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Synthesis and biological evaluation of new active For-Met-Leu-Phe-OMe analogues containing *para*-substituted Phe residues

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In the present study, we report synthesis and biological evaluation of the *N*-Boc-protected tripeptides 4a–l and *N*-For protected tripeptides 5a–l as new For-Met-Leu-Phe-OMe (fMLF-OMe) analogues. All the new ligands are characterized by the *C*-terminal Phe residue variously substituted at position 4 of the aromatic ring. The agonism of 5a–l and the antagonism of 4a–l (chemotaxis, superoxide anion production, lysozyme release as well as receptor binding affinity) have been examined on human neutrophils. No synthesized compounds has higher activity than the standard fMLF-OMe tripeptide to stimulate chemotaxis, although compounds 5a and 5c with -CH_3 and -$C(CH_3)_3$, respectively, in position 4 on the aromatic ring, are better than the standard tripeptide to stimulate the production of superoxide anion, in higher concentration. Compounds 4f and 4i, containing -F and -I in position 4, respectively, on the aromatic ring of phenylalanine, exhibit significant chemotactic antagonism. The influence of the different substitution at the position 4 on the aromatic ring of phenylalanine is discussed.

Supporting information may be found in the online version of this article.

Keywords: biological activity; chemotaxis; receptor binding; human neutrophils; fMLF-OMe analogues; peptidomimetics

Introduction

The chemotactic agent *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (For-Met-Leu-Phe; fMLF) can be isolated from bacterial suspensions and can also be found in disrupted mitochondria [1,2]. This small *N*-acyl tripeptide is the prototype and the most active component of a family of *N*-formyl-methionyl peptides that act as molecular switches capable to trigger the directional migration (chemotaxis) towards the infection sites of human neutrophils, the phagocytic cells specialized in host defense against invading pathogens. The induced chemotactic response is mediated by the interaction of the *N*-formylpeptides with specific G-protein-linked receptors (high-affinity formylpeptide receptor, FPR and its low-affinity subtype, FPRL-1) located on neutrophil cell membrane [3–5]. The FPRs activation initiates the migration of neutrophils along the increasing concentration gradient of chemoattractant agents and is followed by the production of bacterial killing cytotoxic agents triggered by the interaction of the ligand with low-affinity FPRL-1 receptors. Relevant and extensively studied among these functions are the catalyzed conversion of molecular oxygen to superoxide anion (O_2^-) (respiratory burst) [6] and the release of lysosomal enzymes. However, because of the complexity of the intracellular signaling pathways [7] and the peculiar characteristics of the involved receptors, which are rapidly desensitized and internalized soon after the interaction with the ligand, several aspects concerning the involved biochemical mechanisms and the structure–activity relationships concerning *N*-formylpeptides are still scarcely understood and continue to be the object of active investigation [8–12].

Since the Schiffmann original discovery of the chemoattractant activity of *N*-formylpeptides [13], the tripeptide fMLF and its methyl ester For-Met-Leu-Phe-OMe (fMLF-OMe) have been adopted as reference model for structure–activity relationships and conformational studies [8]. Great attention has been dedicated to the role exerted by the *N*-formyl group and the Met residue at the first position. Results clearly indicate that these two *N*-terminal elements are required for optimum activity although different amino acids, with linear or cyclic aliphatic side chains, can suitably replace the central leucine. Comparable less attention has been dedicated to the *C*-terminal third residue.

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Early studies performed on the fMLF-OH analogue For-Nle-Leu-Phe(4-Cl)-OH suggested that the receptor area interacting with the aromatic ring should be spatially restricted [14]. More recently, a series of fMLF-OMe analogues with hydrophilic residues replacing the Phe at position 3 has been examined, and a good interaction with the receptor subtype stimulating the chemotactic migration has been evidenced [15]. However, a systematic replacement of the native residue at position 3 with Phe analogues, characterized by different substituents on the aromatic ring, is not thus far available. This approach should give valuable information on the features of the hydrophobic receptor pocket as well as on the possibility to obtain ligands capable to select the various neutrophil biological functions.

Here, we report results concerning synthesis and biological evaluation of a series of fMLF-OMe analogues in which the *para* hydrogen atom of the *C*-terminal Phe aromatic ring is replaced by a series of substituents possessing different steric and electronic properties (Figure 1). The biological activity (chemotaxis, superoxide anion production and lysozyme release) has been determined on human neutrophils and compared with that of the reference ligand fMLF-OMe. The *N*-formyl tripeptides **5a–l** were examined as agonists, whereas the corresponding *N*-Boc derivatives **4a–l**, obtained as intermediates during the synthesis of **5a–l**, were tested for their activity as antagonists towards the biological functions stimulated by fMLF-OMe. Antagonistic activity at the FPRs is the object of active research due to the great potential as diagnostic and therapeutic agents of this type of ligands [16,17]. The different biochemical behavior of *N*-formyl and *N*-Boc derivatives – agonism *versus* antagonism – is well known and only in part related to the different size of the groups. The key factor resides in the type of the bond joining the *N*-terminal protecting group to the backbone, which is urethanic in the Boc as opposed to amidic in the For group. In following this observation, several potent chemotactic antagonists, characterized by urethanic or ureic protecting group at the amino terminus, have been synthesized [18–22].

