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Hybrid α/β-peptides: For-Met-Leu-Phe-OMe analogues containing geminally disubstituted $\beta^{2,2}$- and $\beta^{3,3}$-amino acids at the central position

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Summary. The two fMLF-OMe analogues For-Met-β^3hAc$_6$c-Phe-OMe (**6**) and For-Met-β^2hAc$_6$c-Phe-OMe (**12**) and their corresponding *N*-Boc derivatives **5** and **11** have been synthesized and their biological activity towards human neutrophils evaluated. The *N*-formyl peptides **6** and **12** exhibit good activity as chemoattractans and **12** is highly active in superoxide anion production. The preferred solution conformation of the two *N*-formyl derivatives has been discussed.

Keywords: Chemotactic peptides – Conformation – Hybrid α/β-peptides – Neutrophils

Introduction

Neutrophils are involved in the first line of defence mechanism against pathogen microorganisms. They migrate from blood to sites of infection by following the concentration gradients of chemoattractants such as the cleavage products of bacterial and mitochondrial proteins. The hydrophobic *N*-formyl tripeptide For-Met-Leu-Phe-OH (fMLF) and its synthetic methyl ester (fMLF-OMe) are among the early recognized and highly potent chemotactic agents. Their binding to specific receptors located on the neutrophil plasma membrane gives rise, in addition to the directed migration (chemotaxis), to a cascade of biochemical responses including activation of radical oxygen production and release of proteolytic enzymes (Prossnitz, 1997; Le, 2002).

A number of primary structure–activity studies, based on structurally modified fMLF analogues, have been performed and several fundamental points concerning the key role of the *N*-terminal protecting group in modulating the activity as well as the nature of the constituent amino acids have been established (Freer, 1980, 1982; Higgins, 1996). A more complex picture emerges from the analysis of the literature data analyzing conformation–activity relationships. Here different tridimensional structures have been proposed as representatives of optimal interaction with the neutrophil formyl-peptide receptors (Toniolo, 1991, and references therein). However, both structure–activity and conformation–activity studies (Dentino, 1991; Dugas, 1993; Prasad, 1996) on fMLF analogues highlight the role exerted on the activity by the nature of the central residue. Suitable replacements of the native Leu can in fact give rise to stabilized folded or extended conformations (Formaggio, 1993; Torrini, 1997) and allow at the same time key interactions with the central hydrophobic site which is known to be an important modulator of the biological functions (Toniolo, 1989; Spisani, 2002) of *N*-formyl chemotactic tripeptides.

In accordance with the above reported observations several papers have examined synthesis and activity of fMLF analogues characterized by replacements at the central position. Particular attention has been paid to For-Met-Xaa-Phe-OMe models incorporating $C^{\alpha,\alpha}$-dialkylated glycines with linear (e.g., α-aminoisobutyric acid (Aib); di-*n*-propylglycine (Dpg)) and cyclic (e.g., 1-aminocycloalkane-1-carboxylic acids (Ac$_n$c)) alkyl side chains at position 2 (Toniolo, 1989; Dentino, 1991; Prasad, 1996) (Fig. 1). These α,α-disubstituted aminoacidic residues significantly restrict the available range of backbone conformations and the derived conformationally constrained peptides represent useful probes to get informations on

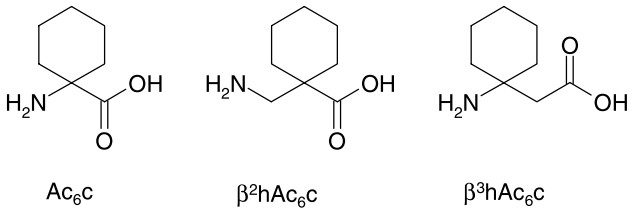


Fig. 1. Geminally disubstituted α- and β-amino acids containing a cyclohexyl side chain

the biological active conformations. In particular, the chemoattractant tripeptides For-Met-Ac_nc-Phe-OMe (with n = 6 and 7), containing 1-aminocycloalkane-1-carboxylic acids in place of native Leu, were found to be very interesting ligands which exhibit both high activity and H-bond stabilized folded conformation (Toniolo, 1989).

