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Synthesis and activity of HCO–Met–Leu–Phe–OMe analogues containing β-alanine or taurine at the central position

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Abstract

New synthetic analogues of the chemotactic *N*-formyltripeptide HCO–Met–Leu–Phe–OMe have been synthesized. The reported new models, namely Boc–Met–β-Ala–Phe–OMe (**1**), HCO–Met–β-Ala–Phe–OMe (**2**), Boc–Met–Tau–Phe–OMe (**3**), HCO–Met–Tau–Phe–OMe (**4**) and HCl·Met–Tau–Phe–OMe (**5**), are characterized by the presence at the central position of a residue of β-alanine or 2-aminoethanesulfonic acid (taurine) replacing the native L-leucine. Whereas tripeptides **1** and **2** have been found quite inactive as chemoattractants, all the three models containing the Tau residue exhibit a remarkable activity. Superoxide anion production and lysozyme release have been also evaluated and the biological results are discussed together with the conformational preferences of the examined models.

Keywords: β-Alanine; Chemotactic peptides; Human neutrophils; Taurine

1. Introduction

In a previous article [1] we reported synthesis and activity of analogues of the chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine methyl ester (fMLF-OMe) characterized by the presence at the central position of achiral ω-amino acid residues $H_2N–(CH_2)_n–COOH$ replacing the central L-leucine. Models incorporating γ-aminobutyric, δ-aminovaleric and ε-aminocaproic acid residues showed good and selective antagonistic activity.

As a prosecution of these studies we started recently a research program aimed at defining synthesis and properties of fMLF-OMe analogues incorporating residues of achiral and chiral 3-aminopropanoic acids (β-amino acids) or of 2-aminoethanesulfonic acids. Here we report the first results concerning the most simple models incorporating at the central position the achiral residues of β-alanine (β-Ala) and of its sulfonic acid analogue taurine (Tau).

In recent years studies on oligopeptides incorporating β-Ala [2–8] or Tau [9–17] residues have received considerable attention in view of the tendency of these models to adopt preferential secondary structures as well as of the stability towards enzymatic degradation [18–21]. Furthermore, it is interesting to note here that taurine, in addition to a variety of biological functions [22,23], has well defined interactions with the activity of neutrophils, the phagocytic cells on which the fMLF-OMe receptors are located. Taurine is, in fact, a recognized modulator of the 'respiratory burst' of neutrophils and this property suggests that Tau derivatives and analogues may be advantageous for the development of antiinflammatory agents [24,25].

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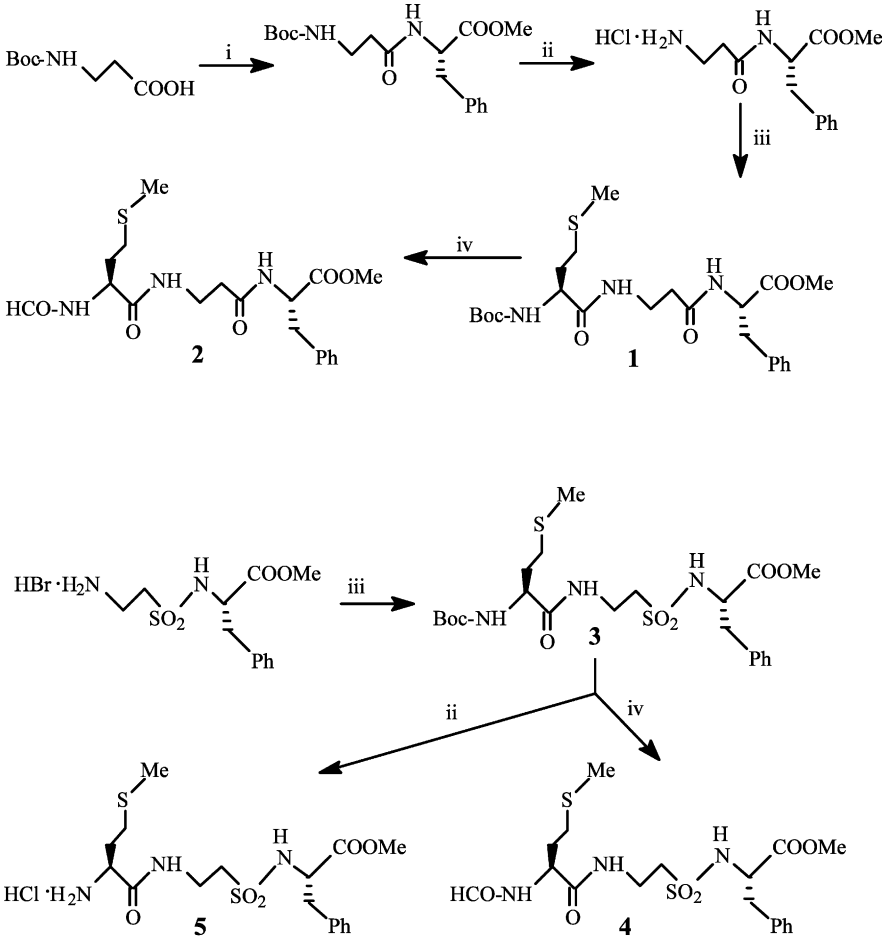
2. Chemistry

