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Synthesis, conformation and biological activity of centrally modified pseudopeptidic analogues of For-Met-Leu-Phe-OMe

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Summary. For-Met-βAlaψ[CSNH]-Phe-OMe (**3**), For-Met-βAlaψ[CH_2NH]-Phe-OMe (**5**), For-Met-NH-*p*C_6H_4-SO_2-Phe-OMe (**8a**), For-Met-NH-*m*C_6H_4-SO_2-Phe-OMe (**8b**) and the corresponding *N*-Boc precursors (**2**, **4**, **7a**, **b**) have been synthesized and their activity towards human neutrophils has been evaluated in comparison with that shown by the reference tripeptide For-Met-Leu-Phe-OMe (fMLF-OMe). Chemotaxis, lysozyme release and superoxide anion production have been measured. ^{1}H NMR titration experiments and IR spectra have been discussed in order to ascertain the preferred solution conformation adopted by the tripeptide **3** with particular reference to the presence of a folded conformation centred at the centrally positioned thionated β-residue.

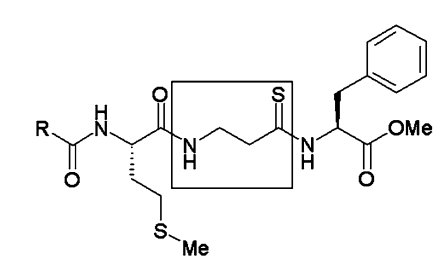
Keywords: Chemotactic peptides – Conformation – Neutrophils – Pseudopeptides – β-Thiopeptides

Introduction

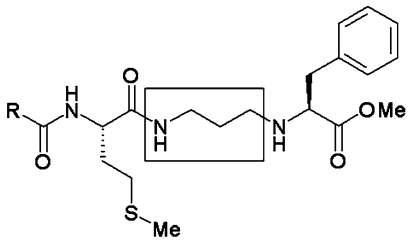
Human neutrophils are phagocytic cells which provide an effective defense against pathogens and play relevant roles in several non-infectious disorders (Malech, 1987; Prossnitz, 1997; Le, 2002). A number of peptides, chemokines and biological components are able to act as chemoattractants towards neutrophils through specific receptors located on the cell surface. Binding of chemoattractants to neutrophil receptors stimulate, in addition to the directed migration of the cell, a variety of coordinated biochemical and cellular responses including production of the cytotoxic and microbicidal superoxide anion radicals and release of lysosomal enzymes (Showell, 1976). Several *N*-formyl oligopeptides derived from bacterial protein degradation, among which the tripeptide For-Met-Leu-Phe-OH, are potent chemoattractants and their activity has stimulated a great deal of interest concerning relationships between molecular structure and biological activity (Dentino, 1991; Rathore, 2005).

In previous papers we reported on the conformational and biochemical consequences connected with structural modifications performed on For-Met-Leu-Phe-OMe (fMLF-OMe), the tripeptide molecule generally adopted as model for studies on chemotactic *N*-formyl peptides. A promising approach concerns the replacement of the native α-aminoacidic residues with β-aminocarboxylic- and β-aminosulfonic-acids (Pagani, 2001; Giordano, 2003, 2004; Mollica, 2006). It was found that, whereas the maintenance of the Met and Phe α-residues at the external positions is of primary importance for receptor recognition, replacement of the central Leu with β-residues produces a profound and often unpredictable alteration of the established requirements for optimal ligand-receptor interaction. In particular, whereas central incorporation of β-aminopropanoic-acid (β-alanine, βAla) generates inactive analogues, the corresponding modification, performed by introducing β-aminoethanesulfonic acid (taurine, Tau) is well tolerated and leads to ligands which are highly active and show selectivity towards the multiple biochemical functions typical of fMLF-OMe. The consequences derived by the replacement of the peptide bond with the sulphonamide was related to the peculiar properties of this junction in terms of both preferred conformation, tetrahedral hybridization of the sulphur atom and hydrogen bonding capacity (Calcagni, 2000; Gennari, 1998; Lowik, 2000).

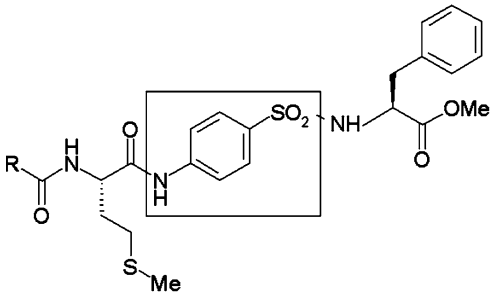
In order to further explore the biochemical and conformational consequences of the structural alteration



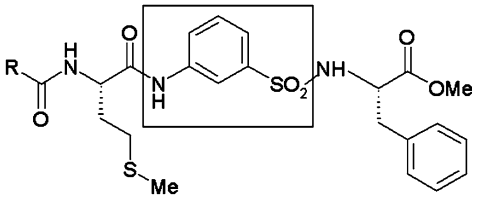
2 R = tBu-O Boc-Met-βAlaψ[CSNH]-Phe-OMe
3 R = H For-Met-βAlaψ[CSNH]-Phe-OMe



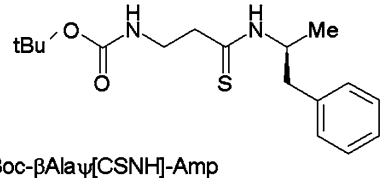
4 R = tBu-O Boc-Met-βAlaψ[CH_2NH]-Phe-OMe
5 R = H For-Met-βAlaψ[CH_2NH]-Phe-OMe



7a R = tBu-O Boc-Met-(*p*)NH-C_6H_4-SO_2-Phe-OMe
8a R = H For-Met-(*p*)NH-C_6H_4-SO_2-Phe-OMe



7b R = tBu-O Boc-Met-(*m*)NH-C_6H_4-SO_2-Phe-OMe
8b R = H For-Met-(*m*)NH-C_6H_4-SO_2-Phe-OMe