By taking into account the above considerations and as part of our previous research aimed at exploring the consequences of incorporating β-residues and higher homologues into short α-peptide sequences (Pagani, 2001; Giordano, 2003, 2004), we report here studies on the two new fMLF analogues For-Met-β^3hAc_6c-Phe-OMe (**6**) and For-Met-β^2hAc_6c-Phe-OMe (**12**) (Fig. 2) in which the central Leu has been replaced by two positional isomers of a geminally disubstituted β-residue (Seebach, 1998) which shares with the Ac_6c the presence of a cyclohexyl side chain (Fig. 1).

It should be noted here that although hybrid α/β-peptides are less studied as compared with the well known class of β-amino acid oligomers (β-peptides: Seebach, 1997; Frackenpohl, 2001; review: Gellman, 1998), they represent an emerging approach for generating analogues of α-peptide endowed with selected resistance to proteolysis and specific conformational properties (Karle, 1997; Gopi, 2002). In particular we have focused our interest on these two unnatural amino acids by taking into account the possibility to combine different points of interest: the improvement of the bioactivity due to the lipophilic side chain; the influence of the different spatial positioning of the side chain on the peptide backbone; the stabilization of folded conformations.

Synthesis

The synthesis of Boc-Met-β^3hAc_6c-Phe-OMe (**5**) and Boc-Met-β^2hAc_6c-Phe-OMe (**11**) has been performed by following two different strategies shown in Schemes 1 and 2. A direct transformation of the *N*-Boc derivatives **5** and **11** into the corresponding *N*-formyl analogues **6** and **12** was performed by following the procedure of Lajoie and Kraus (1984). Tripeptide **5** containing the β^3hAc_6c residue was synthesized starting from methylenecyclohexane by using chlorosulphonyl isocyanate to give the spirolactame **1** (Seebach, 1998) (Scheme 1). A different strategy (Palomo, 1998) was used to obtain the β^2hAc_6c-containing tripeptide **11** by using methyl cyanoacetate as key reagent (Scheme 2).

Biological results

The agonistic activity of the new ligands has been determined on human neutrophils and compared with that of