The synthesis of Boc–Met–β-Ala–Phe–OMe (**1**) and Boc–Met–Tau–Phe–OMe (**3**) and of the corresponding *N*-formyl analogues HCO–Met–β-Ala–Phe–OMe (**2**) and HCO–Met–Tau–Phe–OMe (**4**) has been performed according to the Scheme 1. Compounds **1** and **3** were prepared in solution by coupling Boc–Met–OH with HCl·β-Ala–Phe–OMe (see Scheme 1) or HBr·Tau–Phe–OMe [26] using isobutyl chloroformate for carboxyactivation (mixed anhydride method). Acidolysis of Boc–β-Ala–Phe–OMe and **3**, performed with thionyl chloride in methanol, afforded HCl·β-Ala–Phe–OMe and HCl·Met–Tau–Phe–OMe (**5**). A direct transformation of the *N*-Boc derivatives **1** and **3** into the corresponding *N*-formyl analogues **2** and **4** was performed by following the procedure of Lajoie and Kraus [27]. Thus, treatment of **1** and **3** with formic acid, followed by ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate (EEDQ), gave the formyl derivatives **2** and **4**.

3. Biological results

The agonistic activity of the new ligands has been determined on human neutrophils and compared with that of the standard tripeptide fMLF-OMe; directed migration (chemotaxis), superoxide anion production and lysozyme release have been measured.

While the tripeptides **1** and **2** containing the central β-Ala residue have been found quite inactive as chemoattractants, both the corresponding analogues **3** and **4**, in which the Tau residue replaces the β-Ala, exhibit a remarkable chemotactic activity, comparable to that shown by the reference tripeptide fMLF-OMe (Fig. 1). Furthermore, the Tau containing pseudotripeptide **5**, possessing a free N-terminal amino group, shows an activity which is intermediate between that observed in the case of the corresponding *N*-formyl derivative **4** and *N*-Boc protected model **3**. Concerning the superoxide anion production and the lysozyme release, all the examined compounds, regardless the nature of the



i: *i*-BuOCOCl, NMM, HCl·Phe-OMe; ii: $SOCl_2$, MeOH; iii: Boc-Met-OH, *i*-BuOCOCl, NMM; iv: HCOOH, EEDQ.

Scheme 1.

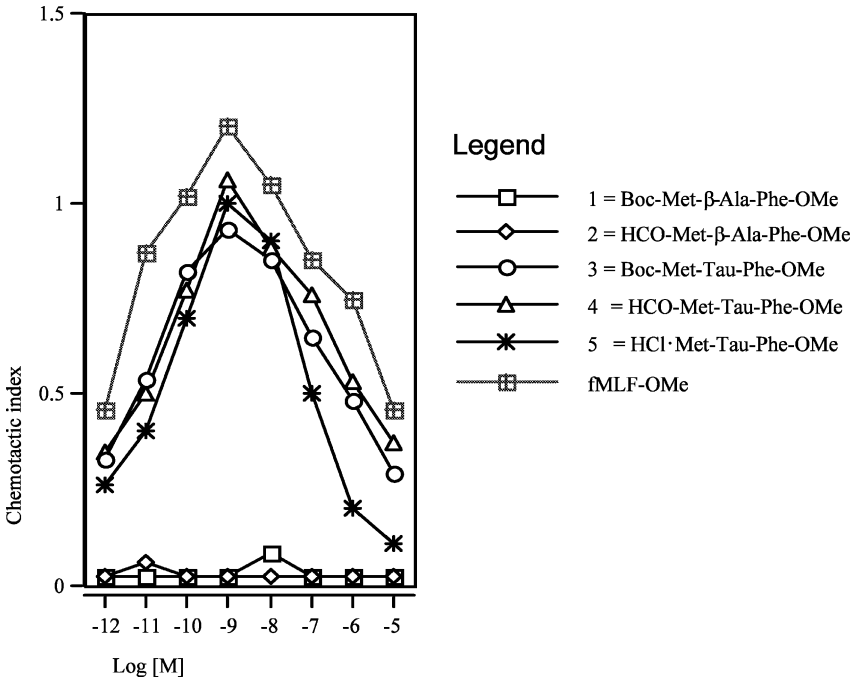


Fig. 1. Chemotactic activity of peptides **1**–**5**.

central residue and the substituent at the N-terminal amino group, are practically inactive, with the exception of weak activity as secretagogue agents observed at high concentration for the β-Ala containing *N*-formyl tripeptide **2** (data not shown).

The antagonistic activity was determined by measuring the ability of the new analogues to inhibit the above cited functions stimulated by an optimal dose of fMLF-OMe. In the case of the chemotaxis, the β-Ala containing derivatives **1** and **2**, which have been found inactive as chemoattractants, have been examined. The influence of increasing concentration of these two compounds on the chemotaxis induced by 10 nM fMLF-OMe is shown in Fig. 2A. A weak inhibition is observed for both the models and the Boc derivative **1** is, as expected, the most efficient antagonist.

The antagonism towards the superoxide anion production exhibited by compounds **1**, **2**, and **5** is reported in Fig. 2B; the *N*-deprotected derivative **5** exerts a statistically significant reduction ($P < 0.05$) on the activity induced by 1 μM fMLF-OMe starting from 10^{-8} M and the inhibition progressively increases with the concentration up to 44%; a weaker antagonist activity is exhibited by **1** and **2**. As shown in Fig. 2C, only a weak inhibitory action on the lysozyme release is observed for compounds **1**, **2**, and **5**; in this case, the formyl derivative **2** is the most efficient antagonist.

