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# Synthesis and activity on human neutrophil functions of fMLF-OMe analogs containing alkyl spacers at the central position

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**Abstract**

We report here the synthesis and activity of new analogs of the *N*-formyl and *N*-*tert*-butyloxycarbonyl (Boc) derivatives of the tripeptide Met-Leu-Phe-OMe containing an achiral ω-amino acid residue replacing the hydrophobic central leucine. The tripeptides HCO-Met-NH-$(CH_2)_n$-CO-Phe-OMe and Boc-Met-NH-$(CH_2)_n$-CO-Phe-OMe ($n = 3–5$) containing the central homomorphic residue of 5-aminopentanoic acid (δ-aminovaleric acid; δ-Ava; $n = 4$) and the two non-homomorphic residues of 4-aminobutanoic acid (γ-aminobutyric acid; γ-Abu; $n = 3$) and 6-aminohexanoic acid (ε-aminocaproic acid; ε-Aca; $n = 5$) have been examined. The activity as agonists and antagonists in chemotaxis, lysozyme release, and superoxide anion production of the new analogs has been determined. The *N*-Boc derivatives **2a** and **2b**, incorporating the γ-Abu and the δ-Ava residues, show good and selective antagonist activity on superoxide anion production. 

*Keywords:* Alkyl spacers; ω-Amino acids; Chemotactic peptides; Human neutrophils

## 1. Introduction

Chemotactic *N*-formyl peptides are involved in the defense mechanism against bacterial infections through binding with specific receptors located on the neutrophil membranes. In addition to the cell-directed migration (chemotaxis), the peptide receptor interaction gives rise to a series of biochemical events including production of superoxide anions and lysosomal enzyme release. The *N*-formyl tripeptide HCO-Met-Leu-Phe-OH (fMLF) and its methyl ester (fMLF-OMe) represent the reference models on which a variety of chemical modifications have been performed in order to gain information on the structure–activity relationships.

An examination of the literature reveals that replacement of the hydrophobic central leucine with $C^{\alpha,\alpha}$-disubstituted glycine residues, bearing two linear side chains (Dxx) [1–3] or a cyclo-aliphatic ring system ($Ac_nc$) [4–8], represents a particularly useful modification strategy.

The occurrence of two main features appears relevant in this approach: (i) restriction of the available range of backbone conformations and induction of predictable conformations on the basis of the typology of the substituents at the $C^{\alpha}$-carbon atom; (ii) possibility to optimize the interaction of the central residue with the corresponding receptor area by modulating side-chain bulkiness and overall hydrophobicity.

By synthesizing the above-mentioned type of tripeptide models, possessing the general formula HCO-Met-Xaa-Phe-OMe (with Xaa = Dxx or $Ac_nc$), it was possible to stabilize both extended and folded backbone conformations and demonstrate that neutrophil receptors can efficiently interact with both types of ligands [6]. The operation of the induced-fit mechanism as well as flexibility at the central position of the tripeptide

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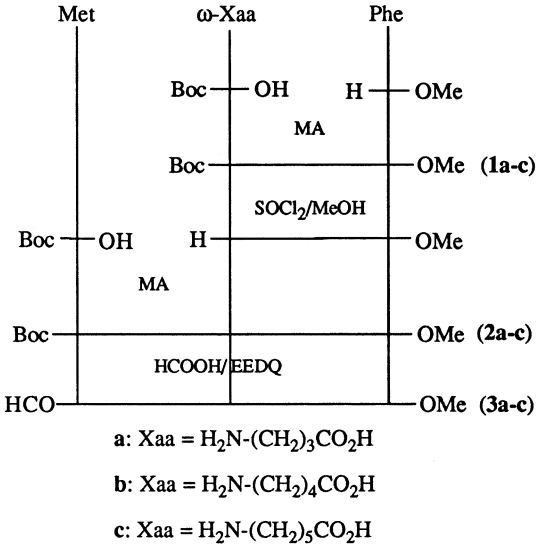



Scheme 1.