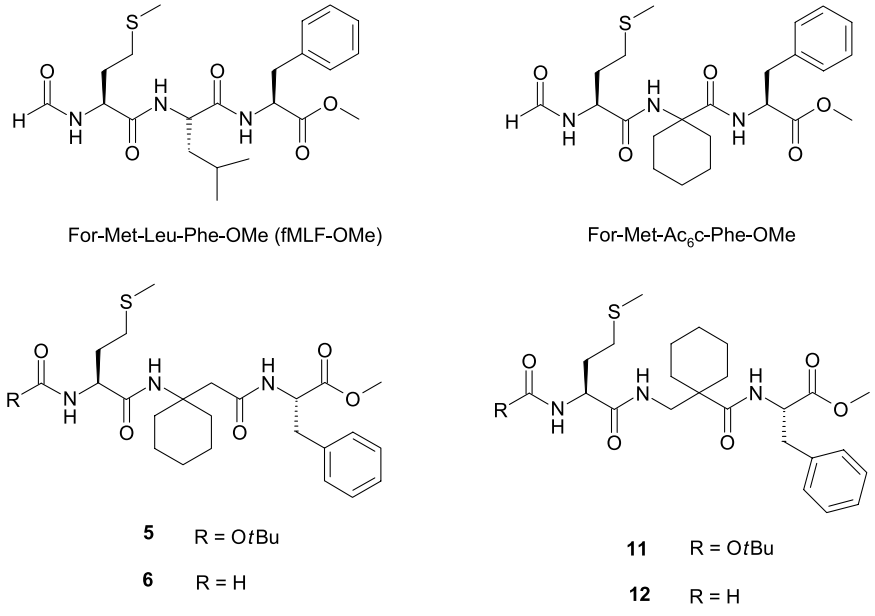
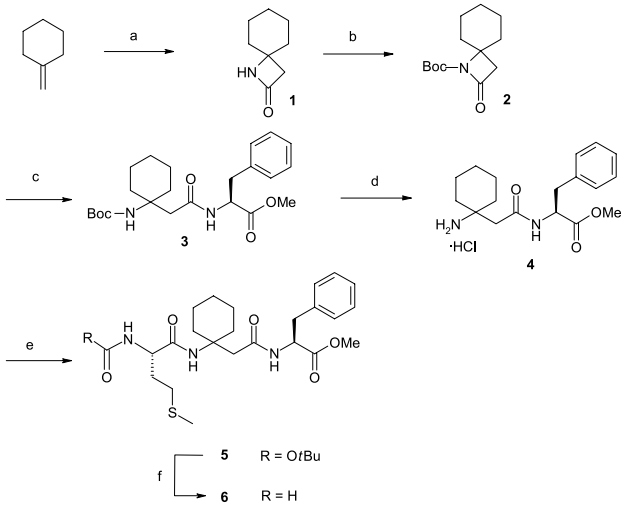
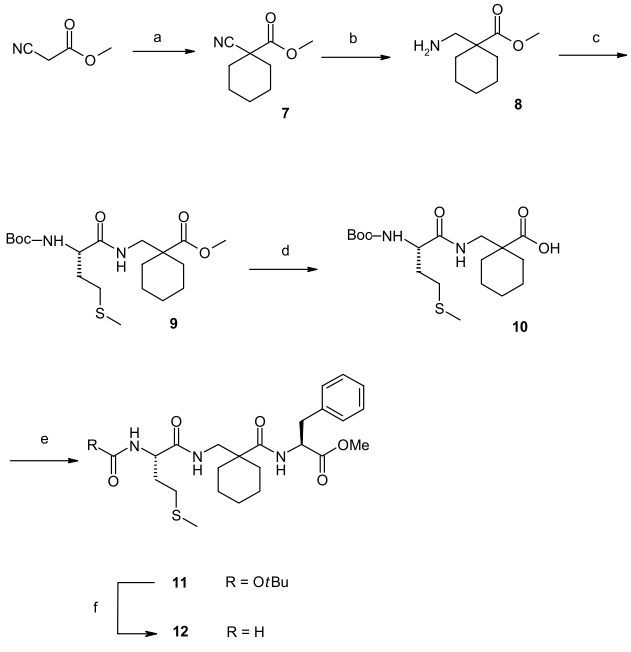


Fig. 2. Structures of the relevant compounds investigated in this study



Scheme 1. Synthesis of the β^3hAc_6c-containing peptide derivatives **5** and **6**. **a** Chlorosulphonyl isocyanate, Et_2O. **b** Boc_2O, DMAP, CH_3CN. **c** NH_2-Phe-OMe, Na_2CO_3, KCN. **d** $SOCl_2$, MeOH. **e** Boc-Met-OH, *i*BuOCOCl, NMM, CH_2Cl_2, DMF. **f** Formic acid, then EEDQ 97%, $CHCl_3$



Scheme 2. Synthesis of the β^2hAc_6c containing peptide derivatives **11** and **12**. **a** K_2CO_3, 1,5-dibromo-pentane, DMF. **b** Pd/C 10%, 10 N HCl, MeOH. **c** Boc-Met-OH, *i*BuOCOCl, NMM, CH_2Cl_2. **d** 1 N LiOH, THF–H_2O (5:1). **e** EDC, HOBT · H_2O, NMM, CH_2Cl_2. **f** Formic acid, EEDQ 97%, dry $CHCl_3$

the standard tripeptide fMLF-OMe; directed migration (chemotaxis), superoxide anion production, and lysozyme release have been measured.

Except for a modest activity shown as chemoattractants (Fig. 3A) and lysozyme releasers (Fig. 3C), the two *N*-Boc derivatives **5** and **11** are completely inactive in the superoxide anion production (Fig. 3B). On the contrary, both the *N*-For-tripeptides **6** and **12** have been found highly active in inducing neutrophil chemotaxis exhibiting an analogous peak which at a concentration of 10^{-10} M reaches the activity of the reference molecule (chemotactic index, 1.0) (Fig. 3A). Analogues **6** and **12** exhibit a comparably weak activity in the superoxide anion production (Fig. 3B). However, for the lysozyme release activity (Fig. 3C) the tripeptide **12** containing the β^2hAc_6c residue is notably more potent and efficient than the positional isomer **6** containing the β^3hAc_6c residue.