Receptor binding experiments have been carried out on derivatives **3** and **4**. Fig. 3 shows the [^{3}H]-fMLF displacements caused by increasing concentrations of the new analogues **3** and **4** and by the reference tripeptide fMLF-OMe. In Table 1 the chemotactic activity, superoxide anion production, lysozyme release, and binding analysis of the new analogues **3** and **4** are reported and compared with those exhibited by the parent fMLF-OMe. The order of potency in [^{3}H]-fMLF displacement assays for the test peptides is: fMLF-OMe > **3** > **4**. Thus, fMLF-OMe is the most potent peptide, with its affinity in the nanomolar range (60 nM), while **3** and **4** display affinities in the micromolar range (15 000 and 20 000 nM, respectively).

The above reported results clearly show that the replacement, in the molecule of protypical ligand fMLF-OMe, of the central leucine with a residue of β-Ala affords the tripeptide **2** which is devoid of activity. On the contrary, an analogous structural alteration of the fMLF-OMe molecule, performed by introducing a Tau residue in place of the native Leu, leads to the *N*-formyl pseudotripeptide **4** which exhibits a chemotactic activity comparable to that of the fMLF-OMe. Furthermore, models **3** and **5**, which contain a bulky *N*-acylating group and a free N-terminal amino group, respectively, are both chemoattractants with activities only slightly lower than those shown by fMLF-OMe and by the corresponding *N*-formyl derivative **4** (see Fig. 1). These latter findings are quite unexpected since the *N*-deacylation of chemotactic *N*-formyltripeptides affords inactive derivatives [28] and the introduction of the bulky *t*-butyloxycarbonyl substituent at the Met NH is associated with antagonistic activity [28,29].

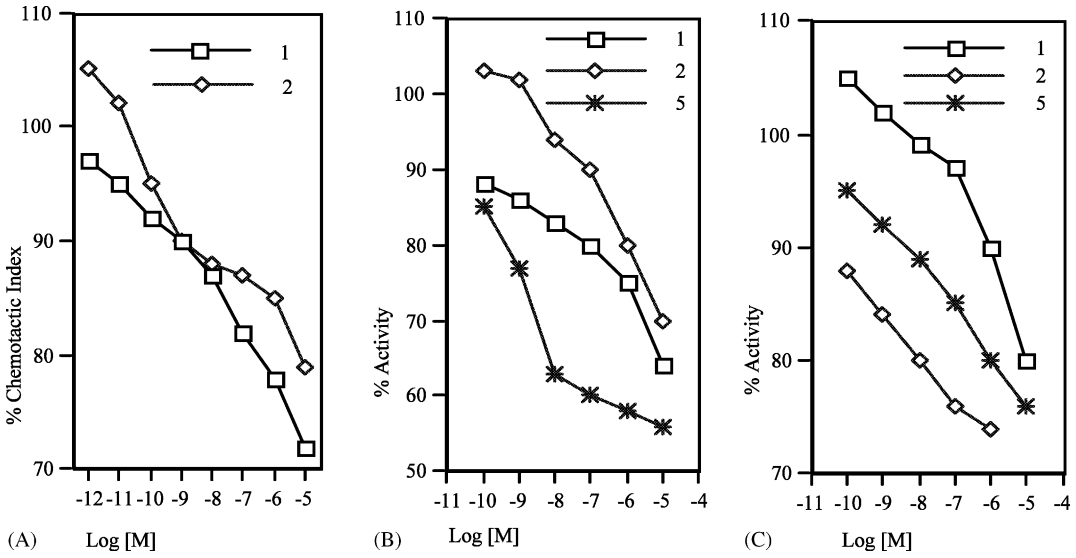


Fig. 2. Antagonistic effect of β-Ala derivatives **1** and **2** and Tau-containing hydrochloride **5** on chemotaxis activated by 10 nM fMLF-OMe (A), on superoxide anion production (B) and on release of granule enzymes (C) triggered by 1 μM fMLF-OMe.

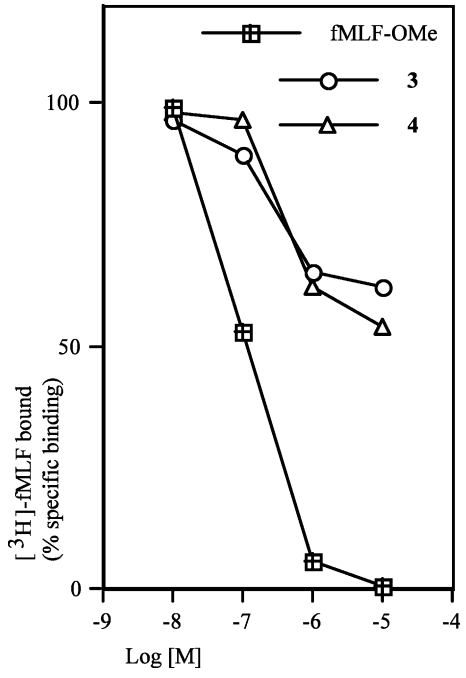


Fig. 3. Competition curves of specific [^{3}H]-fMLF binding to human neutrophils by the test compounds. Curves are representative of a single experiment taken from a series of three independent experiments. Non specific binding was determined in the presence of 100 μM fMLF.