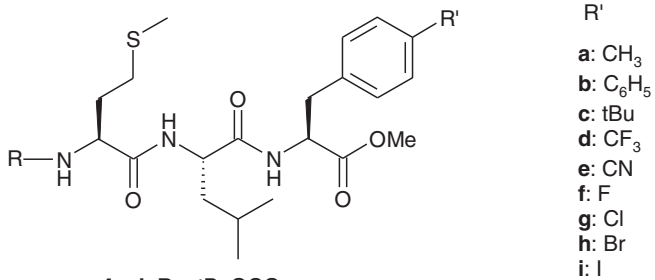


Figure 1. Relevant structures discussed in the text.

Experimental Procedures

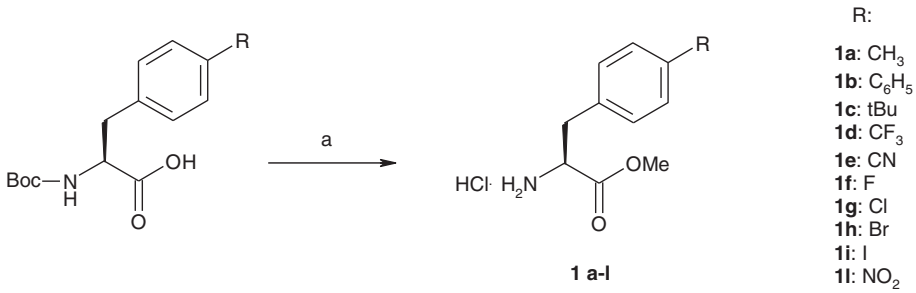
All the starting materials and reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., Saint Louis, MO, USA). ^{1}H NMR spectra were determined in $CDCl_3$ solution with a Varian 300 MHz (Varian Medical Systems, Inc., Palo Alto, CA, USA), and chemical shifts were referred to TMS. The mass spectra were performed on a TOF-ESI spectrometer. Thin layer chromatography was performed on silica gel Merck (Merck & Co., Inc., Whitehouse Station, NJ, USA) 60 F254 plates. Experimental detail and characterization of all new compounds can be found in the supporting information.

Synthesis

The synthesis of the intermediate products **1a-l** and final products **4a-l** and **5a-l** is reported in Schemes 1, 2, 3 respectively. The Boc-protected tripeptides **4a–l** were synthesized starting from Boc-Met-Leu-OMe. The dipeptide methyl ester was hydrolysed to the intermediate Boc-Met-Leu-OH and then coupled with the different HCl·H-Phe(4-R)-OMe (**1a–l**) by using the usual EDC/HOBt procedure (Scheme 2). Aminoesters **1a–l** were obtained in a single step starting from the commercially available *N*-protected aminoacids Boc-Phe(4-R) -OH by treatment with $SOCl_2$/MeOH as reported in Scheme 1. The *N*-Boc tripeptides **4a–l** were then directly converted into the corresponding *N*-formyl derivatives according to the procedure of Lajoie-Kraus [23]. Thus, treatment with formic acid, followed by ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate (EEDQ), gave the derivatives **5a–l** (Scheme 3).

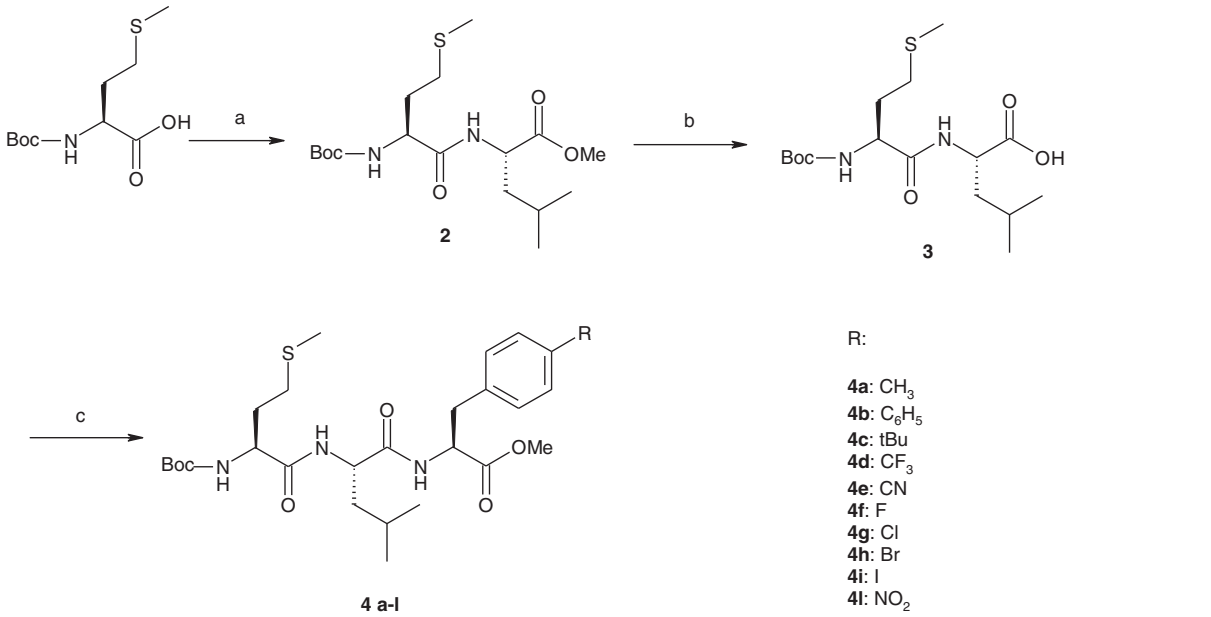
All products were synthesized in solution by using the EDC/HOBt/DIPEA coupling method. The corresponding active esters of additives such as *N*-hydroxy derivatives are less reactive than O-acylisourea. However, these additive increases the efficiency of carbodiimide-mediated reactions. First of all, they suppress the formation of *N*-acylurea. The beneficial effect of HOBt is attributed to its capacity to protonate O-acylisourea, thus preventing the intramolecular reaction from occurring and shifting the reaction to form the corresponding active esters and thereby decreasing the degree of racemization in numerous cases [24].