Conformational studies

In order to ascertain the involvement of the NH groups of *N*-formyl analogues **6** and **12** in intramolecular H-bonds, a ^1H NMR solvent perturbation experiment was undertaken. In Fig. 4, the chemical shift dependence of the three NH groups of each compound is reported as a function of increasing DMSO-d_6 concentration in $CDCl_3$ solution (10 mM). The results clearly show the different behaviors of the two models **6** and **12**. The NH groups of the two external residues of **6** (Fig. 4A) interact freely with the DMSO while the NH of the central β^3hAc_6c is slightly shielded and then probably involved in a weak intramolecular H-bond (the $\Delta\delta$ ppm values shown by the three NH groups are 0.75, 0.31, and 0.50 for Met, β^3hAc_6c, and Phe, respectively). However, when the IR spectrum in $CHCl_3$ of **6** was examined, the absorptions in the 3400–3300 cm^{-1} stretching region, where the intramolecular H-bonds commonly appear, were found to be very weak.

The tripeptide **12** (Fig. 4B) exhibits a strong H-bonding involvement of the NH of the central β^2hAc_6c together with that of the C-terminal Phe, while the NH of the N-terminal Met appears completely free (the $\Delta\delta$ ppm values are 1.21, −0.16, and 0.21 for Met, β^2hAc_6c, and Phe, respectively). In contrast to the IR spectrum of **6**, that of **12** in $CHCl_3$ shows strong absorption bands in the 3400–3300 cm^{-1} stretching region.

For the β^3hAc_6c-containing model **6**, the above reported data suggest, in addition to a large amount of unfolded conformations, the occurrence of a limited population of folded structures centered at the β-residue (C6 conformation) and associated with a weak intraresidue electrostatic interaction which is not clearly detected by IR spectroscopy (Wu, 1998). In the case of the β^2hAc_6c-

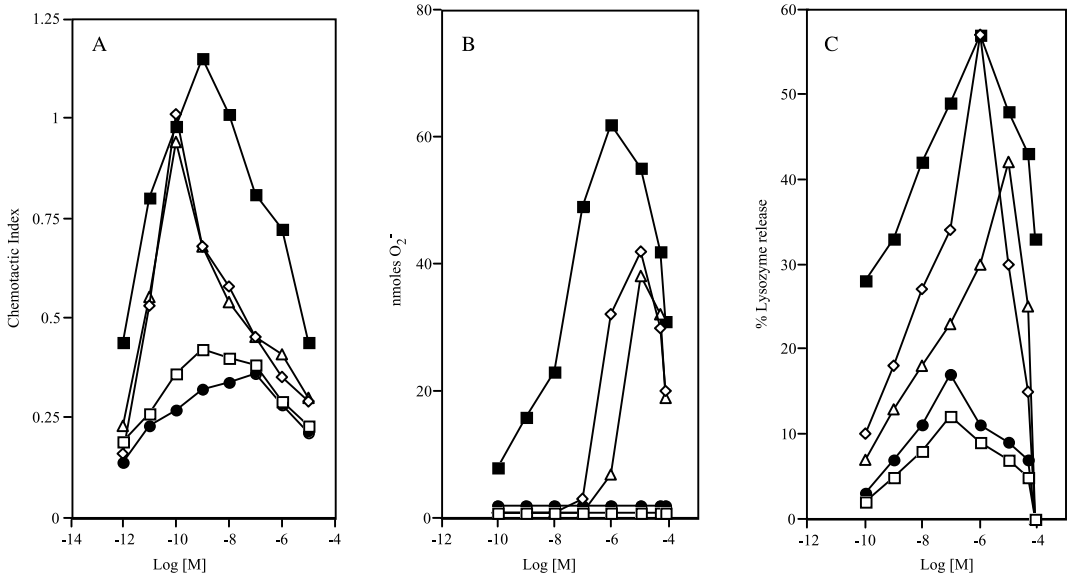


Fig. 3A–C. Biological activity of the tripeptides **5** (●) and **6** (△) and **11** (□) and **12** (◇) compared with the reference ligand fMLF-OMe (■). **A** Chemotactic activity; **B** superoxide anion production; **C** release of neutrophil granule enzymes evaluated by determining lysozyme activity

