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ORIGINAL ARTICLE

Synthesis and bioactivity of chemotactic tetrapeptides: fMLF-OMe analogues incorporating spacer aminoacids at the lateral positions

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Abstract A small library of *N*-For and *N*-Boc tetrapeptidic analogues of the chemotactic tripeptide For-Met-Leu-Phe-OMe (fMLF-OMe), obtained by incorporating three different spacer aminoacids (Gly, βAla and Pro) between the native residues of Met and Leu (*N*-For- and *N*-Boc-Met-Xaa-Leu-Phe-OMe; Xaa^2 series) and Leu and Phe (*N*-For- and *N*-Boc-Met-Leu-Xaa-Phe-OMe; Xaa^3 series), have been synthesized and examined for their biological activity as agonists and antagonists. Chemotaxis, lysozyme release and superoxide anion production have been measured. All the *N*-For analogues maintain good to moderate chemotactic activity with the βAla^3 **15** model reaching the maximum value. All the *N*-Boc tetrapeptides are efficient chemotactic antagonists. Conversely, with the exception of the moderate antagonistic activity exhibited by the *N*-Boc Xaa^2 models against lysozyme release, all the other *N*-Boc analogues do not show significant activity against both superoxide anion and lysozyme release.

Keywords Chemotaxis · fMLF analogues · Formylpeptides · Human neutrophils · Tetrapeptides

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Abbreviations

Boc	*tert*-Butyloxycarbonyl
DMF	Dimethylformamide
EEDQ	Ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate
EDCI	1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride
HOBT	1-Hydroxybenzotriazole
KRPG	Krebs–Ringer phosphate containing 0.1% *w/v* D-glucose
TEA	Triethylamine
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography

Introduction

Natural and synthetic chemoattractant *N*-formyltripeptides continue to stimulate a great deal of interest due to their ability to bind to specific G-protein-coupled receptors (FPRs) primarily expressed on neutrophil and monocyte membranes. In addition to *N*-formyltripeptides several FPR ligands of different origin and molecular structure have been identified over the past years. However, due to its simple structure and high agonistic potency *N*-formyl-Met-Leu-Phe-OH (fMLF) and its methyl ester (fMLF-OMe) remain the reference molecules for studies on peptidic chemoattractants derived from cleavage of bacterial or mitochondrial proteins.

Binding of *N*-formyl peptides to leucocyte FPRs activates a variety of cell functions including migration towards the inflammation sites, generation of free radicals and release of granule contents.

In addition to the above cited biochemical events, which concern the role played by FPR in the host defense against

invading microorganisms, a further relevant point has been recently evidenced. Studies from several groups firmly establish in fact that the distribution of FPR is not limited to neutrophil and monocytes but is widely extended to different tissues and organs (Becker et al. 1998; Le et al. 2000). Thus, it is now clear that the family of chemoattractant receptors, together with a variety of microbial and endogenous ligands, forms a complex system whose pathophysiological functions involve several and probably determinant roles in both inflammatory and immunological diseases. The above reported findings have enlarged the interest on *N*-formylpeptide chemoattractants well beyond the host defence against microbial infection and have underlined, at the same time, the relevance of FPRs as potential target for the development of new therapeutic strategies (Rabiet et al. 2007). However, in spite of these data, several aspects concerning the structural basis of receptor/agonists interaction and the signalling mechanisms (Selvatici et al. 2006) still remain to be elucidated (Cavicchioni et al. 2006).

Previous investigations based on fMLF backbone modification give clear evidence that the sulphur-containing *N*-terminal For-Met-moiety and the aromatic side chain at the *C*-terminal position of the tripeptide are crucial for both binding and receptor activation. The central hydrophobic Leu, on the other hand, appears to be quite tolerant of modification (Mills et al. 1998) and several fMLF analogues modified at position-2 with high activity and selectivity have been described (Giordano et al. 2007; Koksch et al. 2004). Finally, it is now well known that key interactions between ligands and FPRs involve in addition to the Met and Phe hydrophobic side chains specific hydrogen bonding established by the *N*-terminal formylic group. Relevant is the observation that modifications centred at this function can modulate the neutrophil biological responses and open the way to FPR antagonists whose interest for the use in diagnostic and therapeutic applications is well known. In particular both urea and carbamate derivatized fMLF analogues, among which Boc-Met-Leu-Phe-OMe, containing the bulky tBuOCO (Boc) group in place the native H-CO (For), have been studied and found active as antagonists (Derian et al. 1996; Higgins et al. 1996).