9 Boc-βAlaψ[CSNH]-Amp

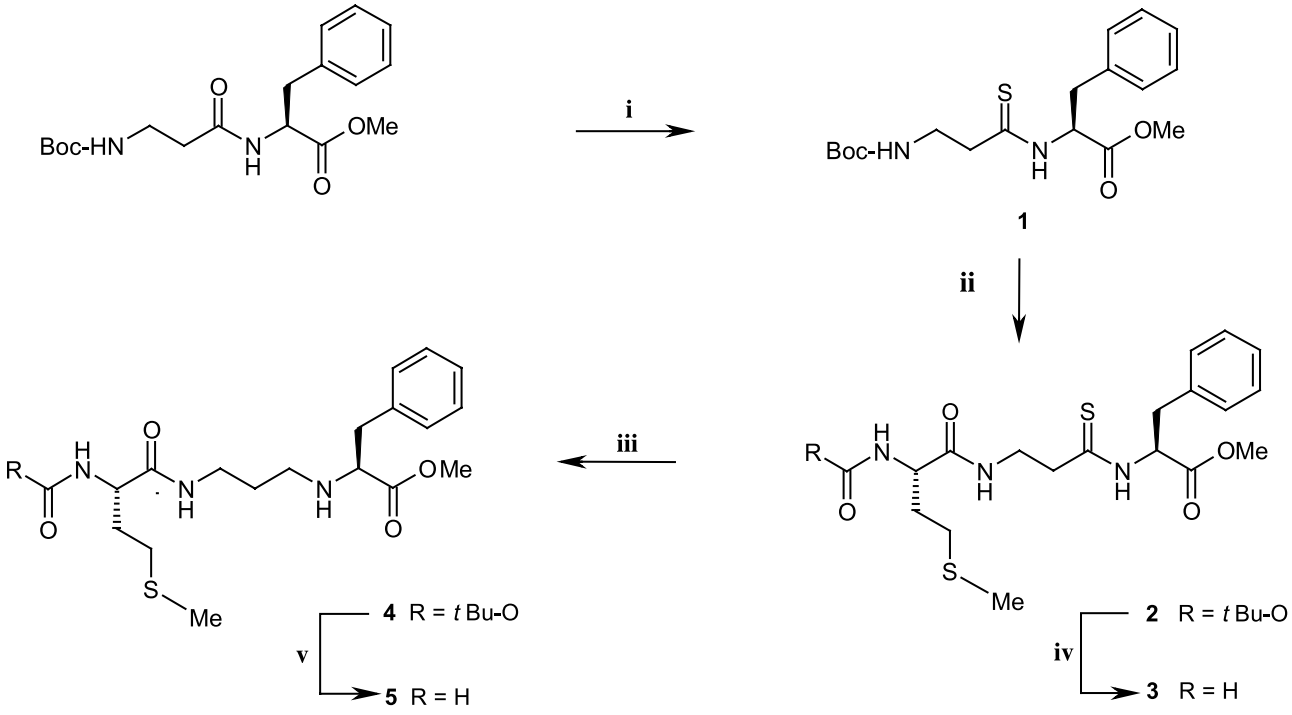
Fig. 1. Structures of the compounds investigated in this study

performed at the central position of fMLF-OMe, with particular reference to Tau containing fMLF-OMe analogues, we report now results obtained by examining the new series of pseudopeptidic ligands **2**–**5** and **7a**, **b**–**8a**, **b** (Fig. 1). Here, the native central Leu has been replaced with achiral residues which, similarly to the βAla and Tau are devoid of side chain and, as compared with α-aminoacids, cause an enhancement of the backbone length.

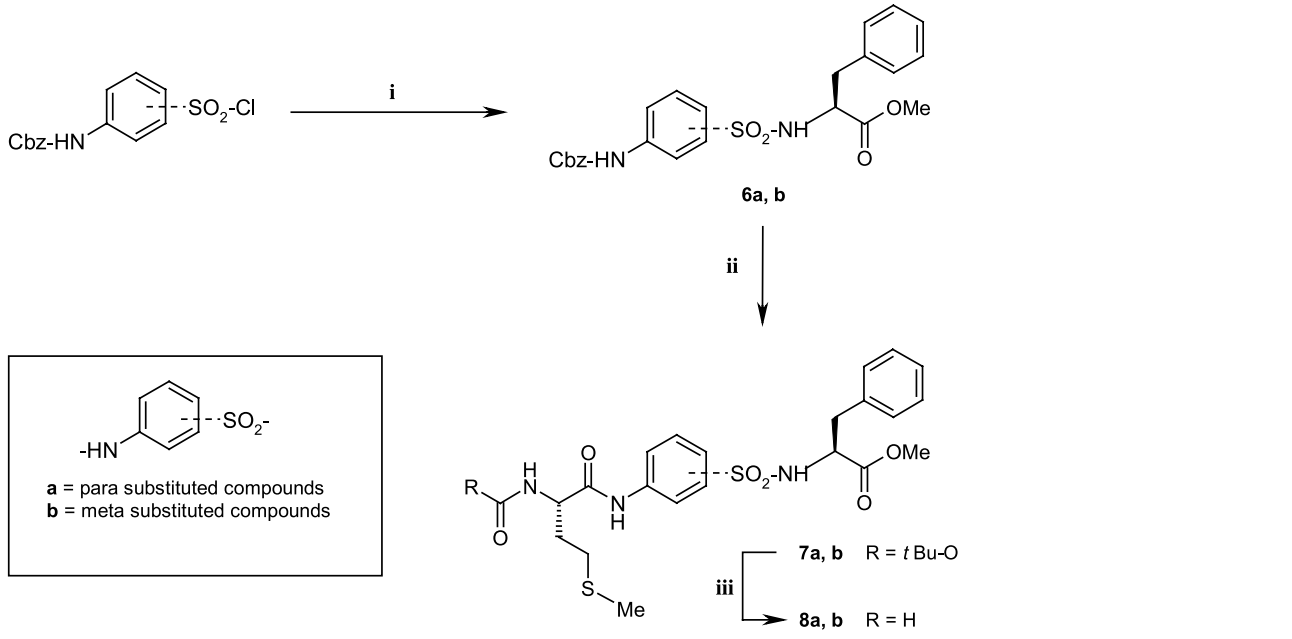
Synthesis

Boc-βAla-Phe-OMe (Giordano, 2003) was used as starting material for the synthesis of Boc-Met-βAlaψ[CSNH]-Phe-OMe (**2**), Boc-Met-βAlaψ[CH_2NH]-Phe-OMe (**4**) and corresponding *N*-formyl analogues HCO-Met-βAlaψ[CSNH]-Phe-OMe (**3**) and HCO-Met-βAlaψ[CH_2NH]-Phe-OMe (**5**) (Scheme 1). Reaction of Boc-βAla-Phe-OMe with the Lawesson reagent (Cava, 1985) gave the corresponding thionated dipeptide **1** which was *N*-deprotected with TFA and coupled with Boc-Met-OH affording the tripeptide **2**. Treatment of this with Raney-Ni by the permanent flow method (Krumme, 1999) afforded the reduced pseudotripeptide **4**.

Preparation of Boc-Met-NH-*p*C_6H_4-SO_2-Phe-OMe (**7a**), Boc-Met-NH-*m*C_6H_4-SO_2-Phe-OMe (**7b**) and of the corresponding *N*-formyl analogues HCO-Met-NH-*p*C_6H_4-SO_2-Phe-OMe (**8a**) and HCO-Met-NH-*m*C_6H_4-SO_2-Phe-OMe (**8b**) is delineated in Scheme 2. Cbz-NH-*p*C_6H_4-SO_2-Cl and Cbz-NH-*m*C_6H_4-SO_2-Cl, obtained by reaction of the parent acids with thionyl chloride, were used as starting material. Coupling of the sulphonyl chlorides with L-phe-



Scheme 1. Synthesis of pseudopeptides **1**–**5**: i) Lawesson reagent, THF; ii) a: TFA, CH_2Cl_2; b: Boc–Met–OH, EDC, HBT, TEA, EtOAc; iii) Ni-Raney, THF/MeOH; iv, v) HCOOH, EEDQ



Scheme 2. Synthesis of pseudopeptides **7a**, **b**–**8a**, **b**: i) HCl · Phe-OMe, TEA, CH_2Cl_2; ii) a: H_2, 10% Pd/C, MeOH, TFA; b: Boc-Met-OH, EDC, HBT, TEA, EtOAc; iii) HCOOH, EEDQ

nylalanine methylester hydrochloride, in the presence of triethylamine, afforded Cbz-NH-*p*C_6H_4-SO_2-Phe-OMe and Cbz-NH-*m*C_6H_4-SO_2-Phe-OMe which, after removal of the Cbz group by catalytic hydrogenation, were coupled with Boc-Met-OH, by the mixed anhydride method, to give **7a** and **7b**, respectively. All the *N*-Boc derivatives were directly transformed (Lajoie, 1984) into the corresponding *N*-formyl analogues.

