). $[\alpha]_D$ = –35°. IR: *ν* 3411, 2959, 1744, 1666, 1557, 1338, 1261, 1151, cm^{-1}. ^1H NMR *δ*: 0.91 [6H, two superimposed d, $CH_2CH(CH_3)_2$], 1.25–1.62 [3H, two m, $CH_2CH(CH_3)_2$], 1.92–2.25 (2H, two m, Met β-CH_2), 2.13 (3H, s, S–CH_3), 2.63 (2H, t, *J* = 5.3 Hz, Met γ-CH_2), 2.98 (2H, m, CH_2SO_2), 3.09 and 3.18 (2H, A and B of an ABX, *J* = 4.2, 5.5 and 10.4 Hz, Phe β-CH_2) 3.76 (3H, s, $COOCH_3$), 4.44 (1H, m, Phe α-CH), 4.49 (1H, m, C*H*CH_2SO_2), 4.64 (1H, m, Met α-CH), 5.85 (1H, d, *J* = 6.7 Hz, Phe NH), 6.41 (2H, m, Met NH and Leu-ψ[CH_2SO_2] NH), 7.21–7.37 (5H, m, aromatics), 8.22 (1H, s, HCO).

6.1.15. Cbz-Tau-Leu-Phe-OMe

Cbz-Tau-Leu-OMe [2] (1.03 g, 2.45 mmol) was C-deprotected according to procedure C. The residue was coupled with HCl.Phe-OMe (0.603 g, 2.8 mmol) according to the general procedure F. White foam (1.31 g, 88%). $[\alpha]_D$ = –22°. IR: *ν* 3434, 2960, 1720, 1689, 1514, 1352, 1146 cm^{-1}. ^1H NMR *δ*: 0.94 [6H, m, $CH_2CH(CH_3)_2$], 1.47 [2H, m, $CH_2CH(CH_3)_2$], 1.76 [1 H, m, $CH_2CH(CH_3)_2$], 2.83–3.03 (2 H, Tau α-CH_2), 3.05 and 3.22 (2 H, A and B of an ABX, *J* = 5.2, 8.8, and 14.0 Hz, Phe β-CH_2), 3.57 (2 H, m, Tau β-CH_2), 3.77 (3 H, s, $COOCH_3$), 3.90 (1 H, m, Leu α-CH), 4.90 (1 H, m, Phe α-CH), 5.11 (3H, m, PhCH_2O and Leu NH), 5.52 (1H, t, *J* = 6.0 Hz, Tau NH), 6.40 (1H, d, *J* = 8.2 Hz, Phe NH), 7.13–7.38 (10H, m, aromatics).

*6.1.16. Boc-Tau-Leu-Phe-OMe (**4a**)*

Cbz-Tau-Leu-Phe-OMe (1.31 g, 2.4 mmol) underwent to N-deprotection according to general procedure B-2. The trifluoroacetate salt (white solid) was *N*-Boc protected according to general procedure D. Silica gel flash chromatography ($CHCl_3$) gave the pure title compound. White crystals (0.225 g, 47%). $[\alpha]_D$ = +18°. M.p. 98–99 °C (hexane). IR: *ν* 3427, 2959, 1741, 1707, 1509, 1368, 1148 cm^{-1}. ^1H NMR *δ*: 0.94 [6H, m, $CH_2CH(CH_3)_2$], 1.46 [9H, s, $C(CH_3)_3$], 1.50 [2H, m, $CH_2CH(CH_3)_2$], 1.77 [1H, m, $CH_2CH(CH_3)_2$], 2.96 (2H, m, Tau α-CH_2), 3.08 and 3.22 (2H, A and B of an ABX, *J* = 5.4, 8.6, and 14.0 Hz, Phe β-CH_2), 3.52 (2H, m, Tau β-CH_2), 3.77 (3H, s, $COOCH_3$), 3.92 (1H, m, Leu α-CH), 4.90 (1H, m, Phe α-CH), 5.18 (2H, m, Leu NH and Tau NH), 6.46 (1H, d, *J* = 8.0 Hz, Phe NH), 7.15–7.35 (5H, m, aromatics).