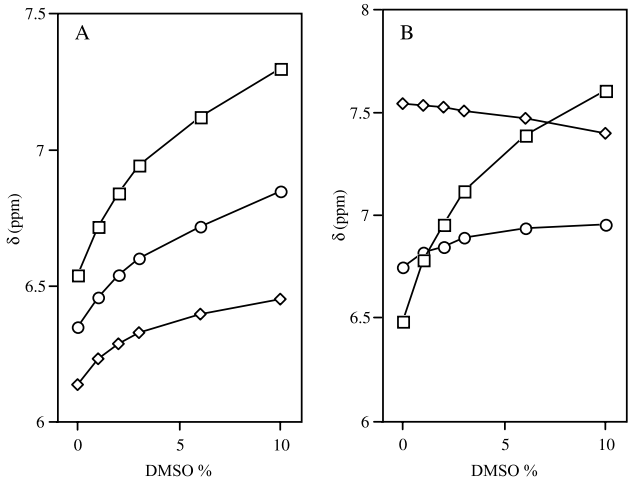


Fig. 4A, B. Delineation of hydrogen-bonded NH groups (□, Met NH; ◇, Ac_6c NH; ○, Phe NH) in tripeptides **6** and **12**. Chemical shift dependence of the NH resonances as a function of the DMSO-d_6 concentration (% v/v) in $CDCl_3$ solution. Peptide concentration 10 mM. **A** HCO-Met-β^3hAc_6c-Phe-OMe (**6**); **B** HCO-Met-β^2hAc_6c-Phe-OMe (**12**)

containing model **12**, the strong involvement in H-bonding of the β-residue NH is accompanied by an analogous, albeit less pronounced behavior of the Phe C-terminal NH. A reasonable interpretation of these data indicates a system of two consecutive local foldings centered at the β^2hAc_6c (C8 conformation; pseudo γ-turn) (Schumann, 2000) and at the Met residue (C7 conformation; γ-turn) as depicted in Fig. 5. In this case, both the intramolecular H-bonds are not highly distorted and clearly revealed by IR spectroscopy.

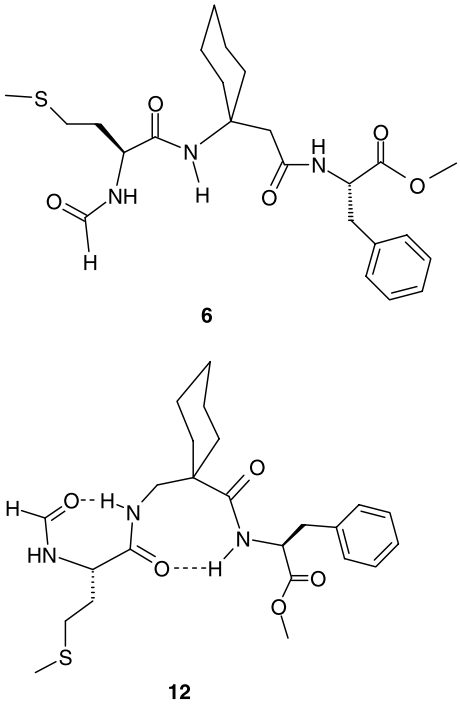


Fig. 5. Representation of the molecular structure of the products **6** and **12**