The N^{α}-terminal Boc-protected peptides were all deprotected by a mixture of TFA in DCM 1 : 1 at r.t. The intermediate TFA salts were used for the following reaction without further purifications. Intermediate products **3**, **5** were purified by precipitation in

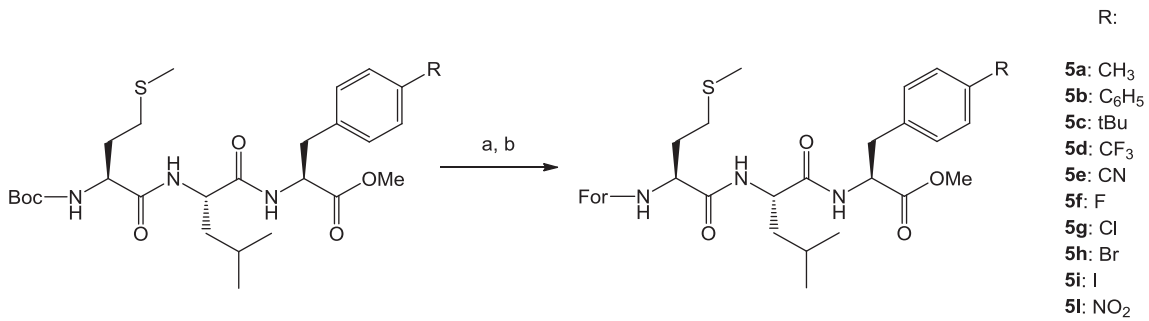


Scheme 1. Synthesis of compounds **1a–l**: (a) $SOCl_2$, MeOH.

Journal of PeptideScience



Scheme 2. Synthesis of compounds **4a–l**: (a) HCl·H-Leu-OMe, DIPEA, HCl·EDC, HOBt, CH_2Cl_2; (b) NaOH 1N, MeOH; (c) HCl·H-Phe(4-R)-OMe, EDC, HOBt·H_2O, DIPEA, CH_2Cl_2.



Scheme 3. Synthesis of compounds **5a–l**: (a) HCOOH; (b) $CHCl_3$, EEDQ.

EtOAc, the filtrate was washed with 3×50 ml of $NaHCO_3$ s.s., 3×50 ml of 5% citric acid, 3×50 ml of deionized water, dried under vacuum, followed by trituration with warm diethyl ether and used in the next step without further purification. All Boc-protected products **1a–l**, **2a–l**, **3a–l** were purified by silica gel column chromatography. All the final products **4a–l** and **5a–l** were purified by RP-HPLC with C_{18} semipreparative column using a linear gradient of H_2O/acetonitrile as eluent from 5% acetonitrile to 90% acetonitrile in 45 min. All the intermediate salts were used in the next step without further purifications.

The purity of the final products, determined by NMR analysis and by analytical RP-HPLC (C_{18}-bonded 4.6×150 mm) at a flow rate of 1 ml/min on a Waters binary pump 1525 (Waters Corporation, Milford, MA, USA) using an isocratic elution of 20% CH_3CN/H_2O 0.1% TFA, monitored with a Waters 2996 Photodiode Array Detector, was found to be >95%.

General Synthetic Procedures

Esterification of Boc-protected amino acids

Thionyl chloride (1.6 mmol) was added dropwise at 0 °C to a stirred solution of Boc-protected amino acid (0.8 mmol) in anhydrous methanol (10 ml), and the resulting solution was stirred at r.t. for 3 h. The oily residue obtained after evaporation under reduced pressure was triturated with Et_2O to give the desired product as chloridrate salt, used for the subsequent reaction.