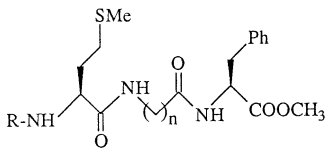
molecule should thus represent factors relevant for the optimization of recognition and binding by the biological target.

In the light of the findings reported above it seemed interesting to incorporate, at the central position of the fMLF-OMe-based chemotactic analogs, residues of ω-amino acids $H_2N–(CH_2)_n–CO_2H$ characterized by an achiral bridge made up of a certain number of methylene units inserted between the amino and the carboxylic group. These residues are largely adopted as useful spacers for designing peptide analogs possessing different degrees of hydrophobicity and enhanced flexibility as compared with the natural counterparts [9,10]. Depending upon the number of methylene units, the ω-amino acid residue may correspond to a dipeptide or tripeptide backbone fragment (e.g. $n = 4$ for a dipeptide; $n = 7$ for a tripeptide) and, on this basis, the spacer is considered homomorphic [11].

In the present paper we report the synthesis and biological activity of the tripeptides Boc-Met-NH-$(CH_2)_n$-CO-Phe-OMe (**2a**–**c**) and HCO-Met-NH-$(CH_2)_n$-CO-Phe-OMe (**3a**–**c**) ($n = 3$–5) containing the central homomorphic residue of δ-aminovaleric acid (δ-Ava; $n = 4$) and the two non-homomorphic residues of γ-aminobutyric acid (γ-Abu; $n = 3$) and ε-aminocaproic acid (ε-Aca; $n = 5$).

## 2. Chemistry

The synthesis of the tripeptides **2a**–**c** and **3a**–**c** was performed according to Scheme 1.




**2**: R = $Bu^tOCO$ **3**: R = H-CO

**a**: n = 3; **b**: n = 4; **c**: n = 5

The peptides **1a**–**c** and **2a**–**c** were prepared in solution via standard coupling reactions which involved isobutyl chloroformate for carboxyactivation (mixed anhydride method) and the *tert*-butyloxycarbonyl (Boc) group for amino protection. Boc-deprotection of compounds **1a**–**c** was performed via acidolysis with thionyl chloride in methanol. Treatment of **2a**–**c** with formic acid followed by ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylaxe (EEDQ) gave the formyl derivatives **3a**–**c**.

Table 1
Physical, analytical, and spectral data of Boc-protected dipeptides **1a**–**c**

| Comp. | Yield (%) | M.p. (°C), crystallization solvent [a] | $[\alpha]_D$ | Formula (MW) [b] | |
|---|---|---|---|---|---|
| **1a** | 83 | 76–77, EA–H | +47° | $C_{19}H_{28}N_2O_5$ (364.44) | |
| **1b** | 100 | 103–104, EA–H | +51° | $C_{20}H_{30}N_2O_5$ (378.47) | |
| **1c** | 96 | 75–76, EA–H | +57° | $C_{21}H_{32}N_2O_5$ (392.49) | |
| *$^1H$ NMR (δ)* | | | | | |
| | Phe | | | ω-Xaa | |
| | α-CH | NH | α-$CH_2$ | ω-$CH_2$ [c] | NH |
| **1a** | 4.84 | 6.65 | 2.20 | 3.10 | 4.84 |
| **1b** | 4.90 | 6.11 | 2.20 | 3.07 | 4.68 |
| **1c** | 4.89 | 6.03 | 2.17 | 3.09 | 4.63 |
| *IR ($cm^{-1}$)* | | | | | |
| **1a** | 3342, 3287, 2981, 1750, 1679, 1643, 1530 | | | | |
| **1b** | 3354, 3326, 2965, 1753, 1739, 1681, 1651, 1532 | | | | |
| **1c** | 3350, 2929, 1743, 1678, 1646, 1524 | | | | |

[a] EA = ethyl acetate; H = *n*-hexane.
[b] Analytical results of C, H, N were within ±0.4% of the calculated values.
[c] Superimposed on the Phe β-$CH_2$ signal.