In accordance with the observed versatility towards structural modifications at position-2 of fMLF we reported previously studies on *N*-For and *N*-Boc analogues characterized by the presence of linear achiral ω-amino acids as spacers, in place of the central Leu (Giordano et al. 2003). These tripeptides maintain the two native Met and Phe side chains but are characterized by different distances on the backbone between side chains at position-1 and position-3 depending upon the ω-amino acid residue used as spacer. As prosecution of these studies we report here the results of a new approach in which *N*-For and *N*-Boc tetrapeptidic analogues of fMLF-OMe are examined. At variance with previous approaches, the here reported analogues have been designed so as to maintain intact all the three native residues present in the reference ligand and to possess a backbone sequence altered by the presence of a spacer aminoacid inserted between the Met and the Leu (tetrapeptide models of the Xaa^2 type) or between Leu and Phe (tetrapeptide models of Xaa^3 type) as depicted in Fig. 1. The aim of this study is to get preliminary information on the consequences that the variation of the disposition and distance, relating the two critical side chains present in the native ligand, has on the interaction with the complementary pockets of the FPR receptor. In order to investigate this point three different spacers have been used. Two of these are the achiral and side chain devoid residues of Gly and βAla while the third is the chiral residue of Pro. This latter shares with the achiral Gly and βAla residues the absence of the characteristic side chain present in all natural aminoacids and, under this aspect, can be related to Gly of which can be considered a ring-constrained analogue.

Materials and methods

General

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. ^1H 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% of the theoretical values.

Protected amino acids were purchased from Bachem and Fluka. Coupling and *N*-Boc deprotection procedures were performed according general protocols (Wunsch 1974) and checked for purity by TLC, HPLC and elemental analysis. *N*-Formylation was attained starting from the corresponding *N*-Boc derivatives (Lajoie and Kraus 1984).

*Boc-Met-Gly-Leu-Phe-OMe (**2**)*

From Boc-Met-OH (0.058 g, 0.23 mmol) and Gly-Leu-Phe-OMe · TFA (0.107 g, 0.23 mmol) (Mladenova-Orlinova

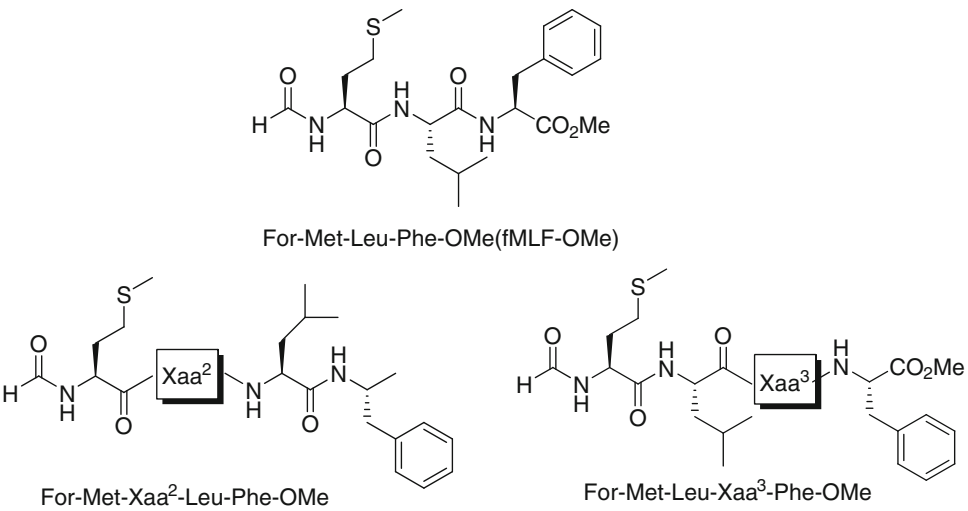


Fig. 1 Structure of fMLF-OMe and its Xaa^2 and Xaa^3 tetrapeptide analogues reported in the present study. The spacer aminoacid Xaa represents in all cases a residue of Gly, Ala or Pro

et al. 1967). Crystallization from Hexane gave the pure product as white solid (0.100 g, 75%).