Coupling reaction

To an ice-cooled mixture containing *N*-protected amino acid or peptide (0.7 mmol) in CH_2Cl_2 (20 ml), EDC·HCl (0.8 mmol), HOBt (0.8 mmol), DIPEA (2.3 mmol) and the required *C*-protected amino acid (0.7 mmol) were added. The reaction mixture was allowed to warm at r.t. overnight and evaporated under reduced pressure. The residue was then dissolved in EtOAc and washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine. The organic phase was dried on Na_2SO_4, and the solvent evaporated under reduced pressure to give the desired product.

Methyl ester hydrolysis

To an ice-cooled solution of methyl ester (5.3 mmol) in methanol (60 ml), 1N NaOH (16 ml) was added dropwise. The mixture was stirred at 0 °C for 15 min and then at room temperature overnight. The solvent was evaporated under reduced pressure, water (15 ml) was added and the solution extracted with diethyl

ether. The aqueous phase was then acidified with 5% citric acid and extracted with AcOEt (3×20 ml). The organic phase was dried on Na_2SO_4 and evaporated under reduced pressure to give the desired compound.

Tripeptide formylation

The *N*-Boc-tripeptide (0.2 mmol) was dissolved in formic acid (5 ml) and the mixture was stirred at room temperature for 24 h. After removal of the excess of formic acid under reduced pressure, the residue was dissolved in 5 ml of dry chloroform. EEDQ (0.2 mmol) was added, and the solution was stirred at room temperature for 24 h. Evaporation under reduced pressure afforded a crude residue, which was purified by silica gel chromatography to give the pure desired compound.

Biological Activity Studies

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 containing 0.1% w/v glucose (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 [25]. 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 (C.I.), 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.

Superoxide anion (O_2^-) production

This anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sigma, St. Louis, MO, USA) modified for microplate-based assays. Tests were carried out in a final volume of 200 μL containing 4×10^5 neutrophils, 100-nmol cytochrome *c* (Sigma) and KRPG. At zero time, different amounts (10^{-10}–5×10^{-5} M) of each peptide were added, and the plates were incubated into a microplate reader (Ceres 900, Bio-TeK Instruments, Inc.; Winooski, VT, USA) 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 a molar extinction coefficient for cytochrome *c* of 18.5 $mmol^{-1}$ 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 $O_2^-/1 \times 10^6$ cells/5 min and are the mean of six separate experiments performed in duplicate.

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}–5×10^{-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 performed in duplicate.

Binding assays

In a final volume of 0.2 ml, 5.10^{-7} cells/ml were incubated in the presence of 6 nM ^{3}H-fMet-Leu-Phe and different concentrations of displacing agents. The incubation was carried out at 37 °C for 15 min, and the bound ligand was separated from the free ligand by rapid filtration of the mixture through Whatman GF/B glass fiber filters. Radioactivity of the filters was then measured in a Beckman LS 1801 spectrometer (Beckman Coulter Inc., Brea, CA, USA). Reported values refer to specific binding, defined as the total amount of ^{3}H-fMLF bound minus the nonspecific. Nonspecific is defined as the amount of binding not inhibited by 10-μM unlabeled fMLF.

Antagonist activity

Antagonist activity was determined by measuring the peptide's ability to inhibit chemotaxis, O_2^- production or granule enzyme release as activated by fMLF-OMe. Antagonist's activity data (percentage of activity) were obtained by comparing the chemotactic index, nanomoles of O_2^- or the percentage of lysozyme release in the absence (100%) and in the presence of analogues. 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.

Statistical analysis

The nonparametric Wicoxon test was used in the statistical evaluation of differences between groups. Differences were considered to be statistically significant at $P \leq 0.05$.

Results and Discussion

Agonism

Biological activity of the new analogues **4a–l** and **5a–l** has been tested on human neutrophils and compared with that shown by fMLF-OMe used as standard ligand. Chemotactic activity data of the new compounds **5a–l** are reported in Figures 2A and 3A, and are expressed in terms of chemotactic index (C.I.). The resulting dose–response curves, as usually found for

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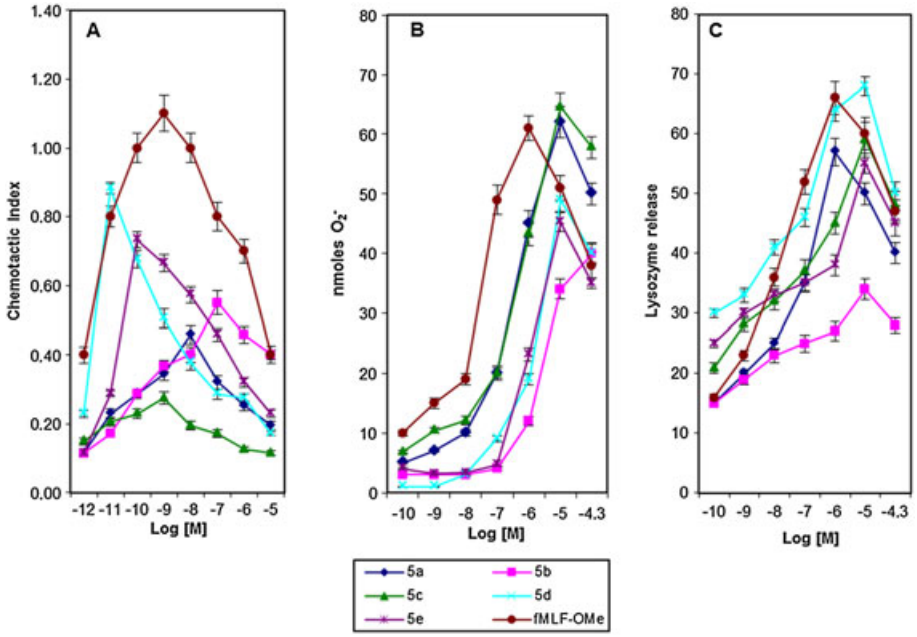