Conformational studies

^{1}H NMR

In order to gain information on the conformational preferences of the pseudotripeptides under study, an ^{1}H NMR analysis of the involvement of the NH groups in intramolecular H-bonds was undertaken. Figure 2 refers to the titration experiments and delineates the chemical shift dependence of the NH resonances as a function of increasing *DMSO*-d_6 concentration in a $CDCl_3$ solution (10 mM). In Table 1 the exposure to *DMSO*-d_6 of the NH groups of the examined pseudotripeptides are reported; the values are expressed as the difference ($\Delta\delta$, ppm) between the NH chemical shift values observed in $CDCl_3$ solution containing 10% *DMSO*-d_6 and in neat $CDCl_3$. In contrast with free NH groups, NH protons engaged in intramolecular H-bonds have low tendency to interact with the sulfoxide and exhibit low values of $\Delta\delta$ (i.e.: high shielding). Data concerning pseudotripeptides **4** and **5**, containing the βAlaψ[CH_2NH] modification, are not reported since the broad band of the aminic proton together with overlapping to the aromatics did not allow any reliable assignment.

The results of the titration experiments performed on For-Met-βAlaψ[CSNH]-Phe-OMe (**3**) (Fig. 2A) clearly show that the NH of the thionated central β-residue presents high solvent inaccessibility while the NH groups of the external Met and Phe residues interact efficiently with *DMSO*-d_6. This puts in evidence the possible involvement of the central NH in a folded conformation characterized by a CO–NH$\cdots$S=C–NH intraresidue interaction. However, larger ring secondary structures, stabilized by H-bonding of the central NH with the C-terminal Phe carbonyl group (C_9 ring) as well as with the preceding formylic CO (C_7 ring, γ-turn), could be responsible for the

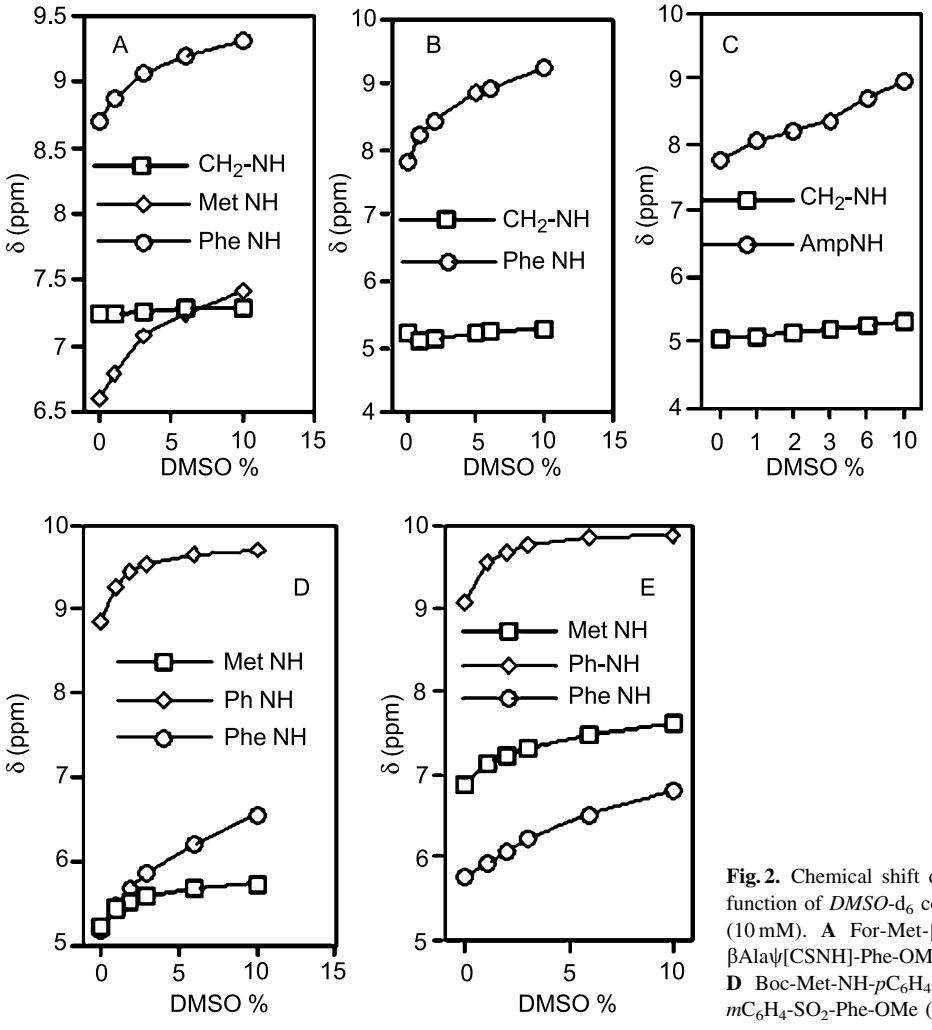


Fig. 2. Chemical shift dependence of the NH resonances as a function of *DMSO*-d_6 concentration (% v/v) in $CDCl_3$ solution (10 mM). **A** For-Met-βAlaψ[CSNH]-Phe-OMe (**3**); **B** Boc-βAlaψ[CSNH]-Phe-OMe (**1**); **C** Boc-βAlaψ[CSNH]-Amp (**9**); **D** Boc-Met-NH-*p*C_6H_4-SO_2-Phe-OMe (**7a**); **E** Boc-Met-NH-*m*C_6H_4-SO_2-Phe-OMe (**7b**)

Table 1. Solvent accessibility of peptide NH groups: differences (Δδ, ppm) between NH chemical shift values observed in $CDCl_3$ solution containing $(CD_3)_2SO$ (10%) and in neat $CDCl_3$

Compound	*N*-terminal NH	Central NH	*C*-terminal NH
For-Met-βAlaψ[CSNH]-Phe-OMe (**3**)	0.81	0.04	0.60
For-Met-βAla-Phe-OMe	0.88	0.19	0.84
Boc-βAlaψ[CSNH]-Phe-OMe (**1**)	0.07	–	1.40
Boc-βAla-Phe-OMe	0.14	–	1.13
Boc-βAlaψ[CSNH]-Amp (**9**)	0.29	–	1.21
Boc-β-Ala-Amp	0.19	–	1.05
Boc-Met-(*p*)$NHC_6H_4SO_2$-Phe-OMe (**7a**)	0.50	0.85	1.38
Boc-Met-(*m*)$NHC_6H_4SO_2$-Phe-OMe (**7b**)	0.74	0.80	1.05