Mp 134.3–136.5°C. $[\alpha]_D$ +2.0 (1% $CHCl_3$). IR *v*: 3,421, 1,710, 1,676 cm^{-1}. ^1H NMR *δ*: 0.87–0.90 [6H, m, Leu $CH(CH_3)_2$], 1.45 [9H, s, $C(CH_3)_3$], 1.48–1.93 [5H, m, Met *β*-CH_2 and Leu CH_2-$CH(CH_3)_2$], 2.12 (3H, s, SCH_3), 2.57 (2H, m, Met *γ*-CH_2), 3.01 and 3.18 (2H, A and B of an ABX, $J = 6$, 5.6 and 14 Hz, Phe *β*-CH_2), 3.71 (3H, s, $COOCH_3$), 3.78–3.99 (2H, m, Gly α-CH_2), 3.98 (1H, m, Met α-CH), 4.44 (1H, m, Leu α-CH), 4.83 (1H, m, Phe α-CH), 5.37 (1H, d, $J = 7.6$ Hz, Met NH), 6.74 (2H, m, Phe NH and Leu NH), 6.96 (1H, br, Gly NH), 7.12–7.29 (5H, m, aromatic). Anal. Calcd for $C_{28}H_{44}N_4O_7S$: C, 57.91; H, 7.64: N, 9.65. Found: C, 58.14; H, 7.61; N, 9.68.

*For-Met-Gly-Leu-Phe-OMe (**3**)*

From Boc-Met-Gly-Leu-Phe-OMe (0.1 g, 0.17 mmol). Crystallization from EtOAc/Hexane. White solid (0.080 g, 93%).

Mp 175–177.9°C. $[\alpha]_D$ +2.0 (1%, $CHCl_3$). IR *v*: 3,671, 3,426, 1,652 cm^{-1}. ^1H NMR *δ*: 0.87–0.90 [6H, m, Leu $CH(CH_3)_2$], 1.48–1.93 [5H, m, Met *β*-CH_2 and Leu CH_2-$CH(CH_3)_2$], 2.12 (3H, s, SCH_3)], 2.57 (2H, m, Met *γ*-CH_2), 3.01 and 3.18 (2H, A and B of an ABX, $J = 6.3$, 5.6, 13.8 Hz, Phe *β*-CH_2), 3.71 (3H, s, $COOCH_3$), 3.78–3.99 (2H, m, Gly α-CH_2), 4.30 (1H, m, Met α-CH), 4.44 (1H, m, Leu α-CH), 4.83 (1H, m, Phe α-CH), 5.37 (1H, d, $J = 8$ Hz, Met NH), 6.74 (2H, m, Phe NH and Leu NH), 6.96 (1H, br, Gly NH), 7.12–7.29 (5H, m, aromatic), 8.04 (1H, s, HCO). Anal. Calcd for $C_{24}H_{36}N_4O_6S$: C, 56.67; H, 7.13; N, 11.02. Found: C, 56.81; H, 7.16; N, 11.05.

*Boc-β-Ala-Leu-Phe-OMe (**4**)*

From Boc-*β*-Ala-OH (0.080 g, 0.42 mmol) and Leu-Phe-OMe · TFA (0.170 g, 0.42 mmol). The title compound was obtained as a white solid, pure on TLC (0.170 g, 87%).

$[\alpha]_D$ −7.0 (1%, $CHCl_3$). IR *v*: 3,427, 3,011, 1,741, 1,631 cm^{-1}. ^1H NMR *δ*: 0.89–0.98 [6H, m, Leu $CH(CH_3)_2$], 1.47 [9H, s, $C(CH_3)_3$], 1.77–1.88 [5H, m, Leu CH_2-$CH(CH_3)_2$ and *β*-Ala α-CH_2], 2.9 and 3.15 (2H, A and B of an ABX, $J = 6.1$, 5.6 and 14 Hz, Phe *β*-CH_2), 3.2–3.5 (2H, m, *β*-Ala *β*-CH_2), 3.7 (3H, s, $COOCH_3$), 4.5 (1H, m, Leu α-CH), 4.75 (1H, m, Phe α-CH), 5.2 (1H, d, $J = 7.2$ Hz, *β*-Ala NH), 6.2 (1H, d, $J = 7.6$ Hz, Phe NH), 6.75 (1H, d, $J = 7.3$ Hz, Leu NH), 7.01–7.45 (5H, m, aromatic). Anal. Calcd for $C_{24}H_{37}N_3O_6$: C, 62.18; H, 8.04; N, 9.06. Found: C, 62.33; H, 8.07; N, 9.09.