*6.1.17. HCO-Tau-Leu-Phe-OMe (**4b**)*

From **4a** (0.125 g, 0.25 mmol) according to the general procedure G. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure title product. White solid (0.080 g, 78%). $[\alpha]_D$ = +34°. M.p. 119–120 °C (EtOAc/hexane). IR: *ν* 3418, 3031, 2958, 1741, 1685, 1517, 1438, 1333, 1236,1141, cm^{-1}. ^1H NMR *δ*: 0.94 [6H, m, $CH_2CH(CH_3)_2$], 1.51 [2H, m, $CH_2CH(CH_3)_2$], 1.77 [1H, m, $CH_2CH(CH_3)_2$], 2.90 and 2.98 (2H, two m, Tau α-CH_2), 3.06 and 3.22 (2H, A and B of an ABX, *J* = 4.9, 9.1 and 14.0 Hz, Phe β-CH_2), 3.66 (2H, m, Tau β-CH_2), 3.77 (3H, s, $COOCH_3$), 3.93 (1H, m, Leu α-CH), 4.90 (1H, m, Phe α-CH), 5.48 (1H, d, *J* = 7.3 Hz, Leu NH), 6.62 (1H, d, *J* = 7.2 Hz, Phe NH), 6.73 (1H, br, Tau NH), 7.16–7.35 (m, 5H, aromatics), 8.14 (1H, s, HCO).

*6.1.18. Boc-Tau-Leu-Phe-Phe-OMe (**5a**)*

From **4a** (0.315 g, 0.63 mmol), which was C-deprotected according to general procedure C. The free acid (white solid) was coupled with HCl.Phe-OMe (0.136 g, 0.63 mmol) according to general procedure F. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure product. White solid (0.28 g, 65%). $[\alpha]_D$ = –10°. M.p. 163–164 °C (EtOAc/hexane). IR: *ν* 3413, 2934, 1743, 1673, 1509, 1368, 1157 cm^{-1}. ^1H NMR *δ*: 0.91 [6H, m, $CH_2CH(CH_3)_2$], 1.44 [9H, s, $C(CH_3)_3$], 1.45–1.80 [3H, m, $CH_2CH(CH_3)_2$], 2.93–3.11 (6H, two m, Tau α-CH_2 and two Phe β-CH_2), 3.51 (2H, m, Tau β-CH_2), 3.70 (3H, s, $COOCH_3$), 3.85 (1H, m, Leu α-CH), 4.64 and 4.75 (2H, two m, two Phe α-CH), 5.18 (1H, m, Tau NH), 6.26 (1H, d, *J* = 8,0 Hz, Phe NH), 6.56 (1H, d, *J* = 7.9 Hz, Leu NH), 7.02 (1H, d, *J* = 7.7 Hz, Phe NH), 7.20–7.31 (10H, m, aromatics).

*6.1.19. HCO-Tau-Leu-Phe-Phe-OMe (**5b**)*

From **5a** (0.216 g, 0.27 mmol) according to general procedure G. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure product. White solid (0.133 g; 81%). $[\alpha]_D$ = –8°. M.p. 164–165 °C (chloroform/hexane). IR: *ν*

3412, 3023,1745, 1685, 1510, 1333, 1142 cm^{-1}. ^1H NMR δ: 0.91 [6H, m, $CH_2CH(CH_3)_2$], 1.46 [2H, m, $CH_2CH(CH_3)_2$], 1.72 (1H, m, $CH_2CH(CH_3)_2$), 2.90–3.11 (6H, two m, Tau α-CH_2 and two Phe β-CH_2), 3.63 (2H, m, Tau β-CH_2), 3.70 (3H, s, $COOCH_3$), 3.93 (1H, m, Leu α-CH), 4.75 (2H, m, two Phe α-CH), 5.69 (1H d, J = 8.1 Hz, Leu NH), 6.66 (1H, d, J = 7.6 Hz, Phe NH), 6.75 (1H, br, Tau NH), 6.98 (1H, d, J = 7.9 Hz, Phe NH), 7.19–7.29 (10H, m, aromatics), 8.09 (1H, s, HCO).

*6.1.20. Boc-Tau-Phe-OMe (**3a**)*

From HBr.Tau-Phe-OMe (0.360 g, 1.0 mmol) according to general procedure D. Silica gel flash chromatography (CH_2Cl_2/MeOH 98:2) gave the pure product. White foam (0.260 g, 67%). $[\alpha]_D$ = –20° (2%, $CHCl_3$). IR: ν 3366, 3018, 1740, 1709, 1508, 1340, 1150 cm^{-1}. ^1H NMR δ: 1.46 [9H, s, $C(CH_3)_3$], 2.87 (2H, m, Tau α-CH_2), 3.04 and 3.18 (2H, A and B of an ABX, J = 5.1, 7.5 and 13.8 Hz, Phe β-CH_2), 3.42 (2H, m, Tau β-CH_2), 3.80 (3H, s, $COOCH_3$), 4.42 (1H, m, Phe α-CH), 5.08 (2H, m, Tau and Phe NH), 7.19–7.36 (5H, m, aromatics).