Values of related, previously studied non thionated analogues, are also reported

observed solvent inaccessibility. As reported in Table 1 the NH shieldings exhibited by the thionated *N*-formyl-tripeptide **3** are similar to those exhibited by the previously examined βAla containing analogue HCO-Met-βAla-Phe-OMe (Giordano, 2003) even if **3** presents higher inaccessibility of the central and *N*-terminal NH groups ($\Delta\delta = 0.04$ and 0.60 as compared with 0.19 and 0.84). In the case of the non thionated tripeptide the observed shielding of the central NH was related to the tendency of the molecule to adopt, in addition to γ-turn structures centred at the Met residue (Aschi, 2006), a folded conformation characterized by the involvement of the βAla into a CO–NH···O=C–NH intraresidue interaction (C_6 conformation).

In order to ascertain if the shielding observed in **3** can be ascribed to an intraresidue interaction analogous to that induced by βAla in non thionated models, we extended the ^{1}H NMR titration experiments to two analogues of **3**, namely: the pseudodipeptide Boc-βAlaψ[CSNH]-Phe-OMe (**1**) which lacks the *N*-terminal-CO-Met-fragment and the new derivative (S)-Boc-βAlaψ[CSNH]-Amp (**9**) where Amp [(S)-2-amino-1-phenylpropane] is the Phe mimicking residue which lacks the *C*-terminal ester carboxyl group. Results of these titrations are reported in Fig. 2B, C and in Table 1. It can be seen that the NH of the β-residue of **1** is practically unaffected by the increase of the *DMSO*-d_6 concentration while the Phe NH interacts freely with the solvent ($\Delta\delta = 0.07$ and 1.40 ppm, respectively). A corresponding behaviour is exhibited by **9** whose *N*-terminal NH results the most solvent inaccessible group (Δδ values are 0.29 and 1.21 ppm for the *N*-terminal and *C*-terminal NH). Thus, although the higher solvent shielding shown by the *N*-terminal NH of **1** ($\Delta\delta = 0.07$ ppm) as compared with the corresponding value of **9** ($\Delta\delta = 0.29$ ppm) indicates that in the case of **1** the Phe CO ester carbonyl contributes to the folding (*i.e.*: presence of a certain population of C_9 ring secondary structure), the overall results from **1** to **9**, which parallel those exhibited by the corresponding non thionated models, clearly support the presence in **3** and in related thionated models of an intraresidue –HN–C=S···HN–CO interaction resembling a local C_6 folding. This behaviour is rather unexpected since is generally accepted that thioamides, as compared with amides, are characterized by a stronger NH donor and a weaker C=S acceptor (Sherman, 1990; Sifferlen, 1999 and references therein). However, it must also be remembered that IR based studies on monothionated α-peptides, while confirming the higher NH acidity and H-bonding capacity of thioamides, clearly indicate that the acceptor capacity of the C=S group may reach or even exceed that of the C=O of amides (Hollòsi, 1990; Shaw, 1995; Min, 1998).

As reported in Fig. 2D, E and Table 1 titration experiments performed on pseudotripeptides **7** and **8**, containing a central *p*-amino- or *m*-amino-benzenesulphonic acid residue, show that all the backbone NH groups interact rather efficiently with the solvent; preference for folded conformers is then disfavoured in this case. It can be noted here the lower accessibility shown by the Met-Boc-NH group of **7a** ($\Delta\delta = 0.50$) as compared with the corresponding HCO-NH group of **8b** ($\Delta\delta = 0.74$). This finding, which is in accordance with previous observations (Aschi, 2003), shows that bulky substituents can significantly hinder the interaction with the solvent of the NH groups; this effect is particularly evident in the present case where the shielding capacity of two acylating groups with highly different size are confronted.

IR

In order to get more information on the conformation based on the β-thioresidue NH solvent shielding experiments, IR spectra ($CHCl_3$; 5 mM) have been performed. In particular, the region of the NH stretching frequencies in the spectrum of the tripeptide For-Met-βAlaψ[CSNH]-Phe-OMe (**3**) has been examined and compared with that of the dipeptide Boc-βAlaψ[CSNH]-Phe-OMe (**1**) and (S)-Boc-βAlaψ[CSNH]-Amp (**9**). In the IR spectrum of **3**, in addition to a band centred at 3430 cm^{-1}, attributable to free NH, two bands at lower

frequencies (3351 and 3290 cm^{-1}), in the region of H-bonded NH groups, are observed. In accordance with the weak shielding of the Phe NH ($\Delta\delta = 0.60$ ppm) the absorption at 3290 cm^{-1} probably represents the NH stretching of a small population of C_8 ring conformers involving the Phe NH and the Met C=O. In the spectrum of the dipeptide **1**, in addition to the expected absorption due to free NH (3453 cm^{-1}) a strong band appears at 3367 cm^{-1} in the region of H-bonded NH. Since the Phe NH of this compound results to be completely free in $CHCl_3$ solution (Table 1; solvent shielding $\Delta\delta =$ 1.40 ppm) the absorbance at 3367 cm^{-1} should be assigned to an intramolecular interaction which the highly shielded *N*-terminal NH establishes, as H-bond donor, with the C=S or the C-terminal ester C=O groups as acceptors. Examination of the spectrum of **9** allows to get information on this point. Here in fact, as already found in the spectrum of the dipeptide **1**, two strong bands (in this case of equal intensity), centred at 3454 and 3381 cm^{-1}, are observed. Thus, since the NH of the Amp residue is not involved in intramolecular H-bonds ($\Delta\delta = 1.21$), the presence of the absorption at 3381 cm^{-1} in the IR spectrum of **9** supports the existence of the above discussed CS···HN C_6 intraresidue interaction both in **9** and in the related thionated peptides **1** and **3** which exhibit analogous absorptions.

As final consideration it can be observed that in the spectrum of **1**, differently from **9**, the band of the bonded NH (3367 cm^{-1}) is sensibly stronger than that of free NH (3453 cm^{-1}). This is in accordance with the ^1H NMR titration experiments according to which the population of conformers with free NH groups in **9** is higher than in **1**. The Boc-NH of **1** is in fact involved as H-bond donor with two acceptors (i.e.: the C=S and C-terminal C=O) and shows a solvent shielding of 0.07 ppm (Table 1); on the contrary, the folded conformers of **9**, only stabilized by Boc–NH···S=C interactions, are less populated. Accordingly, the solvent shielding observed in **9** is lower (0.29 ppm; Table 1) and the intensity ratio between free and bonded NH absorptions is higher in **9** than in **3**.

Biological results

The biological activity of the pseudopeptides under study (**2–5** and **7a**, **b**–**8a**, **b**) 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.