*Boc-Met-β-Ala-Leu-Phe-OMe (**5**)*

From Boc-Met-OH (0.0645 g, 0.26 mmol) and *β*-Ala-Leu-Phe-OMe · TFA (0.123 g, 0.26 mmol), this latter obtained from deprotection of Boc-*β*-Ala-Leu-Phe-OMe. Colourless oil (0.170 g, 87%).

Mp 133.5–135°C. $[\alpha]_D$ +42.0 (1%, $CHCl_3$). IR *v*: 3,427, 1,741, 1,631 cm^{-1}. ^1H NMR *δ*: 0.85–0.91 [6H, m, Leu $CH(CH_3)_2$], 1.45 [9H, s, $C(CH_3)_3$], 1.53–1.66 [3H, m, Leu CH_2-$CH(CH_3)_2$], 1.68–1.84 (2H, m, Met *β*-CH_2), 2.10 (3H, s, SCH_3), 2.28–2.51 (4H, m, *β*-Ala α-CH_2 and *γ*-Met CH_2), 3.04–3.19 (3H, m, *β*-Ala *β*-C*H*H and Phe *β*-CH_2), 3.75 (3H, s, $COOCH_3$), 3.85–3.90 (2H, m, *β*-Ala *β*-C*H*H and Met α-CH), 4.37 (1H, m, Leu α-CH), 4.99 (1H, m, Phe α-CH), 5.21 (1H, d, $J = 8$ Hz, Met NH), 6.68–6.72 (2H, m, Phe NH and Leu NH), 7.14–7.26 (5H, m, aromatic), 7.66 (1H, apparent t, *β*-Ala NH). Anal. Calcd for $C_{29}H_{46}N_4O_7S$: C, 58.56; H, 7.80; N, 9.42. Found: C, 58.79; H, 7.83; N, 9.44.

*For-Met-β-Ala-Leu-Phe-OMe (**6**)*

From Boc-Met-*β*-Ala-Leu-Phe-OMe (0.1 g, 0.168 mmol). Crystallized from hexane. White solid (0.050 g, 57%).

Mp 157.7–161.3°C. $[\alpha]_D$ −16.0 (1%, $CHCl_3$). IR *v*: 3,671, 3,424, 1,734, 1,670 cm^{-1}. ^1H NMR δ: 0.85–0.91 [6H, m, Leu CH(CH_3)$_2$], 1.53–1.66 (3H, m, Leu CH_2-$CH(CH_3)_2$, 1.68–1.84 (2H, m, Met β-CH_2), 2.10 (3H, s, SCH_3), 2.28–2.51 (4H, m, β-Ala α-CH_2 and Met γ-CH_2), 3.04–3.19 (3H, m, β-Ala β C*H*H and Phe β-CH_2), 3.75 (3H, s, $COOCH_3$), 3.85–3.90 (2H, m, β-Ala β-C*H*H and Met α-CH), 4.37 (1H, m, Leu α-CH), 4.99 (1H, m, Phe α-CH), 5.21 (1H, d, $J = 8$ Hz, Met NH), 6.68–6.72 (2H, m, Phe NH and Leu NH), 7.14–7.26 (5H, m, aromatic), 7.66 (1H, br, β-Ala NH), 8.01 (1H, s, HCO). Anal. Calcd for $C_{25}H_{38}N_4O_6S$: C, 57.45; H, 7.33; N, 10.72. Found: C, 57.58; H, 7.30; N, 10.74.

*Boc-Pro-Leu-Phe-OMe (**7**)*

From Boc-Pro-OH (0.07 g, 0.325 mmol) and Leu-Phe-OMe · TFA (0.132 g, 0.325 mmol). White foam, pure on TLC (0.160 g, 90%).