Figure 2. Biological activities of *N*-For tripeptides 5a-e and fMLF-OMe. (A) chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining the lysozyme activity.

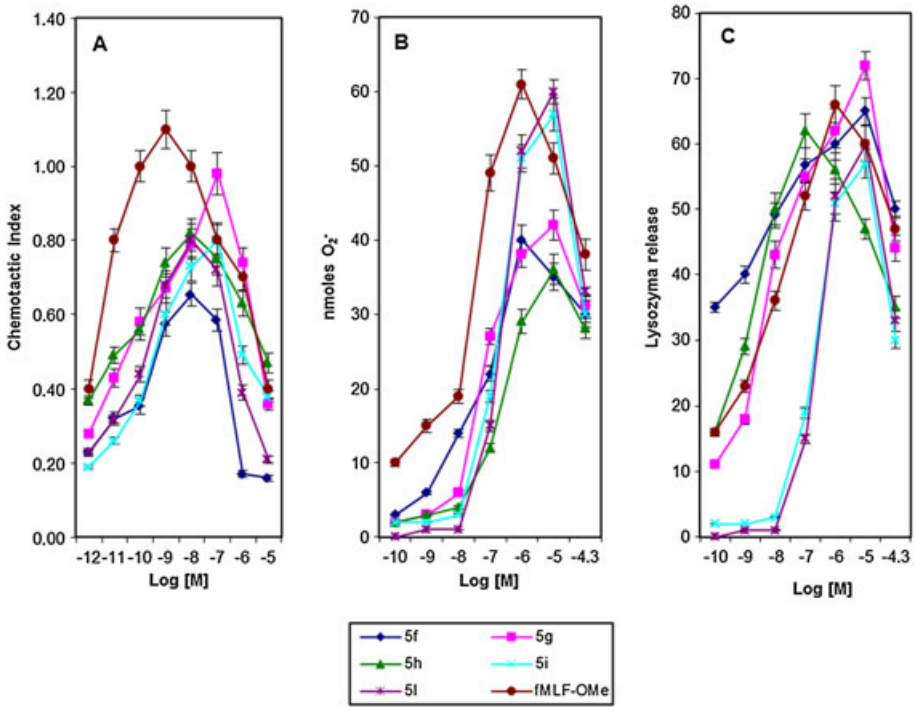

Figure 3. Biological activities of *N*-For tripeptides **5f–l** and fMLF-OMe. (A) chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining the lysozyme activity.

chemoattractants, increase to reach a peak and then decrease rapidly as the concentration of the ligand increases. As shown in the Figures 2A–3A, the new ligands **5a–l** do not reach the efficacy of the standard, which shows a C.I value of 1.10 at a concentration of 10^{-9} M. It is however remarkable that the two tripeptides **5d** and **5e**, characterized by Phe(4-CF_3) and Phe(4-CN) residue at position 3 of the backbone, exhibit the C.I. maximum at a concentration sensibly lower (10^{-11} and 10^{-10} M, respectively) than that required by fMLF-OMe, with a value that, in the case of **5d**, is slightly higher than that exhibited by the standard at the same concentration (C.I. at 10^{-11} M: 0.88 and 0.80 for **5d** and fMLF-OMe, respectively). Thus, both **5d** and **5e**, although less efficacious than the parent, are both the most potent of series, being maximally effective at the concentration of 10^{-10} and 10^{-11} M, respectively. A high activity is also shown by the Phe(4-Cl) containing tripeptide **5g** which, with a C.I. peak of 0.98

at 10^{-7} M, although moderately potent, is the most efficacious among all the here-reported derivatives. As for the other three halogen derivatives, the best effect is exerted by the Phe(4-Br) substituent in **5h** (C.I. maximum = 0.82 at 10^{-8} M) . The Phe(4-I) **5i** is slightly less favorable than **5h** but is clearly better than that of the Phe(4-F) residue in **5f** (C.I. peak 0.65 at 10^{-8} M). Notably, the effect induced by the electronegative and bulky substituent -NO_2 in **5l** is comparable to that of the bromine (**5h**) and sensibly lower to that shown by fluorine in **5f**; thus, this latter substituent remains the less favorable among the tested electronegative substituents. Finally, the three ligands **5a–c**, containing methyl, phenyl or *t*-butyl groups show low activity with the worst value exhibited by the *t*-butyl containing analogue **5c**.