Discussion and conclusion

As previously mentioned, studies on chemotactic tripeptides indicate that the substitution at the central position of fMLF-OMe is critical for the activity. Concerning replacements related with the here examined analogues, it can be recalled that the incorporation of a central βAla

or β^3hLeu leads to inactive or scarcely active ligands. The present results show, however, that the geminal substitution at the β-residue with the cyclohexyl side chain, regardless of the $\beta^{2,2}$ or the $\beta^{3,3}$ location, restores the activity in accordance with the effects produced by the same cyclic side chain on Ac_6c-containing α-peptides. Only in the case of the activity connected with the lysozyme release, the two positional isomers **6** and **12** exhibit clearly different behaviors. In fact, as previously mentioned, the $\beta^{2,2}$ isomer **12** is sensibly both more efficient and more potent than the $\beta^{3,3}$ and at a concentration of 10^{-6} M shows a peak reaching that of the reference molecule (Fig. 3C). Finally, the conformational behavior exhibited in $CHCl_3$ solution by **6** and **12** shows the effects exerted by the different location of the side chain on the β-residue. However, despite the distinct preferred conformations, the two molecules show substantially comparable biological responses, except for the secretagogue activity. This suggests an induced fit mechanism operative during the interaction of this type of ligands with the human neutrophil receptors.

Experimental

Boc-Met-OH (purum, ≥98%) and HCl·Phe-OMe (purissimum, ≥99%) were purchased from Fluka. Spectrograde solvents were purchased from Merck; all the other solvents and reagents were of the purest grade commercially available. 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. ^{1}H NMR spectra were determined in $CDCl_3$ solution with a Bruker AM 400 spectrometer and chemical shifts were indirectly referred to TMS. TLC and PLC were performed on silica gel Merck 60 F_{254} plates. The drying agent was sodium sulphate. Light petroleum refers to the 40–60 °C bp fraction. 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: β^3hAc_6c, (1-aminocyclohexyl)acetic acid; β^2hAc_6c, 1-(aminomethyl)cyclohexanecarboxylic acid; Boc, *tert*-butyloxycarbonyl; Boc_2O, di-*tert*-butyl dicarbonate; DMF, dimethylformamide; EEDQ, ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; Et_2O, diethyl ether; HOBT, 1-hydroxybenzotriazole; *i*BuOCOCl, isobutyl chloroformate 97%; KRPG, Krebs–Ringer phosphate containing 0.1% (w/v) d-glucose; NMM, *N*-methylmorpholine; PLC, preparative-layer chromatography; TEA, triethylamine; TLC, thin-layer chromatography.

Chemistry

N-Boc-β^3hAc_6c-Phe-OMe (**3**): KCN (1 mmol) and freshly prepared NH_2-Phe-OMe (1.2 mmol) were added to a solution of **2** (Seebach, 1998) in dry DMF (6 ml). The mixture was stirred at 50 °C under N_2 atmosphere for 16 h, then the solvent was evaporated in vacuo and the residue diluted in $CHCl_3$, washed with 5% citric acid, $NaHCO_3$ ss, and NaCl ss. The organic layer was dried with Na_2SO_4, filtered and evaporated in vacuo to give crude **3**. The product was purified by silica gel chromatography using a gradient of $CHCl_3$/EtOAc as eluant to afford 0.32 g of pure **3** (76%) as an oil; $[\alpha]_D + 18°$; IR cm^{-1} 3439, 2935, 1742, 1704, 1666, 1498; ^{1}H NMR δ 1.37–1.97 (19H, m, Boc and Ac_6c side chain), 2.63 (2H, m, CH_2–CO), 3.02–3.16 (2H, A and B of an ABX, J = 5.9, 7.2, and 14 Hz, Phe β-CH_2), 3.72 (3H, s, COOCH_3), 4.49 (1H, s, Ac_6c NH), 4.87 (1H, m, Phe α-CH), 6.17 (1H, d, J = 7.8 Hz, Phe NH), 7.15–7.33 (5H, m, aromatics).