$[\alpha]_D$ −49.05 (1%, $CHCl_3$). IR *(*: 3,422, 1,760, 1,676 cm^{-1}. ^1H NMR δ: 0.87–0.98 [6H, m, Leu CH(CH_3)$_2$], 1.51 [9H, s, C(CH_3)$_3$], 1.7–2.5 [(8H, m, Pro β-CH_2, Pro γ-CH_2, Pro δ-C*H*H, and Leu CH_2-$CH(CH_3)_2$)], 3.1–3.3 (2H, m, Phe β-CH_2), 3.3–3.5 (1H, m, Pro δ-C*H*H), 3.7 (3H, s, $COOCH_3$), 4.3–4.6 (3H, m, Pro α-CH, Leu α-CH and Phe α-CH), 6.5–6.7 (2H, m, Leu NH and Phe NH), 7.01–7.5 (5H, m, aromatic). Anal. Calcd for $C_{26}H_{39}N_3O_6$: C, 63.78; H, 8.03; N, 8.58. Found: C, 63.52; H, 8.01; N, 8.54.

*Boc-Met-Pro-Leu-Phe-OMe (**8**)*

From Boc-Met-OH (0.0676 g, 0.27 mmol) and Pro-Leu-Phe-OMe · TFA (0.110 g, 0.27 mmol) this latter obtained from Boc-Pro-Leu-Phe-OMe. Silica gel chromatography (CH_2Cl_2/CH_3OH 98:2) and crystallization from EtOAc/Hexane gave the pure title product as a white solid (0.120 g, 70%).

Mp 112.5–114°C. $[\alpha]_D$ −42.0 (1%, $CHCl_3$). IR *v*: 3,419, 1,741, 1,676 cm^{-1}. ^1H NMR δ: 0.88–0.97 [6H, m, Leu CH(CH_3)$_2$], 1.48 [9H, s, C(CH_3)$_3$], 1.61–1.65 (4H, m, Pro β-CH_2 and Pro γ-CH_2) 1.84–2.14 [8H, m, Met β-CH_2, Leu CH_2-CH-$(CH_3)_2$ and SCH_3 (s at 2.13)], 2.3 (1H, m, Pro δ-C*H*H), 2.58 (2H, m, Met δ-CH_2), 3.10 and 3.21 (2H, A and B of an ABX, $J = 6$, 5.6 and 13.6 Hz, Phe β-CH_2), 3.64 (1H, m, Pro δ-C*H*H) 3.75 (3H, s, $COOCH_3$), 4.35 (1H, m, Met α-CH), 4.53 (1H, m, Pro α-CH), 4.63 (1H, m, Leu α-CH), 4.87 (1H, m, Phe α-CH), 5.30 (1H, d, $J = 9.6$ Hz, Met NH), 6.63 (1H, d, $J = 6.8$ Hz, Phe NH), 7.02 (1H, d, $J = 7.6$ Hz, Leu NH), 7.14–7.33 (5H, m, aromatic). Anal. Calcd for $C_{31}H_{48}N_4O_7S$: C, 59.98; H, 7.79; N, 9.02. Found: C, 60.15; H, 7.82; N, 9.05.

*For-Met-Pro-Leu-Phe-OMe (**9**)*

From Boc-Met-Pro-Leu-Phe-OMe (0.08 g, 0.127 mmol). The mixture was purified on silica gel chromatography ($CHCl_3$/CH_3OH 99:1). Colourless oil (0.068 g, 95%).