Table 2
Physical, analytical, and spectral data of Boc-protected tripeptides **2a–c**

| Comp. | Yield (%) | M.p. (°C), crystallization solvent [a] | $[\alpha]_D$ | Formula (MW) [b] |
|---|---|---|---|---|
| **2a** | 76 | 116–117, EA | +43° | $C_{24}H_{37}N_3O_6S$ (495.64) |
| **2b** | 72 | 120–121, EA | +32° | $C_{25}H_{39}N_3O_6S$ (509.68) |
| **2c** | 73 | 133–136, EA–H | +40° | $C_{26}H_{41}N_3O_6S$ (523.70) |

*$^1H$ NMR ($\delta$)*

| | Met | | ω-Xaa | | | Phe | |
|---|---|---|---|---|---|---|---|
| | α-CH | NH | α-$CH_2$ | ω-$CH_2$ | NH | α-CH | NH |
| **2a** | 4.20 | 5.41 | 2.17 | 3.13 [c] | 7.09 | 4.84 | 7.05 |
| **2b** | 4.25 | 5.46 | 2.20 | 3.12 [c] and 3.37 | 6.85 | 4.89 | 6.72 |
| **2c** | 4.25 | 5.43 | 2.17 | 3.20 [c] | 6.71 | 4.88 | 6.32 |

*IR ($cm^{-1}$)*

| | |
|---|---|
| **2a** | 3323, 3310, 2965, 1747, 1685, 1644, 1525 |
| **2b** | 3309, 2927, 1726, 1683, 1641, 1543, 1524 |
| **2c** | 3329, 3310, 2960, 1711, 1688, 1647, 1524 |

[a] EA = ethyl acetate; H = *n*-hexane.
[b] Analytical results of C, H, N were within ±0.4% of the calculated values.
[c] Superimposed on the Phe β-$CH_2$ signal.

Table 3
Physical, analytical, and spectral data of formyltripeptides **3a–c**

| Comp. | Yield (%) | M.p. (°C) | $[\alpha]_D$ | Formula (MW) [a] |
|---|---|---|---|---|
| **3a** | 95 | 106–107 | +41° | $C_{20}H_{29}N_3O_5S$ (423.54) |
| **3b** | 92 | 133–134 | +27° | $C_{21}H_{31}N_3O_5S$ (437.57) |
| **3c** | 78 | 125–127 | +42° | $C_{22}H_{33}N_3O_5S$ (451.60) |

*$^1H$ NMR ($\delta$)*

| | HCO | Met | | ω-Xaa | | | Phe | |
|---|---|---|---|---|---|---|---|---|
| | | α-CH | NH [b] | α-$CH_2$ | ω-$CH_2$ | NH [b] | α-CH | NH |
| **3a** | 8.14 | 4.64 | 7.08 | 2.17 | 3.18 [c] | 6.90–7.36 | 4.82 | 6.91 |
| **3b** | 8.15 | 4.67 | 6.90–7.35 | 2.17 | 3.11 [c] and 3.34 | 6.90–7.36 | 4.86 | 6.65 |
| **3c** | 8.15 | 4.72 | 6.92–7.35 | 2.13 | 3.25 [c] | 6.92–7.35 | 4.86 | 6.48 |

*IR ($cm^{-1}$)*

| | |
|---|---|
| **3a** | 3291, 3086, 2950, 2921, 1746, 1640, 1547 |
| **3b** | 3282, 3093, 1737, 1639, 1561, 1556, 1449 |
| **3c** | 3287, 3086, 2940, 2923, 1755, 1640, 1549 |

[a] Analytical results of C, H, N were within ±0.4% of the calculated values.
[b] Superimposed on aromatic.
[c] Superimposed on the β-$CH_2$ signal.

Physical, analytical, and spectral data of the three groups of peptides reported in Scheme 1 are summarized in Tables 1–3.

## 3. Biological results

The agonist activity of formyltripeptides **3a–c** and the corresponding Boc-protected tripeptides **2a–c** was determined on human neutrophils in three in vitro assays: directed migration (chemotaxis), superoxide anion production, and lysozyme release. Their activity was compared to that of the standard tripeptide fMLF-OMe.