4. Conformational studies

In order to investigate the role of the backbone conformation on the remarkable biological activity exhibited by pseudotripeptides **3** and **4**, containing the Tau central residue, as compared to the inactivity observed in the case of the corresponding peptides **1** and **2**, incorporating the β-Ala, an ^{1}H NMR study was undertaken to ascertain the involvement of the NH groups in intramolecular H-bonds. In Fig. 4 is reported the chemical shift dependence of the NH resonances as a function of DMSO-d_6 concentration in $CDCl_3$ solution (10 mM).

The results of these titration experiments clearly show that in all the four models **1**–**4** the NH of the central residue (i.e. β-Ala and Tau) presents a pronounced solvent inaccessibility and in particular, in the case of the *N*-Boc protected model **1**, the β-Ala NH is practically unaffected by the increase of the DMSO-d_6 concentration ($\Delta\delta = 0.05$ ppm). On the contrary, the NH groups of the external Met and Phe residues of **1**–**4**, with the exception of the two Boc–NH groups of **1** and **3**, interact efficiently with the solvent and this is particularly evident in the case of the Phe residue of the Tau containing tripeptides **3** and **4** ($\Delta\delta$ values of 1.42 and 1.40 ppm, respectively). As far as the rather low solvent accessibility of the two Boc–NH groups of **1** and **3** is concerned, this is only in part attributable to the participation to intramolecular H-bondings; in fact, as previously observed [30], the bulky *t*-butyloxycarbonyl group significantly hinders the interaction of the NH with the solvent. In accordance with this effect and as can be seen in Table 2, the Met formamido NH groups of **2** and **4** exhibit $\Delta\delta$ values (0.88 and 0.91 ppm, respectively) higher than those of the corresponding Boc protected NH groups (0.41 and 0.46 ppm, respectively).

The presence in the examined tripeptides of an intramolecularly H-bonded NH group is also revealed by the examination of the IR spectra in the NH stretching region. In the spectrum of **1** two bands are observed at 3348 and 3430 cm^{-1}, corresponding to the H-bonded and free NH groups, respectively. The ratio between the intensity of the two absorptions was found

Table 1
Chemotactic activity, superoxide anion production, lysozyme release, and binding analysis of fMLF-OMe, **3**, and **4**

Peptide	Chemotaxis C.I.	Superoxide production O_2^- nmole	% Lysozyme release	Receptor binding IC_{50} (nM)
fMLF-OMe	1.20±0.08 (10^{-9} M)	52±3 (10^{-6} M)	52±3 (10^{-6} M)	60±4
3	0.93±0.07 (10^{-9} M)	2.2±0.1 (10^{-5} M)	10±0.5 (10^{-5} M)	15 000±150
4	1.06±0.08 (10^{-9} M)	10.2±0.3 (10^{-5}M)	14±0.6 (10^{-5}M)	20 000±130

Efficacy data of chemotaxis are expressed as chemotactic index (C.I.), superoxide anion production is expressed as net nanomole of $O_2^-/1 \times 10^6$ cells/5 min, and lysozyme release is expressed in% (±SEM). In parentheses the concentration of the maximum agonistic activity. All data are represented by the mean of six independent experiments performed in duplicate. IC_{50} values represent the mean±SEM of three independent determinations performed in duplicate.

practically concentration independent over the range 10.0–1.0 mM. Thus, peptide self-association occurs, if any, at limited extent under these experimental conditions and the band at 3348 cm^{-1} can be assigned to the intramolecularly H-bonded β-Ala NH group. The IR spectrum of **3** shows a very close outcome in the NH stretching region: two bands are observed at 3346 and 3433 cm^{-1}, and the band at lower frequency can be assigned to an intramolecularly bonded Tau NH.

5. Discussion and conclusion

Although the above reported conformational studies can be interpreted in terms of different foldings, they suggest the presence of a significant population of the tripeptide molecules in a conformation extended at the N- and C-terminal residues and the involvement of the central Tau and β-Ala NH groups in an intraresidue H-bond (C_6 conformation); the $J_{NH-C^{\alpha}H}$ values observed for Met and Phe residues, as reported in Table 2, are in accordance with this hypothesis. In particular, the higher $J_{NH-C^{\alpha}H}$ values found for the C-terminal Phe residue of the pseudopeptides **3** and **4** as compared with **1** and **2**, indicate that the mixture of conformers which populate the $CHCl_3$ solution should be, in this case, more rich of structures adopting an extended C-terminal portion. However, the participation of the central NH to H-bond interaction with the N-terminal carbonyl group (C_7 conformation) cannot be ruled out.

By taking into account the above discussed common conformational features shown by the β-Ala and the Tau containing models, this difference does not seem prevalent to explain the different chemotactic responses. Thus, although more β-sulfonamido pseudopeptides should be examined to clarify the picture, the different activity exhibited by the two *N*-formyltripeptides **2** and **4**, as well as the unexpected activity of **3** and **5**, seem strictly connected with the intrinsic structural and chemical properties of the SO_2–NH as compared with the usual CO–NH, with particular reference to the tetrahedral hybridization of the sulfur atom, the high polar character and the acid–base property of the sulfonamide junction [14–16,31].