$[\alpha]_D$ −13.0 (1% $CHCl_3$). IR *v*: 3,417, 1,743, 1,677 cm^{-1}. ^1H NMR δ: 0.88–0.97 [6H, m, Leu CH(CH_3)$_2$], 1.61–1.65 (4H, m, Pro β-CH_2 and Pro γ-CH_2), 1.84–2.14 [8H, m, Met β-CH_2, Leu CH_2-CH-$(CH_3)_2$ and SCH_3 (s at 2.13)], 2.3 (1H, br, Pro δ-C*H*H), 2.58 (2H, m, Met δ-CH_2), 3.10 and 3.21 (2H, A and B of an ABX, $J = 6.3$, 5.7 and 13.5 Hz, Phe β-CH_2), 3.64 (1H, m, Pro δ-C*H*H) 3.75 (3H, s, $COOCH_3$), 4.35 (1H, m, Leu α-CH), 4.53 (1H, m, Pro α-CH), 4.63 (1H, m, Met α-CH), 4.87 (1H, m, Phe α-CH), 5.30 (1H, d, $J = 8.2$ Hz, Met NH), 6.63 (1H, d, $J = 7.6$ Hz, Phe NH), 7.02 (1H, d, $J = 6.8$ Hz, Leu NH), 7.14–7.33 (5H, m, aromatic), 8.03 (1H, s, HCO). Anal. Calcd for $C_{27}H_{40}N_4O_6S$: C, 59.10; H, 7.35; N, 10.21. Found: C, 59.29; H, 7.38; N, 10.25.

*Boc-Met-Leu-Gly-Phe-OMe (**11**)*

From Boc-Met-Leu-OH (0.107 g, 0.297 mmol) (Torrini et al. 1998), and Gly-Phe-OMe · TFA (**10**) (0.104 g, 0.297 mmol) obtained from Boc-Gly-Phe-OMe (Leleu et al. 2005). The mixture was purified on silica gel chromatography (CH_2Cl_2/EtOAc 2:8). White foam (0.121 g, 70%).

$[\alpha]_D$ +1.0 (1% $CHCl_3$). IR *v*: 3,419, 1,735, 1,678 cm^{-1}. ^1H NMR δ: 0.96–0.99 [6H, m, Leu CH(CH_3)$_2$], 1.45 [9H, s, C(CH_3)$_3$], 1.55–1.69 [3H, m, Leu CH_2-CH-$(CH_3)_2$], 1.88–2.11 [5H, m, Met β-CH_2 and SCH_3 (s, at 2.09)], 2.55 (2H, m, Met γ-CH_2), 3.05 and 3.17 (2H, A and B of an ABX, $J = 6$, 5.6 and 14 Hz, Phe β-CH_2), 3.7 (3H, s, $COOCH_3$), 3.86–4.03 (2H, m Gly α-CH_2), 4.26 (1H, m, Met α-CH), 4.45 (1H, m, Leu α-CH), 4.85 (1H, m, Phe α-CH), 5.45 (1H, br, Met NH), 6.98 (1H, d, $J = 8$ Hz, Leu NH), 7.03 (1H, d, $J = 7.2$ Hz, Phe NH), 7.12–7.29 (6H, m, aromatic and Gly NH). Anal. Calcd for $C_{28}H_{44}N_4O_7S$: C, 57.91; H, 7.64; N, 9.65. Found: C, 58.14; H, 7.61; N, 9.68.

*For-Met-Leu-Gly-Phe-OMe (**12**)*

From Boc-Met-Leu-Gly-Phe-OMe (0.07 g, 0.12 mmol) according to the reported general procedure. The reaction mixture was purified on silica gel ($CHCl_3$/CH_3OH 95:5).White foam (0.059 g, 97%).

$[\alpha]_D$ +0.5 (0.7%, $CHCl_3$). IR *v*: 3,415, 1,740, 1,670 cm^{-1}. ^1H NMR δ: 0.96–0.99 [6H, m, Leu CH(CH_3)$_2$], 1.54–1.68 [3H, m, Leu CH_2-CH-$(CH_3)_2$], 1.87–2.11 [5H, m, Met β-CH_2 and SCH_3 (s, at 2.06)], 2.51 (2H, m, Met γ-CH_2), 3.03 and 3.18 (2H, A and B of an ABX, $J = 6$, 5.7 and 13.6 Hz, Phe β-CH_2), 3.71 (3H, s, $COOCH_3$), 3.96–4.2 (2H, m, Gly

CH_2), 4.94 (1H, m, Met α-CH), 4.71 (1H, m, Leu α-CH), 4.86 (1H, m, Phe α-CH), 7.09–7.31 (6H, m, aromatic and Met NH), 7.42 (1H, br, Phe NH), 7.62 (1H, br, Gly NH), 7.75 (1H, br, Leu NH), 8.14 (1H, s, HCO). Anal. Calcd for $C_{24}H_{36}N_4O_6S$: C, 56.67; H, 7.13; N, 11.02. Found: C, 56.84; H, 7.15; N, 11.05.