Chemotactic activity elicited by the ligands **2–5**, containing a central β-residue with ψ[CSNH] or a ψ[CH_2NH]

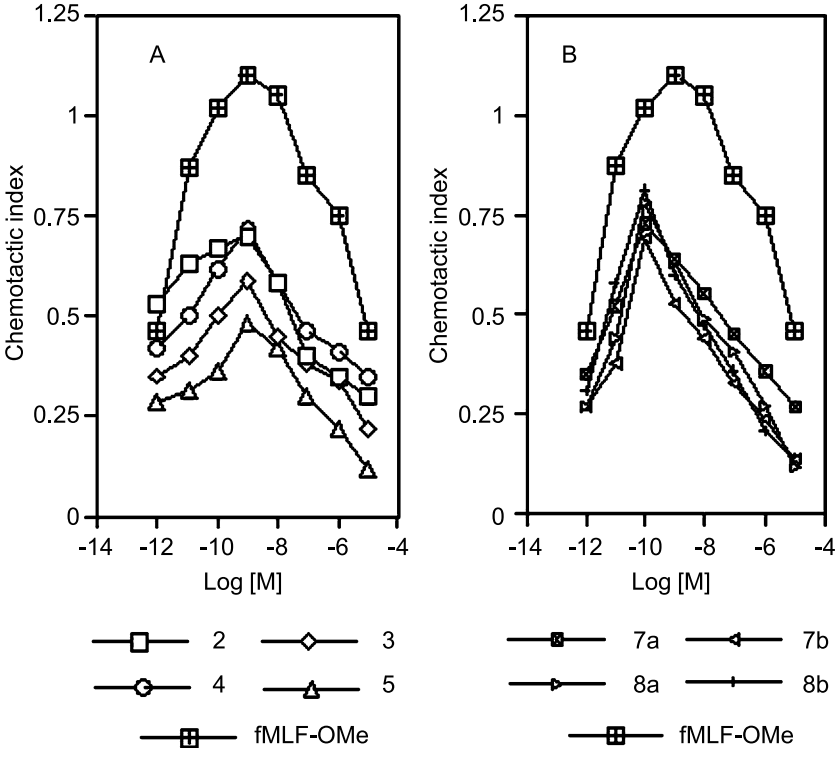


Fig. 3. Chemotactic activity of the pseudotripeptides compared with the reference ligand fMLF-OMe. **A** Analogues **2**, **3** and **4**, **5** containing the ψ[CSNH] and the ψ[CH_2NH] replacement, respectively; **B** analogues **7a**, **8a** and **7b**, **8b** containing the (*p*)NH-C_6H_4-SO_2- and (*m*)NH-C_6H_4-SO_2-replacement, respectively

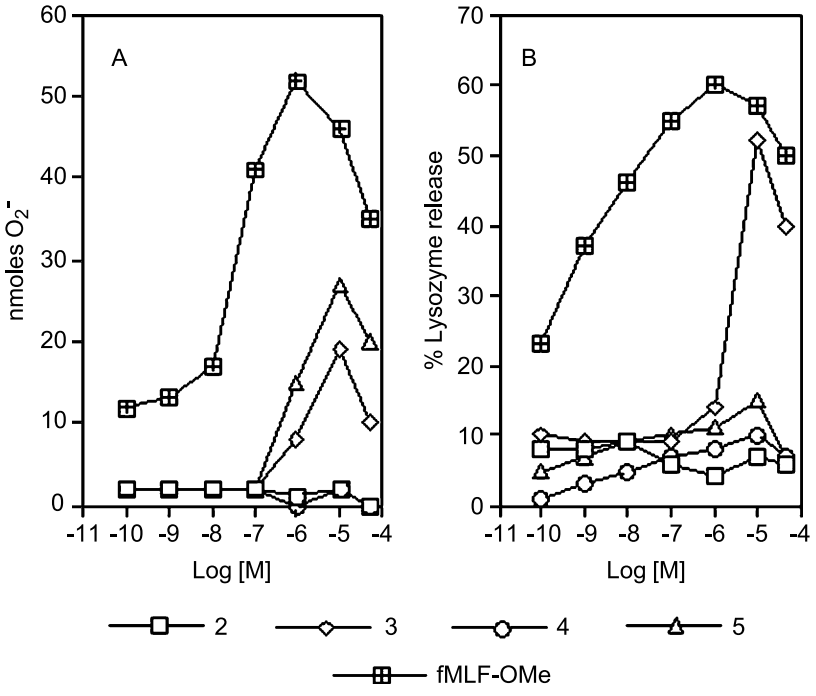


Fig. 4. Biological activity of pseudotripeptides **2**, **3** and **4**, **5** containing the ψ[CSNH] and the ψ[CH_2NH] replacement, respectively. **A** Superoxide anion production; **B** release of neutrophil granule enzymes evaluated by determining lysozyme activity. Values are compared with the reference ligand fMLF-OMe

junctions are reported in Fig. 3A. The dose-response curves, as usually found for chemoattractants, increase to reach a peak and then decrease rapidly as the concentration of the ligand increases. The four analogues are moderately potent as chemoattractants although their maximum of activity appears at the same concentration of the reference ligand (10^{-9} M). Since it has been previously found that the tripeptides *N*-Boc- and *N*-For-Met-βAla-Phe-OMe are practically inactive as chemoattractants (Giordano, 2003) the present results indicate that in the series of fMLF analogues characterized by the replacement of the native Leu with a β-aminoacid, the alteration of the amide nature of the peptide bond restores, at least in part, the chemotactic activity. Furthermore, it can be observed that the *N*-Boc analogues **2** and **4** show higher activity than the corresponding *N*-formyl analogues **3** and **5**. Thus, in accordance with fMLF-OMe analogues containing 2-aminoethanesulfonic acid (taurine; Tau) as central residue (Giordano, 2003), the presence of the bulky *N*-protecting group does not lead to antagonism or inactivity.

In Fig. 3B the chemotactic activity of *N*-Boc and *N*-formyl pseudotripeptides **7a**, **b**–**8a**, **b**, containing a central *p*-amino- or *m*-amino-benzenesulphonic acid residue, is reported. The shape of the curves is similar to that of analogues **2**–**5** (Fig. 3A). In this case, however, the four compounds show a comparable significant activity with a maximum centred at concentration lower (10^{-10} M) than that of the reference ligand. Thus, this group of analogues containing a central sulphonamide junction, although less potent are more efficient (ca. 10 times) than fMLF-OMe. Furthermore, as already found for the analogues **2**–**5** containing ψ[CSNH] or a ψ[CH_2NH] bond, the incorporation at the central position of *para*- or *orto*-aminobenzenesulphonic acid residues maintains the activity of the *N*-Boc derivatives (**7a**–**b**). In this case, however, and unlike the thionated compounds **2**–**5**, the *N*-Boc derivatives are slightly less active than the corresponding *N*-For analogues.