*6.1.21. HCO-Tau-Phe-OMe (**3b**)*

From **3a** (0.120 g, 0.31 mmol) according to general procedure G. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure title compound. Pale yellow foam (0.080 g; 83%). $[\alpha]_D$ = –25°. IR: ν 3367, 3034, 1743, 1686, 1513, 1339, 1144 cm^{-1}. ^1H NMR δ: 2.87 (2H, m, Tau α-CH_2), 3.01 and 3.19 (2H, A and B of an ABX, J = 4.9, 8.0, and 13.8 Hz, Phe β-CH_2), 3.56 (2H, m, Tau β-CH_2), 3.82 (3H, s, $COOCH_3$), 4.39 (1H, m, Phe α-CH), 5.28 (1H, d, J = 9.1 Hz, Phe NH), 6.37 (1H, br, Tau NH), 7.20–7.38 (5H, m, aromatics), 8.09 (1H, s, HCO).

*6.1.22. Boc-Met-Leu-Tau-Phe-OMe (**7a**)*

From Boc-Met-Leu-OH (0.218 g, 0.57 mmol) and HBr.Tau-Phe-OMe (0.228 g, 0.57 mmol) according to general procedure E. White solid (0.228 g; 64%). $[\alpha]_D$ = –25°. M.p. 126–127 °C (hexane). IR: ν 3420, 2960, 1715, 1673, 1498, 1339, 1219, 1146, 1144 cm^{-1}. ^1H NMR δ: 0.93 and 0.96 [6H, two d, J = 6.3 Hz, $CH_2CH(CH_3)_2$], 1.46 [9H, s, $C(CH_3)_3$], 1.69–1.75 [3H, m, $CH_2CH(CH_3)_2$], 1.90–2.13 (2H, m, Met β-CH_2), 2.12 (3H, s, S–CH_3), 2.59 (2H, t, J = 7.1 Hz, Met γ-CH_2), 2.94 (2H, m, Tau α-CH_2), 3.07 and 3.21 (2H, A and B of an ABX, J = 5.1, 8.8 and 14.0 Hz, Phe β-CH_2), 3.45 and 3.68 (2H, two m, Tau β-CH_2), 3.79 (3H, s, $COOCH_3$), 4.23 (1H, m, Met α-CH), 4.33 (1H, m, Leu α-CH), 4.47 (1H, m, Phe α-CH), 5.20 (1H, d, J = 7.2 Hz, Met NH), 5.83 (1H, d, J = 8.9 Hz, Phe NH), 6.69 (1H, d, J = 8.9 Hz, Leu NH), 7.24–7.34 (5H, m, aromatics).

*6.1.23. HCO-Met-Leu-Tau-Phe-OMe (**7b**)*

From **7a** (0.150 g, 0.22 mmol) according to the general procedure G. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure title product. White solid. (0.102 g; 81%). $[\alpha]_D$ = –35° (1%, DMF). M.p. 164–165 °C (hexane). IR (KBr): ν 3288, 2965, 1733, 1638, 1550, 1449, 1339 cm^{-1}. ^1H NMR (d_6-DMSO) δ: 0.83 and 0.88 [6H, two d, J = 6.5 Hz, $CH_2CH(CH_3)_2$], 1.42–1.60 [3H, m, $CH_2CH(CH_3)_2$], 1.75–1.95 (2H, m, Met β-CH_2), 2.03 (3H, s, S–CH_3), 2.43 (2H, t, J = 7.2 Hz, Met γ-CH_2), 2.81–3.06 (4H, m, Phe β-CH_2 and Tau α-CH_2), 3.20 (2H, m, Tau β-CH_2), 3.63 (3H, s, $COOCH_3$), 4.15 (1H, m, Phe α-CH), 4.22 (1H, m, Leu α-CH), 4.41 (1H, m, Met α-CH), 7.20–7.31 (5H, m, aromatics), 7.90 (1H, poorly resolved t, Tau NH), 8.00–8.07 [3H, m, Phe NH, Leu NH and HCO (s at 8.02)], 8.28 (1H, d, J = 8.1 Hz, Met NH).

*6.1.24. Boc-Met-Tau-Phe-Phe-OMe (**6a**)*

From Boc-Met-Tau-Phe-OMe [5] (0.231 g, 0.44 mmol) which underwent to C-deprotection according to procedure C. The residue was coupled with HCl.Phe-OMe (0.095 g, 0.44 mmol) according to general procedure F. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure title product as oil which solidified by treatment with hexane. White solid (0.335 g; 82%). $[\alpha]_D$ = +35° (2%, $CHCl_3$). M.p. 98–99 °C. IR: ν 1421, 2961, 1718, 1680, 1497, 1289, 1145 cm^{-1}. ^1H NMR δ: 1.46 [9H, s, $C(CH_3)_3$], 1.90–2.15 (2H, m, Met β-CH_2), 2.11 (3H, s, S–CH_3), 2.55 (2H, m, Met γ-CH_2), 2.72 (2H, m, Tau α-CH_2), 2.92–3.21 (4H, m, two Phe CH_2), 3.32 and 3.68 (2H, two m, Tau β-CH_2), 3.72 (3H, s, $COOCH_3$), 4.19 (2H, m, Met and Phe α-CH), 4.85 (1H, m, Phe α-CH), 5.32 (1H, d, J = 8.2 Hz, Met NH), 6.00 (1H, m, Phe NH), 6.55 (1H, d, J = 7.5 Hz, Phe NH), 6.90–7.34 (11H, m, aromatics and Tau NH).