Concerning the above reported binding studies, the results are in accordance with the weak activity in relation to O_2^- production and lysosomal enzyme release but are not consistent with the remarkable and selective chemotactic activity exhibited by pseudopeptides **3** and **4**. However, an analogous behaviour has already been observed in the case of fMLF-OMe analogues containing unnatural residues such as 4-amino-tetrahydrothiopyran-4-carboxylic acid (Thp). In these cases, full agonists such as fMLF-OMe, are effective in displacing the labelled peptide from its binding sites, while selective chemoattractants, as [Thp^1]fMLF-OMe or **3** and **4**, are much less efficacious [32]. This different behaviour can be rationalized on the basis of the existence of at least two different functional receptor subtypes or isoforms [33–35]; low doses of a full agonist (or a 'pure' chemoattractant) are required to interact with a high-affinity receptor subtype that activates transduction pathway responsible for chemotactic response, while the increase of the full agonist concentration allows binding with the low-affinity subtype, able to activate the trasduction pathways responsible for O_2^- production and lysozyme release.

As a consequence a peptide selective for chemotaxis, which preferentially interacts with the high-affinity subtype is not efficient in effectively displacing the full agonist [^{3}H]-fMLF, whose dose is sufficiently high to interact also with the low-affinity receptor subtype.

6. Experimental

6.1. Chemistry

Melting points were determined with a Büchi oil bath 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 $CHCl_3$). IR spectra (KBr

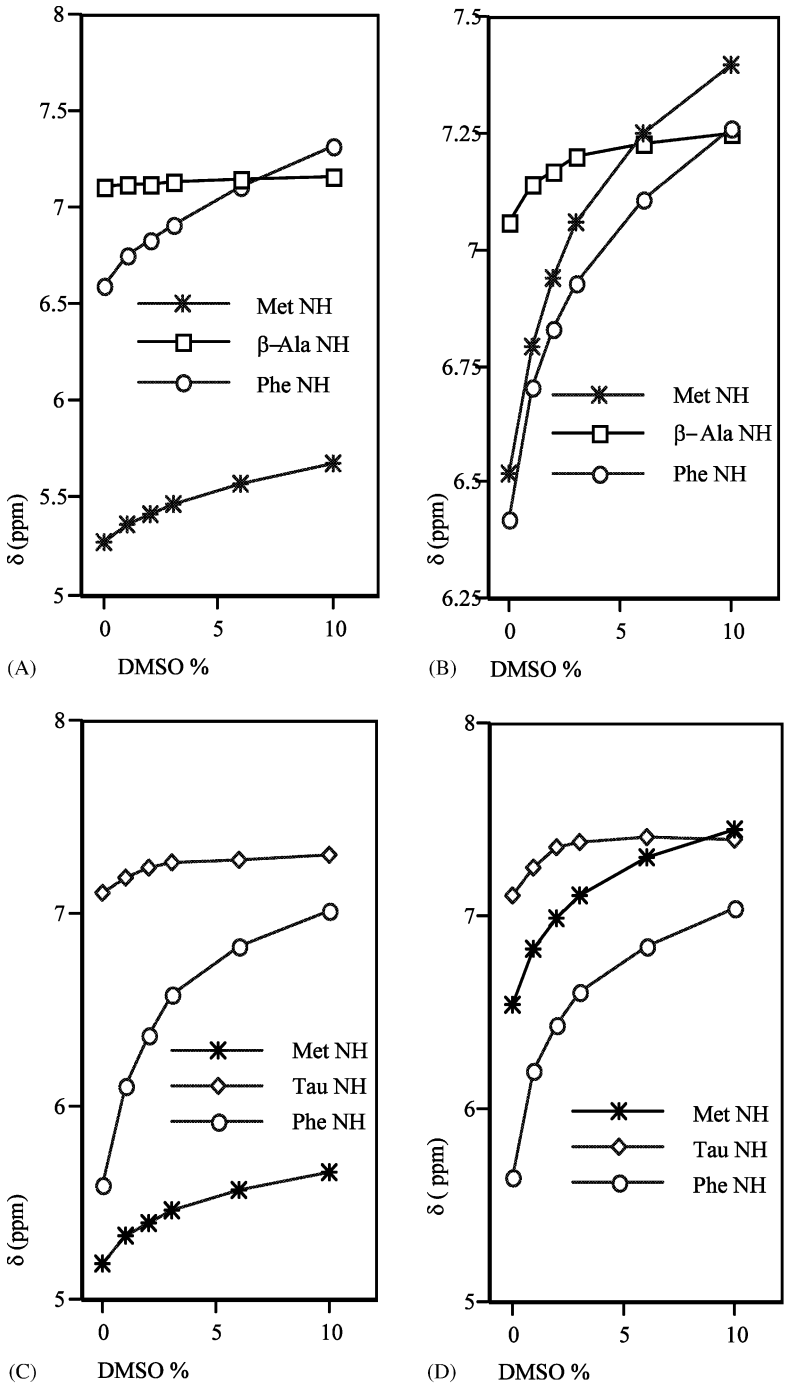


Fig. 4. Delineation of hydrogen-bonded NH groups in tripeptides **1**–**4**. Chemical shift dependence of the NH resonances as a function of the DMSO-d_6 concentration (% v/v) in $CDCl_3$ solution. Peptide concentration 10 mM. (A) Boc–Met–β-Ala–Phe–OMe (**1**), (B) HCO–Met–β-Ala–Phe–OMe (**2**), (C) Boc–Met–Tau–Phe–OMe (**3**), (D) HCO–Met–Tau–Phe–OMe (**4**).