Concerning the production of superoxide anions (Fig. 4A), a modest activity has been observed for the formyl derivatives **3** and **5** at high concentration; all the remaining derivatives under study are in fact completely inactive. An analogous result concerns the lysozyme release induction (Fig. 4B). Here only the formylated pseudotripeptide **3** exhibits an activity peak which, at high concentration (10^{-5} M) is comparable to that observed for the parent fMLF-OMe.

Conclusion

Our results confirm that the replacement of the native central residue with different types of spacers represents a promising and flexible approach in order to modulate the activity of the reference ligand fMLF-OMe. In particular, the critical role exerted by the nature of the junction

placed between the central spacer and the *N*-terminal residue appears evident. While the usual amide bond of βAla derivatives leads to inactive ligands (Giordano, 2003), models, containing the bulkier thioamide or junctions with tetrahedral hybridization as sulphonamides or ψ[CH_2NH] analogues, elicit in different degree significant chemotactic activity and maintain selectivity towards superoxide anion production and lysozyme release. Furthermore, as already shown by pseudotripeptides containing a central 2-aminoethanesulfonic acid residue, the *N*-Boc protected derivatives **2**, **4**, **7a** and **7b** exhibit chemotactic activity comparable or higher than that shown by the *N*-For counterparts **3**, **5**, **8a** and **8b** (Fig. 3A, B).

The data on the preferred conformation of tripeptide **3** containing a thionated β-residue are of interest. It is well known that although thioamides maintain some key features of amides (e.g.: planarity; *trans*-geometry), several physical and chemical properties are deeply different (Le et al., 2002; Tran, 2002) and may lead, on passing from α-peptides to α-thiopeptides, to unexpected conformational changes, enhanced stability toward proteases and altered biological activity (Sherman, 1990). Thus, it is surprising that despite the interest on α-thiopeptides and the extensive studies on β-peptides and their foldamers (Hill, 2001 and references therein), the new family of thionated β-peptides (i.e., β-thiopeptides; Sifferlen, 1999) and α-peptides containing thionated β-residues are still largely unexplored. At the best of our knowledge the here reported studies on tripeptide **3** provide the first literature data on the conformational preferences induced by a thionated β-aminoacid inserted into an α-peptide backbone. In this context the analogy between the –HN–C=S···HN–CO intraresidue interaction evidenced in the tripeptide **3** and the C_5 ring structure found in α-thiopeptides (Shaw, 1995), appears a stimulating observation fur further studies in the field of peptide secondary structure stabilization.

Experimental

All starting materials and reagents were obtained commercially and used without further purification. 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% $CHCl_3$ solution (unless otherwise specified) 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 400 spectrometer and chemical shifts were indirectly referred to TMS. TLC 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. Boc, *tert*-butyloxycarbonyl; DMF, dimethylformamide; TFA, trifluoroacetic acid; EEDQ, ethyl-2-ethoxy-1,2-dihydro-1-quinolinecarboxylate; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; Et_2O, diethyl ether; HOBT, 1-hydroxybenzotriazole; KRPG, Krebs-Ringer phosphate containing 0.1% w/v D-glucose; TEA, triethylamine; TLC, thin-layer chromatography.

Chemistry

Boc-βAlaψ[CSNH]-Phe-OMe (**1**)

Boc-βAla-Phe-OMe (1.35 g, 3.8 mmol) and Lawesson reagent (770 mg, 1.9 mmol) were suspended in THF (30 ml) and the reaction mixture was stirred at room temperature overnight. The clear solution was evaporated to dryness and the residue was subjected to silica gel flash chromatography (CH_2Cl_2) to give the pure product **1** as a pale yellow oil (1.27 g, 91%).

$[\alpha]_D + 99$; IR 3689, 3370, 2982, 1740, 1705, 1504, 1166 cm^{-1}; ^{1}H NMR: δ = 1.44 [9H, s, $C(CH_3)_3$], 2.80 (2H, t, *J* = 6.0 Hz, β-Ala α-CH_2), 3.20 and 3.38 (2H, m, Phe β-CH_2), 3.72 (2H, m, β-Ala β-CH_2), 3.76 (3H, s, OCH_3), 5.10 (1H, bs, NH), 5.42 (1H, m, Phe α-CH), 7.11 and 7.31 (5H, two m, aromatic), 7.82 (1H, bs, NH).

Anal. Calcd for $C_{18}H_{26}N_2O_4S$: C, 58.99; H, 7.15. Found: C, 59.14; H, 7.08.

Boc-Met-βAlaψ[CSNH]-Phe-OMe (**2**)

TFA deprotection of Boc-βAlaψ[CSNH]-Phe-OMe (**1**) gave the corresponding trifluoroacetate as a white foam. To an ice-cooled suspension of this dipeptide salt in EtOAc (15 mL), Boc-Met-OH (204 mg, 0.82 mmol), HOBt (166 mg, 1.23 mmol), TEA (0.34 ml, 2.46 mmol) and EDC (173 mg, 0.90 mmol) were consecutively added and the reaction mixture was allowed to warm slowly to room temperature overnight. The reaction mixture was then diluted with CH_2Cl_2 (20 ml) and washed with 1 M $KHSO_4$ (2 × 15 ml), saturated $NaHCO_3$ (2 × 15 ml) and brine (15 ml). The organic phase was dried and evaporated under reduced pressure. The crude product was triturated with hexane to give **2** as pale yellow foam (354 mg, 87%).

$[\alpha]_D + 131$; IR 3690, 3359, 2956, 1729, 1689, 1498, 1164 cm^{-1}; ^{1}H NMR: δ = 1.42 [9H, s, $C(CH_3)_3$], 1.78–2.15 (2H, m, Met β-CH_2), 2.12 (3H, s, S–CH_3), 2.58 (2H, t, *J* = 6.0 Hz, β-Ala α-CH_2), 2.80 (2H, m, Met γ-CH_2), 3.15–3.42 (4H, m, Phe β-CH_2 and β-Ala β-CH_2), 3.79 (3H, s, OCH_3), 4.18 (1H, m, Met α-CH), 5.25 (1H, d, *J* = 6.0 Hz, NH), 5.46 (1H, m, Phe α-CH), 7.28 (6H, m, aromatic and NH), 8.72 (1H, bs, NH).

Anal. Calcd for $C_{23}H_{35}N_3O_5S_2$: C, 55.51; H, 7.09. Found: C, 55.48; H, 7.12.

Boc-Met-βAlaψ[CH_2NH]-Phe-OMe (**4**)

A solution of **2** (190 mg, 0.38 mmol) in 5 ml of THF/MeOH (100:1 v/v) was passed through a small chromatography column (5 × 10 mm) filled with Raney nickel according to reported procedure (Krumme, 1999). The reduced product was purified by silica gel flash chromatography (CH_2Cl_2/MeOH 98:2) to give **4** as a white foam (136 mg, 77%).