Boc-Met-β^3hAc_6c-Phe-OMe (**5**): The mixture of Boc-Met-OH (0.67 mmol) in 5 ml of CH_2Cl_2 was cooled at −15 °C for 10 min, then *i*BuOCOCl (0.75 mmol) and NMM (0.75 mmol) were added and the mixture was stirred for 10 min at the same temperature. To the solution **4** (0.67 mmol) in CH_2Cl_2, 10% DMF and NMM (0.75 mmol) were added. After 15 min at −15 °C, the mixture was kept at room temperature overnight. The mixture was diluted with $CHCl_3$, washed with 5% citric acid, $NaHCO_3$ ss and NaCl ss. The organic layers were put together, dried with Na_2SO_4, and filtered and the solvent was removed in vacuo to afford 0.35 g of crude **5**. This was purified by silica gel column chromatography using a gradient of $CHCl_3$–EtOAc as eluant to give 0.240 g of pure **5** as colorless oil (65%); $[\alpha]_D + 12°$ ($CHCl_3$ c = 0.5); IR cm^{-1} ($CHCl_3$ c = 0.5%) 3420, 3360, 2936, 1737, 1671; ^{1}H NMR δ 1.27–1.90 (19H, m, Boc and Ac_6c side chain), 1.87–2.58 (6H, m, β^3hAc_6c CH_2CO, Met β-CH_2 and Met γ-CH_2), 2.13 (3H, s, SCH_3), 3.00–3.25 (2H, m, Phe β-CH_2), 3.74 (3H, s, $COOCH_3$), 4.20 (1H, m, Met α-CH), 4.86 (1H, m, Phe α-CH), 5.34 (1H, d, J = 7.7 Hz, Met NH), 6.27 (1H, s, β^3hAc_6c NH), 6.40 (1H, d, J = 7.3, Phe NH), 7.15–7.34 (5H, m, aromatics).

Boc-Met-β^2hAc_6c-OMe (**9**): The mixture of Boc-Met-OH (0.814 mmol) in 5 ml of CH_2Cl_2 was cooled at −15 °C for 15 min, then *i*BuOCOCl (0.82 mmol) and NMM (0.9 mmol) were added and the mixture was stirred for 15 min at the same temperature. Then a solution of **7** (0.814 mmol) in CH_2Cl_2 (4 ml) plus DMF (0.4 ml) was added. After 10 min at −15 °C, the mixture was kept at room temperature overnight. The mixture was diluted with CH_2Cl_2 and washed with of 10% citric acid, $NaHCO_3$ ss, and NaCl ss. The organic layers were put together, dried over Na_2SO_4, and filtered and the solvent removed in vacuo to obtain crude **9**. The product was purified by PLC, with EtOAc–$CHCl_3$ (1:1) as eluant to give pure **9** (90%). $[\alpha]_D$ −15°; IR cm^{-1} ($CHCl_3$ c = 0.5%) 3433, 3029, 1712, 1678, 1496. ^{1}H NMR δ 1.26–1.58 (19H, m, Boc [s at 1.46] and β^2hAc_6c side chain), 1.95–2.58 (7H, m, SCH_3, [s at 2.13] Met β-CH_2 and Met γ-CH_2), 3.39 (2H, m, β^2hAc_6c NHCH_2), 3.71 (3H, s, $COOCH_3$), 4.22 (1H, m, Met α-CH), 5.21 (1H, d, J = 7.3 Hz, Met NH), 6.52 (1H, m, β^2hAc_6c NH).

Boc-Met-β^2hAc_6c-OH (**10**): 1 N LiOH (1.43 ml) was added to the solution of **9** (0.7 mmol) in THF–H_2O (5:1) (3 ml), stirred at 0 °C for 15 min and then left at room temperature overnight. The mixture was concentrated in vacuo and extracted with Et_2O. The aqueous phase was acidified with 1 N HCl and extracted with EtOAc. The organic layer was washed with NaCl ss, dried over Na_2SO_4 and evaporated in vacuo to give 0.13 g (50%) of crude product. The obtained **10** was used into the next step without further purification.