All Boc-derivatives were found to be unable to induce chemotaxis and to trigger superoxide anion production. Concerning the lysozyme release the derivatives **2a–c** show a slight activity, never statistically significant, in the concentration range $10^{-10}$–$10^{-5}$ M (not shown). The formyltripeptides **3a–c**, although unable to elicit superoxide anion production, did show significant activity as chemoattractants (Fig. 1A) and segretagogue agents (Fig. 1B) even if with a lesser potency than the standard tripeptide. In particular, **3a**, containing the γ-Abu residue, is the most active formylpeptide reaching the maximum activity (ca. 0.80 chemotactic index; 7% decrease) at a concentration of $10^{-7}$ M, whereas the ε-Aca derivative **3c** is the least active agonist (ca. 0.30 chemotactic index; 60% decrease

at $10^{-7}$ M). An intermediate activity was observed for **3b** (ca. 0.60 chemotactic index; 32% decrease at $10^{-7}$ M). On the contrary, the maximum value of the lysozyme release activity is shown by **3c** at $10^{-7}$ M (ca. 30% lysozyme release).

The antagonist activity of the Boc-tripeptides **2a–c** was determined by measuring the ability to inhibit the above-cited human neutrophil responses stimulated by the optimal dose of fMLF and compared to that shown by Boc-Met-Leu-Phe-OMe (**4**). The influence of increasing concentration of the examined derivatives on chemotaxis induced by 10 nM fMLF is shown in Fig. 2A. A dose-dependent inhibition of the chemotactic activity can be observed for all the three compounds and the inhibition becomes statistically significant ($P < 0.05$) at the concentration of $10^{-6}$ M for **2b** and **2c** and at $10^{-10}$ M for **2a**, indicating that **2a** is the most efficient analog. The influence on superoxide anion production is reported in Fig. 2B; compounds **2a** and **2b** exert a statistically significant reduction ($P < 0.05$) on the activity induced by 1 μM fMLF starting from $10^{-9}$ M and the inhibition progressively increases with the concentration up to ca. 60 and 45% for **2a** and **2b**, respectively; a weaker antagonist activity is exhibited by **2c**. As shown in Fig. 2C, only a weak inhibitory action on the lysozyme release is observed for the three new compounds.

## 4. Discussion and conclusions

The results reported above indicate that the introduction of an alkyl spacer at the central position of fMLF-OMe based analogs leads to agonists which, although less potent than the parent tripeptide, are able to discriminate the different biological responses associated with the stimulation of human formylpeptide receptors or receptor subtypes. All the three new analogs are, in fact, active as chemoattractants and degranulating agents, but practically inactive as superoxide anion producers. Furthermore, the finding that **3c**, the least-active analog as a chemotactic agent, is found to be the most active secretagogue and that **3a**, the least-active secretagogue, is the most active as a chemoattractant, underlines the receptor functional heterogeneity and the complex control systems of the signal transduction mechanisms [12–15].

The selectivity shown by the *N*-formyltripeptide agonists **3a–c** is not maintained in the *N*-*tert*-butyloxycarbonyl derivatives **2a–c** which inhibit, although with different intensity, all the three examined biological functions. While only a slight inhibition has been found on chemotaxis and lysozyme release (Fig. 2A and C), a marked inhibitory activity, well superior to that associated with the *N*-Boc tripeptide **4** containing a central leucine residue, is observed for the two new analogs **2a** and **2b** on superoxide anion production. In this case, the most efficient inhibitor **2a** is obtained when the shortest spacer (i.e. γ-Abu) has been introduced and the inhibition reaches values which are comparable to those shown by *N*-Boc-MLF-OMe (**4**) in the chemotactic activity (Fig. 2A). High and selective affinity for the receptor subtype responsible for $O_2^-$ production is then associated with the new *N*-Boc analogs **2a** and **2b** incorporating alkyl spacers with three (γ-Abu) or four methylene groups (δ-Ava). It should also be noted that