Figures 2B and 3B report the capacity of the new ligands to stimulate superoxide anion production. With the only exception of the fluorine containing analogue **5f**, whose peak of activity appears at the same concentration of the standard (10^{-6} M), all the analogues, regardless of the substituents at the Phe aromatic ring, show peaks grouped at a higher concentration (around 10^{-5} M). Compounds **5a** and **5c**, containing -CH_3 and -$C(CH_3)_3$ substituents, show, in this range of concentration, the highest activity with peaks of 62 and 65 nmoles of O_2^-, which are slightly higher than the standard, thus exhibiting practically comparable potency. Among the ligands **5e–l**, containing electron withdrawing substituents, the best results are shown by **5i** and **5l**, which are characterized by the presence of -NO_2 and -I groups

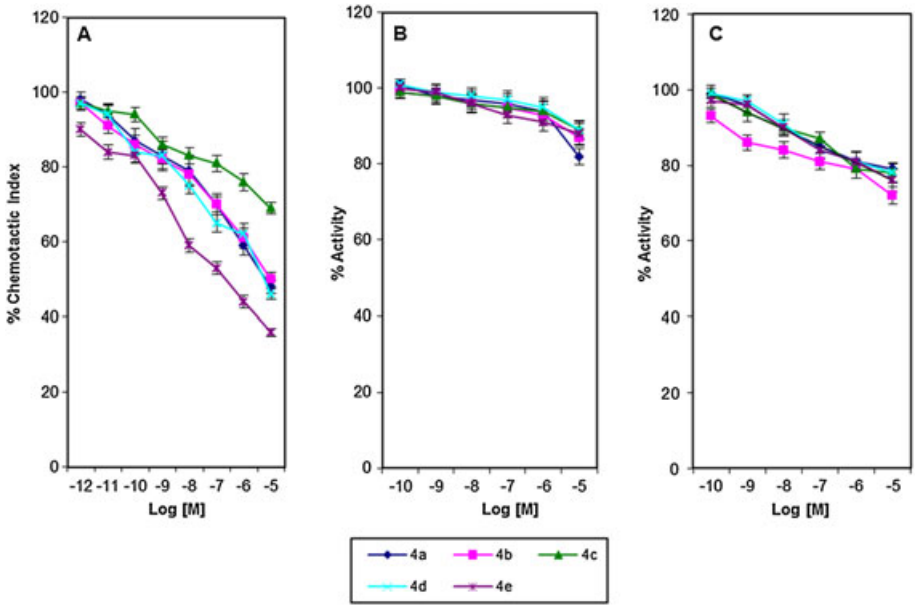

Figure 4. Effect of *N*-Boc-protected tripeptides **4a–e** on the neutrophil activities triggered by fMLF-OMe. (A) chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining the lysozyme activity.

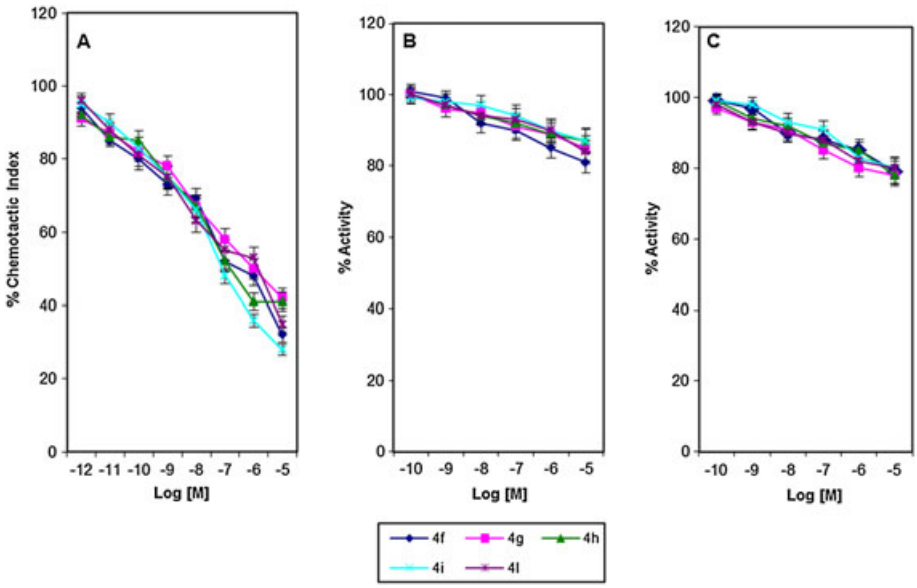

Figure 5. Effect of *N*-Boc-protected tripeptides **4f–l** on the neutrophil activities triggered by fMLF-OMe. (A) chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining the lysozyme activity.