disks) were recorded employing a Perkin–Elmer FT-IR Spectrum 1000 spectrometer. ^{1}H NMR spectra were determined in $CDCl_3$ solution (unless otherwise specified) with a Bruker AM 200 spectrometer using Me_4Si as internal standard. Thin-layer and preparative layer chromatographies were performed on silica gel Merck 60 F_{254} plates. The drying agent was sodium sulfate. Parent fMLF-OMe and HBr·Tau–Phe–OMe were prepared as described in the literature [36,26]. Boc–Met–OH (Fluka Chemie, AG, Switzerland), Boc–β-Ala–OH (Fluka Chemie), HCl·Phe–OMe (Fluka Chemie), and fMLF-OH (Sigma, USA) were employed without purification. 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 abbrevia-

Table 2
Solvent accessibility of peptide NH groups: differences ($\Delta\delta$, ppm) between NH chemical shift values observed in $CDCl_3$ solution containing $(CD_3)_2SO$ (10%) and in neat $CDCl_3$; see also Fig. 4

Compound	Met NH	β-Ala NH	Phe NH
1	0.41 (7.3)	0.05	0.72 (7.3)
2	0.88 (7.4)	0.19	0.84 (7.8)
	Tau NH		
3	0.46 (7.9)	0.20	1.42 (8.8)
4	0.91 (8.1)	0.29	1.40 (9.1)

In parentheses the $J_{NH-C^{\alpha}H}$ values (Hz) for Met and Phe residues.

tions used are as follows: β-Ala, 3-aminopropanoic acid; Boc, *tert*-butyloxycarbonyl; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EEDQ, ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate; KRPG, Krebs-Ringer-phosphate containing 0.1% w/v D-glucose; NMM, *N*-methylmorpholine.

6.1.1. Boc-β-Ala–Phe–OMe

Isobutyl chloroformate 98% (0.13 ml, 1 mmol) was added at −15 °C to a stirred solution of Boc–β-Ala–OH (0.189 g, 1 mmol) and NMM (0.13 ml, 1.2 mmol) in dry CH_2Cl_2 (5 ml). The temperature was maintained at −15 °C for 10 min, and HCl·Phe–OMe (0.216 g, 1 mmol), NMM (0.11 ml, 1 mmol) and dry CH_2Cl_2 (3.6 ml) were then added. The mixture was stirred at −15 °C for 15 min and then allowed to warm to room temperature. Dry DMF (20 drops) was added and stirring was continued for 1 day. Ethyl acetate was added in excess and the organic layer washed with 2 N HCl, brine, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried and evaporated under reduced pressure to give pure oily title compound (0.35 g, 100%), which slowly crystallized at room temperature. M.p. 91–92 °C. $[\alpha]_D = +51°$. IR: 3369, 3317, 2984, 1740, 1683, 1650, 1524 cm^{-1}. ^1H NMR δ (ppm): [1.43 9H, s, $C(CH_3)_3$], 2.37 (2H, t, $J = 6$ Hz, β-Ala α-CH_2), 3.07 and 3.15 (2H, A and B of an ABX, $J = 6.3$, 5.7 and 13.8 Hz, Phe β-CH_2), 3.36 (2H, m, β-Ala β-CH_2), 3.73 (3H, s, $COOCH_3$), 4.87 (1H, m, Phe α-CH), 5.10 (1H, br, β-Ala NH), 6.11 (1H, d, $J = 7.3$ Hz, Phe NH), 7.05–7.33 (5H, m, aromatic). *Anal*. (C, H, N) for $C_{18}H_{26}N_2O_5$.

6.1.2. Boc–Met–β-Ala–Phe–OMe (1)

Thionyl chloride (0.076 ml, 1.05 mmol) was added to a solution of Boc–β-Ala–Phe–OMe (0.35 g, 1 mmol) in dry methanol (1 ml), cooled at −15 °C. After stirring at −15 °C for 30 min and at 45 °C for 2.5 h, the solution was evaporated under vacuum to give intermediate hydrochloride as a foam. This salt was used without further purification. Boc–Met–OH (0.249 g, 1 mmol) was activated with isobutyl chloroformate 98% (0.133 ml, 1 mmol) and NMM (0.13 ml, 1.2 mmol) in dry CH_2Cl_2 (5 ml) and equivalent amounts of the above hydrochloride and NMM in dry CH_2Cl_2 (3.6 ml) were added as described for Boc–β-Ala–Phe–OMe. Usual work up afforded a residue (0.485 g), which was purified by preparative layer chromatography [$CHCl_3$–MeOH (95:5)] to give the pure title compound **1** (0.381 g, 79%) as a foam. M.p. 105–106 °C (EtOAc). $[\alpha]_D = +62°$. IR: 3356, 3319, 2949, 1736, 1686, 1647, 1526, 1168 cm^{-1}. ^1H NMR δ (ppm): 1.44 [9H, s, $C(CH_3)_3$], 1.78–2.17 (2H, m, Met β-CH_2), 2.11 (3H, s, S–CH_3), 2.34 (2H, m, β-Ala α-CH_2), 2.55 (2H, t, $J = 7.3$ Hz, Met γ-CH_2), 3.06 and 3.16 (2H, A and B of an ABX, $J = 7.8$, 5.5, and 13.8 Hz, Phe β-CH_2), 3.30 and 3.78 (2H, 2 m, β-Ala β-CH_2), 3.76 (3H,s, $COOCH_3$), 4.17 (1H, m, Met α-CH), 4.84 (1H, m, Phe α-CH), 5.27 (1H, d, $J = 7.3$ Hz, Met NH), 6.58 (1H, d, $J = 7.3$ Hz, Phe NH), 7.10 (1H, br, β-Ala NH), 7.16-7.40 (5H, m, aromatic). *Anal*. (C, H, N) for $C_{23}H_{35}N_3O_6S$.