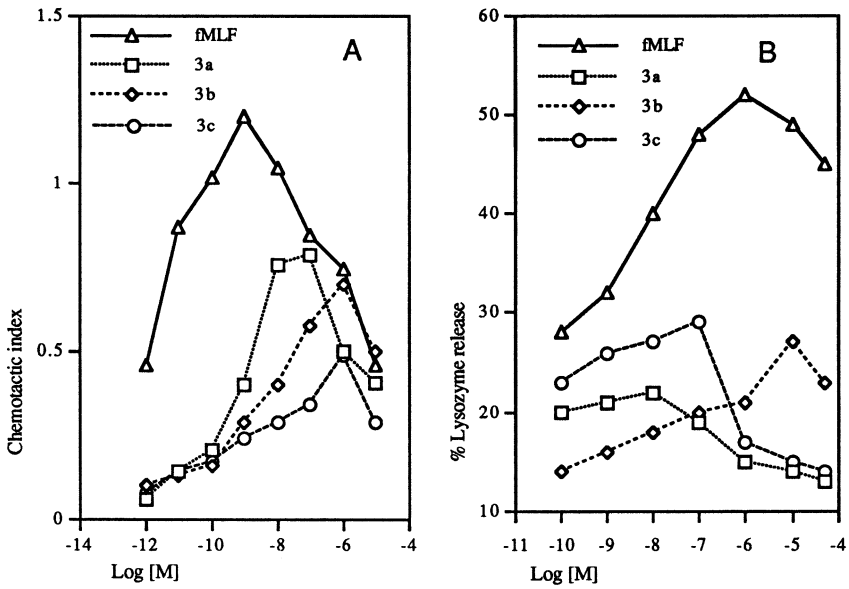



Fig. 1. Biological activity of formyltripeptides **3a–c** towards human neutrophils: (A) chemotactic activity; (B) release of neutrophil granule enzymes evaluated by determining lysozyme activity.