Journal of PeptideScience

at the Phe *para* position, respectively. Notably, these latter substituents are less effective than the alkyl -CH_3 and -$C(CH_3)_3$ groups in **5a** and **5c**.

As reported in the experimental section, the release of neutrophil granule enzymes was evaluated by determining the lysozyme activity expressed as % of lysozyme release and is reported in Figures 2C and 3C. The analogues **5e–l**, containing electron withdrawing substituents at the Phe residue, show good release values (in the range 55–72% at a 10^{-5} M) for all the analogues of this series except for **5h** whose maximum (62%) appears at 10^{-7} M. Thus, as compared with the standard, **5h** shows lower efficacy and higher potency, whereas **5d** has higher efficacy but lower potency. Among the three nonhalogenated derivatives, **5a–c** the C_6H_5- containing analogue **5b** is almost inactive, whereas the Me- and tBu- containing ligands (**5a** and **5c**) show good activity, comparable to that induced by the presence of electron withdrawing substituents at the phenylalanine *para* position.

With the consideration of the entire group of the analogues here under study, it can be seen that most of them show a selective ability to stimulate neutrophils. In fact, as general feature, some of them are able to induce chemotaxis although this property is generally accompanied by scarce superoxide anion production or lysozyme release. These data are not surprising as it is well recognized that the transduction pathway underlying the chemotactic response is different from those responsible for superoxide anion production and lysozyme release [26]. Distinct mechanisms are in fact involved in each of the examined neutrophil responses, and a distinctive imprint of signal protein activation, for a single set of cellular responses, may exist. Moreover, it has been shown that each physiological function shows different requirements for receptor occupancy.

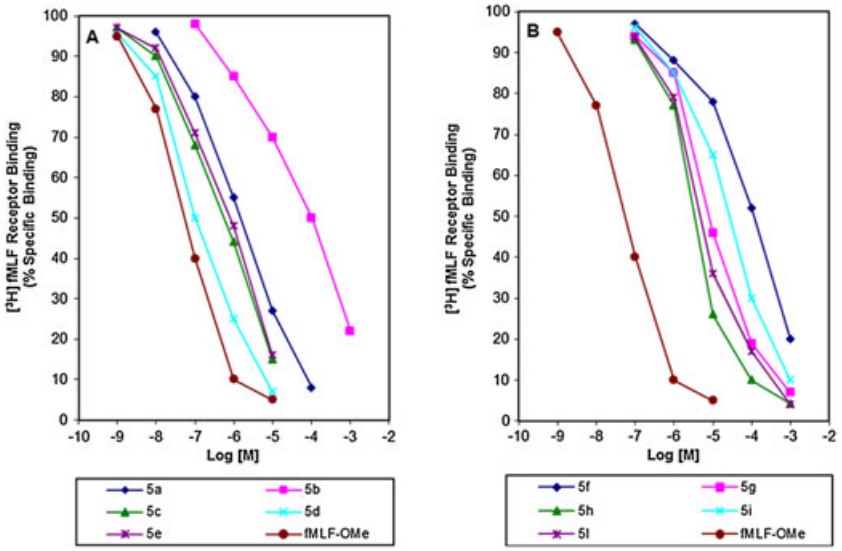

Figure 6. Competition curves of specific [^{3}H] fMLF binding to human neutrophils by agonists **5a–e** (A) and **5f–l** (B). Curves are a representative experiment taken from a series of three independent experiments. Nonspecific binding was determined in the presence of 10-μL fMLF-OMe.

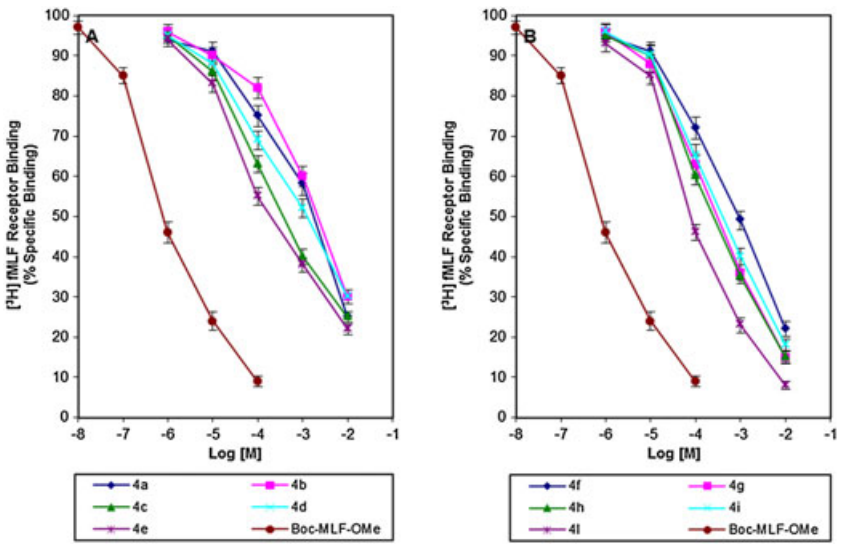

Figure 7. Competition curves of specific [^{3}H] fMLF binding to human neutrophils by antagonists **4a–e** (A) and **4f–l** (B). Plot is a representative experiment replicate three times using different cell preparations. Nonspecific binding was determined in the presence of 10-μL fMLF-OMe.