*Boc-Met-Leu-β-Ala-Phe-OMe (**14**)*

From Boc-Met-Leu-OH (0.128 g, 0.354 mmol) and β-Ala-Phe-OMe · TFA (13) (0.129 g, 0.354 mmol) this latter obtained from Boc-β-Ala-Phe-OMe (Giordano et al. 2003). Amorphous solid, pure on TLC (0.155 g, 74%).

$[\alpha]_D$ +23.0 (1% $CHCl_3$). IR *v*: 3,421, 1,738, 1,673 cm^{-1}. ^1H NMR δ: 0.93–0.96 [6H, m, Leu CH(CH_3)$_2$], 1.41 [9H, s, $C(CH_3)_3$], 1.48–1.70 [3H, m, Leu CH_2-CH-$(CH_3)_2$], 1.91–2.15 [5H, m, Met β-CH_2 and SCH_3 (s, at 2.12)], 2.30 (2H, m, β-Ala α-CH_2), 2.63 (2H, m, Met γ-CH_2), 2.99–3.22 (3H, m, β-Ala β-C*H*H and Phe β-CH_2), 3.78 (3H, s, $COOCH_3$), 3.79–3.86 (1H, m, β-Ala β-C*H*H), 4.25 (1H, m, Met α-CH), 4.27 (1H, m, Leu α-CH), 4.84 (1H, m, Phe α-CH), 5.18 (1H, br, Met NH), 6.72 (1H, br, Leu NH), 7.05 (1H, d, $J = 8$ Hz, Phe NH), 7.22–7.33 (6H, m, aromatic and β-Ala NH). Anal. Calcd for $C_{29}H_{46}N_4O_7S$: C, 58.56; H, 7.80; N, 9.42. Found: C, 58.79; H, 7.83; N, 9.44.

*For-Met-Leu-β-Ala-Phe-OMe (**15**)*

From Boc-Met-Leu-β-Ala-Phe-OMe (0.056 g, 0.095 mmol). The mixture was purified on silica gel flash chromatography ($CHCl_3$/CH_3OH 95:5). Amorphous solid (0.039 g, 79%).

$[\alpha]_D$ −55.3 (0.38%, CH_3OH). IR *v*: 3,421, 1,743, 1,648 cm^{-1}. ^1H NMR δ: 0.84–0.91 [6H, Leu CH(CH_3)$_2$], 1.34–1.60 [3H, m, Leu CH_2-CH-$(CH_3)_2$], 1.70–2.05 [5H, m, Met β-CH_2 and SCH_3 (s, at 2.02)], 2.22 (2H, m, β-Ala α-CH_2), 2.42 (2H, m, Met γ-CH_2), 3.05–3.25 (2H, m, β-Ala β-CH_2), 2.83–3.04 (2H, m, Phe β-CH_2), 3.58 (3H, s, $COOCH_3$), 4.21 (1H, m, Leu α-CH), 4.40 (1H, m, Met α-CH), 4.46 (1H, m, Phe α-CH), 7.17–7.30 (5H, m, aromatic), 7.87 (1H, br, β-Ala NH), 8.00 (1H, br, Leu NH), 8.01 (1H, s, HCO), 8.29 (1H, br, Met NH), 8.39 (1H, d, $J = 7.2$ Hz, Phe NH),). Anal. Calcd for $C_{25}H_{38}N_4O_6S$: C, 57.45; H, 7.33; N, 10.72. Found: C, 57.30; H, 7.30; N, 10.67.

*Boc-Met-Leu-Pro-Phe-OMe (**17**)*

From Boc-Met-Leu-OH (0.115 g, 0.319 mmol) and Pro-Phe-OMe · TFA (**16**) (0.124 g, 0.319 mmol) this latter obtained from Boc-Pro-Phe-OMe (Dugas et al. 1993). The mixture was purified on silica gel chromatography (CH_2Cl_2/EtOAc 1:1). Colourless oil (0.115 g, 58%).