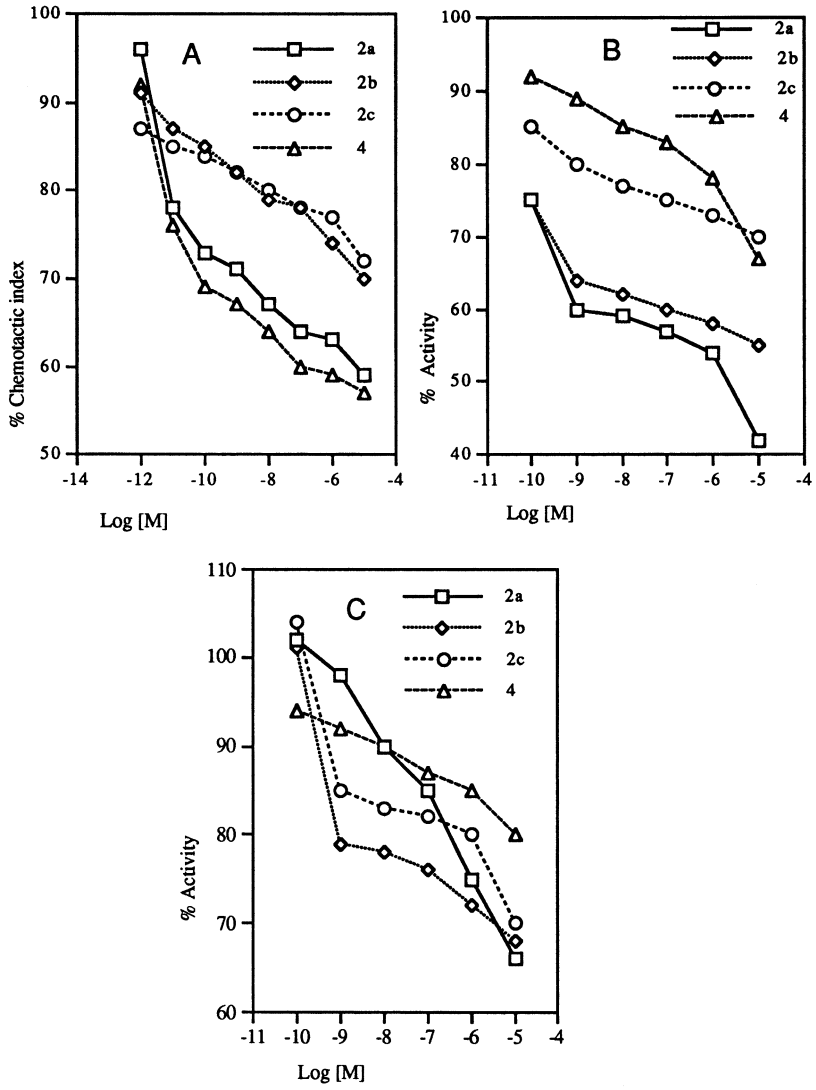



Fig. 2. Effect of Boc derivatives **2a**–**c** and **4** on chemotaxis activated by 10 nM fMLF (A), on superoxide anion production (B), and on release of neutrophil granule enzymes (C) triggered by 1 μM fMLF.

the most active antagonist **2a** contains the shortest alkyl spacer γ-Abu as it is found in the case of the most active agonist **3a**.

In conclusion, the strategy adopted here, based on the replacement of the central leucine with achiral residues of ω-amino acids in chemotactic formyltripeptides, leads to moderately active and selective agonists and, in the case of the *N*-Boc derivatives, to new efficient antagonists of superoxide anion production. By taking into account the considerable interest associated with the development of high-affinity ligands for the human neutrophil receptor, in view of their potential use as diagnostic and therapeutical agents [16,17], the results reported here indicate that proper structural modifications at the central position of *N*-Boc-MLF-OMe based ligands can lead to potent and selective antagonists. Further studies are in progress in our laboratories to better define this point.

## 5. Experimental

### 5.1. Chemistry

Melting points were determined with a Kofler hot-stage 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 disks) were recorded employing a Perkin–Elmer 983 spectrophotometer. $^1H$ NMR spectra were determined in $CDCl_3$ solution with a Bruker AM 200 spectrometer using $Me_4Si$ as internal standard. TLC and PLC were performed on Merck 60 $F_{254}$ silica gel plates. The drying agent was sodium sulfate. Parent fMLF-OMe, Boc-Met-Leu-Phe-OMe, Boc-δ-Ava-OH, and Boc-ε-Aca-OH were prepared as described in the literature [18,19]. Boc-Met-OH (Fluka Chemie AG, Switzerland), HCl·Phe-OMe (Fluka Chemie AG,

Switzerland), Boc-γ-Abu-OH (Sigma, USA), 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. The abbreviations used are as follows: γ-Abu, γ-aminobutyric acid (4-aminobutanoic acid); ε-Aca, ε-aminocaproic acid (6-aminohexanoic acid); δ-Ava, δ-aminovaleric acid (5-aminopentanoic acid); Boc, *tert*-butyloxycarbonyl; EEDQ, ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate; KRPG, Krebs–Ringer-phosphate containing 0.1% w/v D-glucose (pH 7.4); PLC, preparative layer chromatography.

#### 5.1.1. General procedure for the synthesis of dipeptides *1a–c*

Isobutyl chloroformate 95% (0.14 ml, 1 mmol) was added at −15 °C to a stirred solution of Boc-ω-Xaa-OH (1 mmol) and *N*-methylmorpholine (0.13 ml, 1.2 mmol) in dry $CH_2Cl_2$ (4.8 ml). The temperature was kept at −15 °C for 10 min, and then HCl·Phe-OMe (0.216 g, 1 mmol), *N*-methylmorpholine (0.11 ml, 1 mmol), and dry $CH_2Cl_2$ (3.6 ml) were added. The mixture was stirred at −15 °C for 15 min and then at room temperature (r.t.) 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 to give a pure solid residue in the case of **1b** and **1c**. Crude **1a** was purified by PLC [$CH_2Cl_2$–EtOAc (8:2)].