*6.1.25. HCO-Met-Tau-Phe-Phe-OMe (**6b**)*

From **6a** (0.200 g, 0,30 mmol) according to the general procedure G. Silica gel flash chromatography ($CHCl_3$/MeOH 98:2) gave the pure title product. White solid (0.102 g, 57%). $[\alpha]_D$ = +26°. M.p. 168–169 °C (EtOAc/hexane). IR: ν 3412, 2955, 1742, 1673, 1521, 1335, 1145 cm^{-1}. ^1H NMR δ: 1.97–2.18 (2H, m, Met β-CH_2), 2.13 (3H, s, S–CH_3), 2.55 (2H, t, J = 7.1 Hz, Met γ-CH_2), 2.68–3.22 (6H, m, Tau α-CH_2 and two Phe β-CH_2), 3.30 and 3.60 (2H, two m, Tau β-CH_2), 3.73 (3H, s, $COOCH_3$), 4.20 (2H, m, Phe α-CH), 4.58 (1H, m, Met α-CH), 4.85 (1H, m, Phe α-CH), 5.96 (1H, d, J = 8.1 Hz, Phe NH), 6.79 (1H, d, J = 8.0 Hz, Met NH), 7.11 (1H, d, J = 8.1 Hz, Phe NH), 7.21–7.33 (11H, m, aromatics and Tau NH), 8.07 (1H, s, HCO).

6.2. Biological assays

6.2.1. Cell preparation

Cells were obtained from the blood of healthy subjects, and human peripheral blood neutrophils were purified by using the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Ficoll-Paque (Pharmacia), and hypotonic lysis of contaminating red

cells. Cells were washed twice and resuspended in Krebs–Ringer phosphate (KRPG), pH 7.4, at final concentration of 50×10^6 cells/ml and kept at room temperature until used. Neutrophils were 98–100% viable, as determined using the Trypan Blue exclusion test.

6.2.2. Random locomotion

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Milan, Italy) and migration into the filter was evaluated by the leading-front method [21]. The actual control random movement is 35 ± 3 μm SE of 10 separate experiments performed in duplicate.

6.2.3. Chemotaxis

Each peptide was added to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution with KRPG containing 1 mg/ml of bovine serum albumin (BSA; Orha Behringwerke, Germany) and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (CI), which is the ratio: (migration toward test attractant minus migration toward the buffer)/migration toward the buffer; the values are the mean of six separate experiments performed in duplicate. Standard errors are in the 0.02–0.09 CI range.

6.2.4. Superoxide anion (O_2^-) production

This anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sigma, USA) modified for microplate-based assays. Tests were carried out in a final volume of 200 ml containing 4×10^5 neutrophils, 100 nmoles cytochrome *c* and KRPG. At zero time different amounts (10^{-10}–8×10^{-5} M) of each peptide were added and the plates were incubated into a microplate reader (Ceres 900, Bio-TeK Instruments, Inc.) with the compartment temperature set at 37 °C. Absorbance was recorded at 550 and 468 nm. The difference in absorbance at the two wavelengths was used to calculate nmoles of O_2^- produced using an absorptivity for cytochrome *c* of 18.5 mM^{-1} cm^{-1}. Neutrophils were incubated with 5 μg/ml cytochalasin B (Sigma) for 5 min prior to activation by peptides. Results were expressed as net nmoles of O_2^- per 1×10^6 cells per 5 min and are the mean of six separate experiments performed in duplicate. Standard errors are in 0.1–4 nmoles O_2^- range.

6.2.5. Enzyme assay

The release of neutrophil granule enzymes was evaluated by determination of lysozyme activity, modified for microplate-based assays. Cells, 3×10^6/well, were first incubated in triplicate wells of microplates with 5 μg/ml cytochalasin B at 37 °C for 15 min and then in the presence of each peptide at a final concentration of 10^{-10}–8×10^{-5} M for a further 15 min. The plates were then centrifuged at $400 \times g$ for 5 min and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus*. The reaction rate was measured using a microplate reader at 465 nm. Enzyme release was expressed as a net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 mg per 1×10^7 cells/min. The values are the mean of five separate experiments done in duplicate. Standard errors are in the range 1–6%.

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