$[\alpha]_D - 4$; IR: 3688, 3432, 3007, 1731, 1670, 1496, 1169 cm^{-1}; ^{1}H NMR: δ = 1.43 [9H, s, $C(CH_3)_3$], 1.62 (2H, m, N-CH_2*CH_2*CH_2–N), 1.73–2.18 (2H, two m, Met β-CH_2), 2.15 (3H, s, S–CH_3), 2.45–2.78 (4H, two m, N–CH_2CH_2*CH_2*–N and Met γ-CH_2), 3.00 (2H, m, Phe β-CH_2), 3.33 (2H, m, N–*CH_2*CH_2CH_2–N), 3.55 (1H, m, Phe α-CH), 3.71 (3H, s, OCH_3), 4.20 (1H, m, Met α-CH), 5.26 (1H, poorly resolved d, NH), 7.06 (1H, poorly resolved d, NH), 7.16–7.42 (6H, m, aromatic and NH).

Anal. Calcd for $C_{23}H_{37}N_3O_5S$: C, 59.08; H, 7.98. Found: C, 59.16; H, 7.93.

Cbz-NH-*p*C_6H_4-SO_2-Cl and Cbz-NH-*m*C_6H_4-SO_2-Cl

A solution of the appropriate aminobenzensulfonic acid (2.0 g, 11.5 mmol) in $NaHCO_3$ saturated solution (7.0 ml) was adjusted to pH 8.5 by using an

aqueous solution of 1 M NaOH. Under vigorous stirring, benzylchloroformate (1.96 g, 11.5 mmol) was added in five portions at room temperature, maintaining the pH around the above value by addition of small amounts of NaOH, then the reaction mixture was stirred overnight. After dilution with water (50 ml), the aqueous phase was washed with Et_2O (2 × 20 ml) and evaporated. The crude solid residue was coevaporated three times with toluene and dried under high vacuum over P_2O_5 overnight. To a suspension of the crude sodium sulfonate in anhydrous benzene (10 ml) and DMF (2 ml), thionyl chloride (2.73 g, 23.0 mmol) was added under stirring at room temperature. After 1 h, the mixture was washed with water and the organic phase dried and evaporated. The product was flash chromatographed on SiO_2 (CH_2Cl_2) and the purified sulphonyl chloride, obtained as pale yellow oil, was used as such for the subsequent reaction (Cbz-NH-*p*C_6H_4-SO_2-Cl: 1.9 g, 48%; Cbz-NH-*m*C_6H_4-SO_2-Cl: 1.19 g, 30%).

Cbz-NH-*p*C_6H_4-SO_2-Phe-OMe (**6a**)

To an ice-cooled solution of Cbz-NH-*p*C_6H_4-SO_2-Cl (900 mg, 2.77 mmol) and HCl · Phe-OMe (1.19 g, 5.54 mmol) in dry THF (20 ml), TEA (559 mg, 5.54 mmol) in dry THF (10 ml) was added dropwise under nitrogen. The resulting mixture was stirred overnight allowing to warm to room temperature. After dilution with EtOAc (50 ml), the mixture was washed with 1 M HCl, saturated $NaHCO_3$ and brine. The organic phase was dried and evaporated under reduced pressure. Crystallization from EtOAc/hexane gave **6a** as white solid (1.20 g, 92%).

Mp 120–121 °C; $[\alpha]_D + 6$; IR 3691, 3345, 2955, 1741, 1594, 1521, 1161 cm^{-1}; ^1H NMR: $\delta = 3.05$ (2H, m, Phe β-CH_2), 3.52 (3H, s, OCH_3), 4.41 (1H, m, Phe α-CH), 5.19 (1H, d, $J = 8.5$ Hz, Phe NH), 5.43 (2H, s, Cbz CH_2), 6.98 (1H, s, Ar–NH), 7.02–7.43 (9H, m, aromatic).

Anal. Calcd for $C_{24}H_{24}N_2O_6S$: C, 61.53; H, 5.16. Found: C, 61.71; H, 5.09.

Cbz-NH-*m*C_6H_4-SO_2-Phe-OMe (**6b**)

By following the above reported procedure and starting from Cbz-NH-*m*C_6H_4-SO_2-Cl (900 mg, 2.77 mmol) and phenylalanine methylester hydrochloride (1.19 g, 5.54 mmol), **6b** was obtained as a white solid (1.10 g, 85%).

$[\alpha]_D + 8$; IR 3690, 3356, 2955, 1740, 1601, 1525, 1161 cm^{-1}; ^1H NMR: $\delta = 3.03$ (2H, m, Phe β-CH_2), 3.42 (3H, s, OCH_3), 4.23 (1H, m, Phe α-CH), 5.21 (2H, s, Cbz-CH_2), 5.47 (1H, d, $J = 8.0$ Hz, Phe NH), 7.00–7.68 (9H, m, aromatic), 7.35 (1H, bs, NH).

Anal. Calcd for $C_{24}H_{24}N_2O_6S$: C, 61.53; H, 5.16. Found: C, 61.64; H, 5.18.

Boc-Met-NH-*p*C_6H_4-SO_2-Phe-OMe (**7a**)

Hydrogenolysis of Cbz-NH-*p*C_6H_4-SO_2-Phe-OMe (**6a**) followed by coupling with Boc-Met-OH gave **7a** as white solid (88%).

Mp 124–125 °C; $[\alpha]_D - 14$; IR 3630, 3441, 2944, 1741, 1696, 1595, 1335, 1160 cm^{-1}; ^1H NMR: $\delta = 1.46$ [9H, s, $C(CH_3)_3$], 2.00 and 2.16 (2H, two m, Met β-CH_2), 2.12 (3H, s, S–CH_3), 2.62 (2H, m, Met γ-CH_2), 3.01 (2H, m, Phe β-CH_2), 3.52 (3H, s, OCH_3), 4.20 (1H, m, Phe α-CH), 4.41 (1H, m, Met α-CH), 5.18 (1H, d, $J = 9.1$, Phe NH), 5.24 (1H, d, $J = 8.4$, Met NH), 7.05–7.67 (9H, m, aromatic), 8.87 (1H, bs, Ar–NH).

Anal. Calcd for $C_{26}H_{35}N_3O_7S_2$: C, 55.20; H, 6.24. Found: C, 55.37; H, 6.22.

Boc-Met-NH-*m*C_6H_4-SO_2-Phe-OMe (**7b**)

From Cbz-NH-*m*C_6H_4-SO_2-Phe-OMe (430 mg, 0.92 mmol) and Boc-Met-OH (229 mg, 0.92 mmol) according to the usual procedure. White foam (420 mg; 74%).

$[\alpha]_D - 14$; IR 3691, 3431, 2984, 1742, 1700, 1600, 1497, 1351, 1159 cm^{-1}; ^1H NMR: $\delta = 1.43$ [9H, s, $C(CH_3)_3$], 1.98–2.25 (2H, two m, Met β-CH_2), 2.16 (3H, s, S–CH_3), 2.65 (2H, m, Met γ-CH_2), 3.02 (2H, m, Phe β-CH_2), 3.52 (3H, s, OCH_3), 4.21 (1H, m, Phe α-CH), 4.42 (1H, m, Met α-CH), 5.21 (2H, m, Phe and Met NH), 7.00–7.78 (9H, m, aromatic), 8.65 (1H, bs, Ar–NH).