6.1.3. HCO–Met–β-Ala–Phe–OMe (2)

The Boc-tripeptide **1** (0.169 g, 0.35 mmol) was dissolved in formic acid (2.1 ml) and the mixture stirred at room temperature for 1 day. After removal of the excess of formic acid in vacuo, the residue was dissolved in 2.1 ml of dry DMF. EEDQ 97% (0.107 g, 0.42 mmol) was added and the solution stirred at room temperature for 24 h. Evaporation under reduced pressure afforded a residue which was dissolved in dry chloroform and the product was precipitated by *n*-hexane. Washing with dry ether afforded the pure formylpeptide **2** (0.133 g, 93%) as a white powder. M.p. 123 °C. $[\alpha]_D = +66°$. IR: 3293, 3084, 2951, 2919, 1739, 1646, 1538, 1443, 1384, 1223 cm^{-1}. ^1H NMR δ (ppm): 1.85–2.20 (2H, m, Met β-CH_2), 2.13 (3H, s, S–CH_3), 2.36 (2H, m, β-Ala α-CH_2), 2.56 (2H, m, Met γ-CH_2), 3.08 and 3.14 (2H, A and B of an ABX, $J = 7.9$, 5.5, and 13.5 Hz, Phe β-CH_2), 3.34 and 3.72 (2H, 2 m, β-Ala β-CH_2), 3.77 (3H, s, $COOCH_3$), 4.56 (1H, m, Met α-CH), 4.85 (1H, m, Phe α-CH), 6.75 (1H, d, $J = 7.8$ Hz, Phe NH), 6.91 (1H, d, $J = 7.4$ Hz, Met NH), 7.14–7.38 (6H, m, aromatic and β-Ala NH), 8.17 (1H, s, H–CO). *Anal*. (C, H, N) for $C_{19}H_{27}N_3O_5S$.

6.1.4. Boc–Met–Tau–Phe–OMe (3)

Boc–Met–OH (0.272 g, 1.09 mmol) was activated with isobutyl chloroformate 98% (0.14 ml, 1.09 mmol) and NMM (0.14 ml, 1.3 mmol) in dry tetrahydrofuran (8 ml) as described for compound **1**. Addition of a solution of HBr·Tau–Phe–OMe [26] (0.41 g, 1.09 mmol) and NMM (0.12 ml, 1.09 mmol) in dry tetrahydrofuran (5 ml) and usual work up afforded an oily residue which was crystallized from diethyl ether-*n*-hexane (0.484 g, 84%). M.p. 119–121 °C. $[\alpha]_D = -13°$. IR: 3343, 2985, 1738, 1661, 1521, 1344, 1149 cm^{-1}. ^1H NMR δ (ppm): 1.43 [9H, s, $C(CH_3)_3$], 1.80–2.18 (2H, m, Met β-CH_2), 2.11 (3H, s, S–CH_3), 2.55 (2H, t, $J = 7.3$ Hz, Met γ-CH_2), 2.87 (2H, m, Tau α-CH_2), 2.99 and

3.19 (2H, A and B of an ABX, $J = 5$, 8, and 13.9 Hz, Phe β-CH_2), 3.55 (2H, m, Tau β-CH_2), 3.78 (3H,s, $COOCH_3$), 4.20 (1H, m, Met α-CH), 4.43 (1H, m, Phe α-CH), 5.18 (1H, d, $J = 7.9$ Hz, Met NH), 6.92 (1H, d, $J = 8.8$ Hz, Phe NH), 7.10 (1H, br, Tau NH), 7.21–7.43 (5H, m, aromatic). *Anal*. (C, H, N) for $C_{22}H_{35}N_3O_7S_2$.

6.1.5. HCO–Met–Tau–Phe–OMe (4)

The Boc-derivative **3** (0.263 g, 0.5 mmol) was dissolved in formic acid (3 ml) and the mixture stirred at room temperature for 1 day. After removal of the excess of formic acid in vacuo, the residue was dissolved in 3 ml of dry DMF. EEDQ 97% (0.153 g, 0.6 mmol) was added and the solution stirred at room temperature for 24 h. Evaporation under reduced pressure afforded a residue which was purified by preparative layer chromatography [$CHCl_3$–MeOH (98:2)] to give the pure title compound **4** (0.2 g, 88%) as a foam. $[\alpha]_D = -19°$. IR ($CHCl_3$): 3340, 1743, 1674, 1339, 1145 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm): 1.85–2.18 (2H, m, Met β-CH_2), 2.11 (3H, s, S–CH_3), 2.55 (2H, t, $J = 7.3$ Met γ-CH_2), 2.89 (2H, m, Tau α-CH_2), 2.99 and 3.19 (2H, A and B of an ABX, $J = 4.9$, 8.8, and 13.9 Hz, Phe β-CH_2), 3.51 (2H, m, Tau β-CH_2), 3.80 (3H, s, $COOCH_3$), 4.42 (1H, m, Phe α-CH), 4.62 (1H, m, Met α-CH), 6.18 (1H, d, $J =$ 9.1 Hz, Phe NH), 6.92 (1H, d, $J = 8.1$ Hz, Met NH), 7.20–7.40 (6H, m, aromatic and Tau NH), 8.12 (1H, s, H–CO). *Anal*. (C, H, N) for $C_{18}H_{27}N_3O_6S_2$.