In the case of the superoxide anion production, continuous occupancy of almost 100% of the receptors to achieve and maintain an optimal response is required, and this is not necessary for the chemotactic response [20].

Antagonism

All Boc-protected tripeptides **4a–l** have been tested to evaluate the antagonist activity on FPR receptor. The antagonism was determined by measuring the inhibition of the activity by derivatives on human neutrophils stimulated by standard tripeptide fMLF-OMe (Figures 4 and 5). A significant dose-dependent inhibition of chemotactic index, which reaches the value 70% at 10^{-9} M concentration, is observed for compounds **4f** and **4i**, with -F and -I in position 4, respectively, on the aromatic ring of phenylalanine, whereas tripeptide **4c**, with -C$(CH_3)_3$ in the same position on the aromatic ring, shows the lower ability to inhibit the activity of standard tripeptide fMLF-OMe (30%). None of the Boc-protected tripeptides **4a–l** show significant antagonism towards both superoxide anion production and in lysozyme release (Figures 4 and 5).

Binding

Competition binding assays were carried out to gain information regarding receptor affinity of formylated (**5a–l**) and Boc-protected analogues (**4a–l**). fMLF-OMe and Boc-MLF-OMe were used as reference compounds, respectively, for agonists and antagonists.

For-Met-Leu-Phe-OMe showed the highest binding affinity, in agreement with its overall activity in the displacing of [^{3}H]-fMLF, followed by analogues **5d** > **5c** > **5e** ≥ **5a** (Figure 6). As regards the other compounds, the rank order of binding affinity is generally similar to that obtained in chemotaxis experiments with the exception of tripeptide **5c** that shows a considerable receptor affinity, which is 44% of [^{3}H] fMLF inhibition at 10^{-6} M concentration; no significant chemotactic activity was revealed, probably due to the presence of a bulky group (4-tBu) on the Phe residue.

All Boc derivatives **4a–l** (antagonists) show lower receptor affinity, Boc-MLF-OMe was used as standard [27] (Figure 7). Compounds **4e** and **4l** have the best affinity, followed by **4c**, **4d** and **4h**. As expected, Boc-protected compounds **4a–l** bind to the formylpeptide receptors at concentration of 10^3 times higher of their respective formylated compounds; this reflects the obtained inhibition activity data (Figures 3 and 4). The presence of Boc group, which is a key feature to exhert antagonism activity at the formyl peptide receptors [16], also determines the observed weak affinity of these compounds. The poor binding shown by the Boc-MLF analogues are not surprising and agree with the scarce data referred by Derian *et al.* [16]. The agonist and antagonist modification seen in this series of Phe^3 modified-chemotactic-peptides in general suggests that considerable changes at the aromatic ring of the Phe residues in position 3 are unfavorable for binding affinity.

Conclusion

In the present paper, we have examined the fMLF-OMe analogues **4a–l** and **5a–l**, which combine the presence of various substituents at the *para* position of the Phe aromatic ring with two *N*-terminal protecting groups, namely H-CO- and *t*Bu-OCO-, which are characterized by different size and chemical properties. None of the examined compounds is more active than fMLF-OMe to stimulate chemotaxis, thus confirming that the presence of a shallow binding pocket scarcely adapts to accommodate *para*-substituted aromatic rings. In conclusion, the here-described fMLF-OMe analogues provide new information on structure–activity relationship concerning chemotactic peptides and formyl-peptide receptors, leading to molecules which, depending upon the nature of acylating groups at the *N*-terminal position and substitution in 4 position on the aromatic ring of phenylalanine, exhibit significant chemotactic agonism or antagonism with selectivity towards the neutrophil biological functions. The different behavior can be rationalized on the basis of the existence of at least two different states and/or with different subtypes of FPRs [22,23,25]. Low doses of a full agonist (or a 'pure' chemoattractant) are required to interact with a high-affinity receptor subtype, which activates the transduction pathway responsible for chemotactic response, whereas an increase in agonist concentration allows binding with the low-affinity subtype, able to activate the transduction pathways responsible for O_2^- production and lysozyme release [28]. As a consequence, a peptide selective for killing mechanisms, which preferentially interacts with the low-affinity subtypes, is efficient in effectively displacing the full agonist [^{3}H]-fMLF [29] only at high concentration. A further investigation using specific receptor inhibitors of FPR or FPRL1 should give useful information and will be the object of incoming research. However, the here-reported results, obtained by using simple and structurally strongly related fMLF-OMe analogues, represent an interesting contribution toward the comprehension of the subtle mechanisms involved in the different neutrophil biological functions. Furthermore, the good versatility and good efficacy of the here-reported new molecules are a promising characteristic particularly as concerns pharmacological application.

The analytical data of all synthesized products are provided in the Supplemental Information section associated with this article.

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