Anal. Calcd for $C_{26}H_{35}N_3O_7S_2$: C, 55.20; H, 6.24. Found: C, 55.31; H, 6.28.

Boc-βAlaψ[CSNH]-Amp (**9**)

Boc-βAla-Amp (306 mg, 1.0 mmol) was thionated according to the above procedure. The crude product was purified by silica gel flash chromatography (CH_2Cl_2). Pale yellow oil (280 mg, 87%).

$[\alpha]_D + 5$; IR 3380, 2981, 2337, 1698, 1509, 1164 cm^{-1}; ^1H NMR: $\delta = 1.17$ (2H, d, $J = 6.5$ Hz, Amp CH_3), 1.39 [9H, s, $C(CH_3)_3$], 2.74 (3H, m, β-Ala α-CH_2 and 1H of Amp CH_2Ph), 3.01 (1H, A part of the Amp CH_2Ph ABX; $J = 5.4$ and 13.5 Hz;), 3.44 (2H, m, β-Ala β-CH_2), 4.81 (1H, m, Amp α-CH), 5.05 (1H, bs, NHCO), 7.23 (5H, m, aromatic), 7.77 (1H, bs, NHCS).

Anal. Calcd for $C_{17}H_{26}N_2O_2S$: C, 63.32; H, 8.13. Found: C, 63.58; H, 8.06.

Synthesis of the *N*-formyl derivatives **3**, **5**, **8a**, **8b** (general procedure)

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 removal of the excess of formic acid under vacuum, the residue was dissolved in dry DMF (2.0 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 products which were purified by silica gel flash chromatography (CH_2Cl_2/MeOH 98:2).

HCO-Met-βAlaψ[CSNH]-Phe-OMe (**3**)

From **2** (170 mg, 0.34 mmol) according to general procedure. White foam (75 mg, 52%).

$[\alpha]_D + 40$; IR 3690, 3351, 3011, 1730, 1674, 1498, 1232 cm^{-1}; ^1H NMR: $\delta = 1.95$–2.14 (2H, m, Met β-CH_2), 2.13 (3H, s, S–CH_3), 2.58 (2H, m, Met γ-CH_2), 2.74 (2H, m, β-Ala α-CH_2), 3.20–3.33 (4H, m, Phe β-CH_2 and β-Ala β-CH_2), 3.80 (3H, s, OCH_3), 4.00 (1H, m, β-Ala NH), 4.42 (1H, m, Met α-CH), 5.37 (1H, m, Phe α-CH), 6.61 (1H, Met NH), 7.24–7.30 (5H, m, aromatic), 8.17 (1H, s, HCO), 8.71 (1H, poorly resolved d, NH).

Anal. Calcd for $C_{19}H_{27}N_3O_4S_2$: C, 53.63; H, 6.39. Found: C, 53.67; H, 6.43.

HCO-Met-βAlaψ[CH_2NH]-Phe-OMe (**5**)

From **4** (111 mg, 0.24 mmol) according to general procedure. Pale yellow oil (50 mg, 53%).

$[\alpha]_D - 6$; IR 3688, 3418, 2927, 1741, 1668, 1602 cm^{-1}; ^1H NMR: $\delta = 1.62$ (2H, m, N–CH_2*CH_2*CH_2–N), 1.94–2.10 (2H, two m, Met β-CH_2), 2.13 (3H, s, S–CH_3), 2.51, 2.60 and 2.71 (4H, three m, N–CH_2CH_2*CH_2*–N and Met γ-CH_2), 2.92–3.07 (2H, m, A and B of an ABX, $J = 7.7$, 5.9, and 14.1 Hz, Phe β-CH_2), 3.34 (2H, m, N–*CH_2*CH_2CH_2–N), 3.57 (1H, m, Phe α-CH), 3.75 (3H, s, OCH_3), 4.57 (1H, m, Met α-CH), 6.43 (1H, d, $J = 7.5$ Hz, NH), 7.06 (1H, poorly resolved d, NH), 7.15–7.36 (6H, m, aromatic and NH), 8.19 (1H, s, HCO).

Anal. Calcd for $C_{19}H_{29}N_3O_4S$: C, 57.70; H, 7.39. Found: C, 57.79; H, 7.44.

HCO-Met-NH-*p*C_6H_4-SO_2-Phe-OMe (**8a**)

From **7a** (200 mg, 0.35 mmol) according to general procedure. White solid (160 mg, 93%).

$[\alpha]_D - 10$; IR 3690, 3344, 3009, 1742, 1678, 1600, 1348, 1159 cm^{-1}; ^1H NMR (d_6-*DMSO*): $\delta = 1.85$ and 2.04 (2H, two m, Met β-CH_2), 2.03 (3H, s, S–CH_3), 2.48 (2H, m, Met γ-CH_2), 2.44–2.87 (2H, m, A and B of an ABX, $J = 9.0$, 6.5, and 13.5 Hz, Phe β-CH_2), 3.34 (3H, s, OCH_3), 3.89 (1H, m, Phe α-CH), 4.54 (1H, m, Met α-CH), 7.08–7.66 (9H, m, aromatic), 8.06 (1H, s, HCO), 8.38 (1H, d, $J = 8.5$, Phe NH), 8.50 (1H, d, $J = 7.7$, Met NH), 10.44 (1H, bs, Ar–NH).

Anal. Calcd for $C_{22}H_{27}N_3O_6S_2$: C, 53.53; H, 5.51. Found: C, 53.48; H, 5.68.

HCO-Met-NH-*m*C_6H_4-SO_2-Phe-OMe (**8b**)

From **7b** (200 mg, 0.35 mmol) according to general procedure. White foam (153 mg; 89%).

$[\alpha]_D - 20$; IR 3688, 3323, 3009, 1738, 1678, 1594, 1351, 1161 cm^{-1}; ^1H NMR: $\delta = 2.05$ and 2.23 (2H, two m, Met β-CH_2), 2.20 (3H, s, S–CH_3), 2.63 (2H, m, Met γ-CH_2), 3.02 (2H, m, Phe β-CH_2), 3.52 (3H, s, OCH_3), 4.27 (1H, m, Phe α-CH), 4.92 (1H, m, Met α-CH), 5.78 (1H, d, $J = 8.5$, Phe NH), 6.87 (1H, d, $J = 7.7$, Met NH), 7.06–7.94 (9H, m, aromatic), 8.27 (1H, s, HCO), 9.08 (1H, s, Ar–NH).

Anal. Calcd for $C_{22}H_{27}N_3O_6S_2$: C, 53.53; H, 5.51. Found: C, 53.35; H, 5.57.

Biological assays

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. The study was approved by the local Ethics Committee and informed consent was obtained from all participants.

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 (Zigmond, 1973). The actual control random movement is 35 ± 3 μm SE of 10 separate experiments performed in duplicate.

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.

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 μl containing 4×10^5 neutrophils, 100 nmoles cytochrome *c* and KRPG. At zero time different amounts ($10^{-10} - 8 \times 10^{-5}$ 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 nmol O_2^- range.

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 $10^{-10} - 8 \times 10^{-5}$ 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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