Boc-Met-β^2hAc_6c-Phe-OMe (**11**): Product **10** (0.4 mmol) was added at r.t. to a mixture of EDC (0.4 mmol) and HOBT (0.4 mmol) and NMM (0.8 mmol) in 20 ml of dry CH_2Cl_2. After 10 min, HCl·NH_2-Phe-OMe (0.36 mmol) was added. The reaction flask was kept at room temperature under N_2 atmosphere overnight. The mixture was concentrated in vacuo, diluted with EtOAc, and washed with 5% citric acid, $NaHCO_3$ ss, and NaCl ss. The organic layers were put together, dried with Na_2SO_4, filtered and the solvent was removed in vacuo to obtain the crude **11**. The product was purified by PLC using CH_2Cl_2–MeOH (95:5) as eluant to give 0.16 g of a pure **11** as colorless oil (72%). ^{1}H NMR δ 1.25–1.90 (21H, m, Boc, [s at 1.46] Met β-CH_2 and β^2hAc_6c side chain), 2.10 (3H, s, SCH_3), 2.53 (2H, m, Met γ-CH_2), 3.10–3.30 (2H, A and B of an ABX, J = 4.4, 11.0 and 14 Hz, Phe β-CH_2), 2.70 and 3.70 (2H, two m, β^2hAc_6c NHCH_2), 3.81 (3H, s, $COOCH_3$), 4.08 (1H, m, Met α-CH), 4.88 (1H, m, Phe α-CH), 5.16 (1H, d, J = 8.4 Hz, Met NH), 7.01 (1H, d, J = 8.4 Hz, Phe NH), 7.10–7.30 (5H, m, aromatics), 7.70 (1H, d, $NHCH_2$).

For-Met-β^3hAc$_6$c-Phe-OMe (**6**): $[\alpha]_D$ +16° ($CHCl_3$ c = 0.5); IR cm^{-1} 3423, 3032, 2400, 1739, 1675, 1510; ^{1}H NMR δ 1.27–2.32 (17H, m, β^3hAc$_6$c side chain and CH_2CO, Met β-CH_2, SCH_3 [s at 2.15]), 2.57 (2H, m, Met γ-CH_2), 3.07 and 3.11 (2H, A and B of ABX, J = 5.5 Hz, 7.5 Hz, 14 Hz, Phe β-CH_2), 3.75 (3H, s, $COOCH_3$), 4.59 (1H, m, Met α-CH), 4.83 (1H, m, Phe α-CH), 6.02 (1H, s, β^3hAc$_6$c NH), 6.35 (1H, d, J = 8.2 Hz, Phe NH), 6.55 (1H, d, J = 7.5 Hz, Met NH), 7.15–7.35 (5H, m, aromatics), 8.20 (1H, s, HCO).

For-Met-β^2hAc$_6$c-Phe-OMe (**12**): $[\alpha]_D$ −29.5° ($CHCl_3$ c = 0.17); IR cm^{-1} (CHCl3 c = 0.5%) 3429, 3369, 3337, 2934, 1728, 1673, 1498. ^{1}H NMR δ 1.15–2.05 (12H, m, Met β-CH_2 and β^2hAc$_6$c side chain), 2.15 (3H, s, SCH_3), 2.60 (2H, m, Met γ-CH_2), 3.15–3.27 (2H, A and B of an ABX, J = 4.6, 10, 14 Hz, Phe β-CH_2), 2.86 and 3.75 (2H, m, β^2hAc$_6$c NH*CH$_2$*), 3.84 (3H, s, $COOCH_3$), 4.48 (1H, m, Met α-CH), 4.83 (1H, m, Phe α-CH), 6.50 (1H, d, J = 7.6, Met NH), 6.77 (1H, d, J = 8 Hz, Phe NH), 7.20–7.35 (5H, m, aromatics), 7.56 (1H, m, *NH*CH_2), 8.18 (1H, s, HCO).

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 (mean with standard error) 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 of the difference of migration toward test attractant minus migration toward the buffer to the migration toward the buffer; the values are the means of six separate experiments performed in duplicate. Standard errors are in the 0.02–0.09 CI range.

Superoxide anion production

Superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sigma) 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 (1×10^{-10} to 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 nanomoles 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 nanomoles of O_2^- per 1×10^6 cells per 5 min and are the means of six separate experiments performed in duplicate. Standard errors are in 0.1–4 nanomoles 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 1×10^{-10} to 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 the 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 means of five separate experiments done in duplicate. Standard errors are in the range of 1–6%.

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