6.1.6. HCl·Met–Tau–Phe–OMe (5)

Thionyl chloride (0.044 ml, 0.60 mmol) was added to a solution of Boc–Met–Tau–Phe–OMe (**3**) (0.3 g, 0.57 mmol) in dry methanol (3 ml), cooled at −15 °C. After stirring at −15 °C for 30 min and at 45 °C for 2.5 h, the solution was evaporated under vacuum to give the hydrochloride **5** as a foam in quantitative yield. $[\alpha]_D = +10°$. IR ($CHCl_3$): 3035, 1738, 1710, 1680, 1437, 1361, 1144 cm^{-1}. ^1H NMR (DMSO-d_6): δ (ppm): 2.01 (2H, m, Met β-CH_2), 2.06 (3H, s, S–CH_3), 2.51 (2H, t, $J = 7.1$ Hz, Met γ-CH_2), 2.85–3.06 (4H, m, Phe β-CH_2 and Tau α-CH_2), 3.22 and 3.37 (2H, 2 m, Tau β-CH_2), 3.64 (3H, s, $COOCH_3$), 3.81 (1H, poorly resolved t, Met α-CH), 4.17 (1H, m, Phe α-CH), 7.21–7.33 (5H, m, aromatic), 8.08 (1H, d, $J = 6.6$ Hz, Phe NH), 8.41 (2H, s, Met NH_2), 8.80 (1H, poorly resolved t, Tau NH). *Anal*. (C, H, N) for $C_{17}H_{28}ClN_3O_5S_2$.

6.2. Biological assays

6.2.1. Peptides

Stock solutions of fMLF-OMe and peptide analogues, 10^{-2} M, were prepared in DMSO and diluted in KRPG, pH 7.4, before use. At the concentration used, DMSO did not interfere with any of the biological assays performed.

6.2.2. Cell preparation

Cells were obtained from the blood of healthy subjects, and human peripheral blood neutrophils were purified 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 KRPG, pH 7.4, at a final concentration of 50×10^6 cells/ml and kept at room temperature until used. Neutrophils were 98–100% pure and 99% viable, as determined using the Trypan blue exclusion test.

6.2.3. Random locomotion

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Milan, Italy) and migration into the filter was evaluated by the method of leading-front [37]. The actual control random movement is 35 μm ± 3 SE of 10 separate experiments performed in duplicate.

6.2.4. Chemotaxis

In order to study the potential chemotactic activity, 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 (Orha Behringwerke, Germany) and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index, 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 chemotactic index range.

6.2.5. Superoxide anion (O_2^-) production

The superoxide 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 μl containing 4×10^5 neutrophils, 100 nmole cytochrome *c* and KRPG. At zero time different amounts (10^{-9}–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 wavelenghts of 550 and 468 nm. The difference in absorbance at the two wavelenghts was used to calculate nmole 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 nmole 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 nmole O_2^- range.

6.2.6. 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 in a final concentration of 10^{-9}–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 μg/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%.

6.2.7. Antagonist assay

Antagonist activity was determined by measuring the ability of a derivative to inhibit chemotaxis, superoxide anion production or granule enzyme release as induced by fMLF-OMe. Antagonist activity data (% of activity) were obtained by comparing the chemotactic index, nmole of O_2^- or percentage of lysozyme release in the absence (100%) and in the presence of the derivative. Chemotactic index of 10 nM fMLF-OMe was 1.15 ± 0.10 SE. O_2^- generation produced by 1 nM fMLF-OMe was 43 ± 2 nmol/1×10^6 cells/5 min. Enzyme activity triggered by 1 μM fMLF-OMe was $57 \pm 5\%/3 \times 10^6$ cells/min.

Derivatives were added to neutrophils 10 min before the incubation step for cellular functionality. Each value represents an average of six separate experiments performed in duplicate. Standard errors are within 10% of the mean value.

6.2.8. Statistical analysis

The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups.

6.2.9. Receptor binding assay

Binding assays were carried out essentially according to Spisani et al. [38]. In competition experiments were carried out to determine the IC_{50} values, 6 nM [^{3}H]-fMLF (specific activity = 71.5 Ci/mmol, NEN Research Products, Du Pont de Nemours, Milan, Italy) was incubated with 100 μl of human neutrophils (5×10^6) at different concentrations of the test compounds at 37 °C for 15 min. Non specific binding was measured in the presence of 100 μM fMLF, and was about 20% of total binding. Bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/C glass-filters using a Micro-Mate 196 Cell Harvester (Packard Instrument Company). The filter-bound radioactivity was counted on Top Count (efficiency 57%) with Micro-Scint-20 (30 μl in 96-well plates).

The cold drug concentrations displacing 50% of labelled ligand (IC_{50}) were obtained by computer analysis of displacement curves. All data were analysed using the non-linear regression curve fitting computer program Graph Pad Prism (Graph Pad, San Diego, CA). All the values obtained are the mean of three independent experiments performed in duplicate.

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