#### 5.1.2. General procedure for the synthesis of tripeptides *2a–c*

Thionyl chloride (0.076 ml, 1.05 mmol) was added to a solution of Boc-ω-Xaa-Phe-OMe (**1a–c**) (1 mmol) in dry methanol (1 ml), cooled at −15 °C. After stirring at −15 °C for 30 min and at 45 °C for 4 h, the solution was evaporated under vacuum to give HCl·H-ω-Xaa-Phe-OMe as a foam. This salt was used without further purification. Boc-Met-OH (0.249 g, 1 mmol) was activated with isobutyl chloroformate 95% (0.14 ml, 1 mmol) and *N*-methylmorpholine (0.13 ml, 1.2 mmol) in dry $CH_2Cl_2$ (4.8 ml) as described above for Boc-ω-Xaa-OH. Addition of a solution of HCl·H-ω-Xaa-Phe-OMe and *N*-methylmorpholine (0.11 ml, 1 mmol) in dry $CH_2Cl_2$ (3.6 ml) and usual work-up afforded an oily residue which was purified by PLC [$CH_2Cl_2$–EtOAc (1:1)].

#### 5.1.3. General procedure for the synthesis of formylpeptides *3a–c*

The *N*-Boc-protected peptide (1 mmol) was dissolved in formic acid (6 ml) and the mixture was stirred at r.t. for 24 h. After removal of the excess of formic acid in vacuo, the residue was dissolved in dry chloroform (6 ml) and EEDQ 97% (1.2 mmol) was added. The solution was stirred at r.t. for 24 h. Evaporation under reduced pressure afforded an oily residue, in the case of **3a** and **3b**, which was dissolved in dry chloroform, and the product was precipitated by *n*-hexane. Washing with dry ether afforded the pure title compounds **3a** and **3b**. The solid residue, obtained by evaporation of the reaction mixture containing **3c**, was filtered and washed with dry ether.

### 5.2. Biological assays

#### 5.2.1. Peptides

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

#### 5.2.2. Cell preparation

Cells were obtained from the blood of healthy subjects, and human peripheral blood neutrophils were purified employing the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Ficoll–Paque (Pharmacia), and hypotonic lysis of contaminating red cells. The cells were washed twice and resuspended in KRPG, pH 7.4, at a final concentration of $50 \times 10^6$ cells/ml and kept at r.t. until used. Neutrophils were 98–100% pure and 99% viable, as determined by the Trypan blue exclusion test.

#### 5.2.3. Random locomotion

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Milan, Italy) and the migration into the filter was evaluated by the method of leading-front [20]. The actual control random movement is 35 μm ± 3 SE of ten separate experiments performed in duplicate.

#### 5.2.4. 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 (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 chemotactic index range.

#### 5.2.5. 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 $4 \times 10^5$ neutrophils, 100 nmol cytochrome *c* and KRPG. At zero time different amounts ($10^{-10}$–$5 \times 10^{-5}$ 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 wavelengths of 550 and 468 nm. 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 are expressed as net nanomoles of $O_2^-/1 \times 10^6$ cells/5 min and are the mean of six separate experiments done in duplicate. Standard errors are in the 0.1–4 nmol $O_2^-$ range.

*5.2.6. Enzyme assay*

The release of neutrophil granule enzymes was evaluated by determining the lysozyme activity, modified for microplate-based assays. Cells, $3 \times 10^6$ per 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 $10^{-10}$–$2 \times 10^{-5}$ M for a further 15 min. The plates were then centrifuged at 400*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 the total enzyme content released by 0.1% Triton X-100. Total enzyme activity was $85 \pm 1$ μg/$1 \times 10^7$ cells/min. The values are the mean of five separate experiments done in duplicate. Standard errors are in the 1–6% range.

*5.2.7. 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. Antagonist activity data (percentage of activity) were obtained by comparing the chemotactic index, nanomoles 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 was $1.15 \pm 0.10$ SE. $O_2^-$ generation produced by 1 μM fMLF was $43 \pm 2$ nmol/$1 \times 10^6$ cells/5 min. Enzyme activity triggered by 1 μM fMLF 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 done in duplicate. Standard errors are within 10% of the mean value.

*5.2.8. Statistical analysis*

The non-parametric Wilcoxon test was used in the statistical evaluation of differences between groups.

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