$[\alpha]_D$ −52.0 (0.94% $CHCl_3$). IR *v*: 3,421, 1,743, 1,678 cm^{-1}. ^1H NMR δ: 0.92–0.96 [6H, m, Leu CH(CH_3)$_2$], 1.46 [9H, s, $C(CH_3)_3$], 1.56–1.72 [3H, m, Leu CH_2-CH-$(CH_3)_2$], 1.86–2.36 [9H, m, Met β-CH_2, SCH_3 (s, at 2.12) and Pro β-CH_2 and Pro γ-CH_2], 2.58 (2H, m, Met γ-CH_2), 3.02–3.2 (2H, m, Phe β-CH_2), 3.42–3.51 (1H, m, Pro δ-C*H*H), 3.61–3.69 (1H, m, Pro δ-C*H*H), 3.72 (3H, s, $COOCH_3$), 4.29 (1H, m, Met α-CH), 4.55 (1H, m, Pro α-CH) 4.73 (1H, m, Leu α-CH), 4.78 (1H, m, Phe α-CH), 5.17 (1H, d, $J = 8.4$ Hz, Met NH), 6.74 (1H, br, Leu NH), 6.99 (1H, d, $J = 7.6$ Hz, Phe NH), 7.11–7.33 (5H, m, aromatic). Anal. Calcd for $C_{31}H_{48}N_4O_7S$: C, 59.98; H, 7.79; N, 9.02. Found: C, 59.80; H, 7.76; N, 9.00.

*For-Met-Leu-Pro-Phe-OMe (**18**)*

From Boc-Met-Leu-Pro-Phe-OMe (0.071 g, 0.114 mmol). The mixture was purified on silica gel flash chromatography ($CHCl_3$/CH_3OH 98:2). Colourless oil (0.058 g, 93%).

$[\alpha]_D$ −43.2 (0.95%, $CHCl_3$). IR *v*: 3,417, 1,743, 1,673 cm^{-1}. ^1H NMR δ: 0.93–0.95 [6H, m, Leu CH(CH_3)$_2$], 1.25–1.40 [3H, m, Leu CH_2-CH-$(CH_3)_2$], 1.58–2.31 [9H, m, Met β-CH_2, SCH_3 (s, at 2.11), Pro β-CH_2 and Pro γ-CH_2], 2.58 (2H, m, Met γ-CH_2), 3.02 and 3.15 (2H, A and B of an ABX, $J = 6.4$, 5.6 and 13.6 Hz, Phe β-CH_2), 3.51 (1H, m, Pro δ-C*H*H), 3.67 (1H, m, Pro δ-C*H*H), 3.69 (3H, s, $COOCH_3$), 4.62 (1H, m, Pro α-CH), 4.76 (1H, m, Leu α-CH), 4.77 (1H, m, Phe α-CH), 4.85 (1H, m, Met α-CH), 6.55 (1H, d, $J = 8$ Hz, Met NH), 7.09 (1H, d, $J = 7.2$ Hz, Phe NH), 7.23 (1H, d, $J = 7.6$ Hz, Leu NH), 7.08–7.30 (5H, m, aromatic), 8.18 (1H, s, HCO). Anal. Calcd for $C_{27}H_{40}N_4O_6S$: C, 59.10; H, 7.35; N, 10.21. Found: C, 59.29; H, 7.38; N, 10.25.

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 ten 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 towards test attractant minus migration towards the buffer/migration towards 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}–8×10^{-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 a molar extinction coefficient 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 nmoles 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}–8×10^{-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 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%.

Antagonist assay

Antagonist activity was determined by measuring the ability of a derivative to inhibit chemotaxis, superoxide anion production or granule enzyme release as induced by fMLF-OMe. Antagonist activity data (percentage of activity) were obtained by comparing the CI, nanomoles of O_2^- or percentage of lysozyme release in the absence (100%) and in the presence of the derivative. CI of 10 nM fMLF-OMe was 1.15 ± 0.10 SE. O_2^- generation produced by 1 μM fMLF-OMe was 62 ± 2 nmol/1×10^6 cells/5 min. Enzyme activity triggered by 1 μM fMLF-OMe was 54 ± 5%/3×10^6 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.

Statistical analysis

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

Synthesis

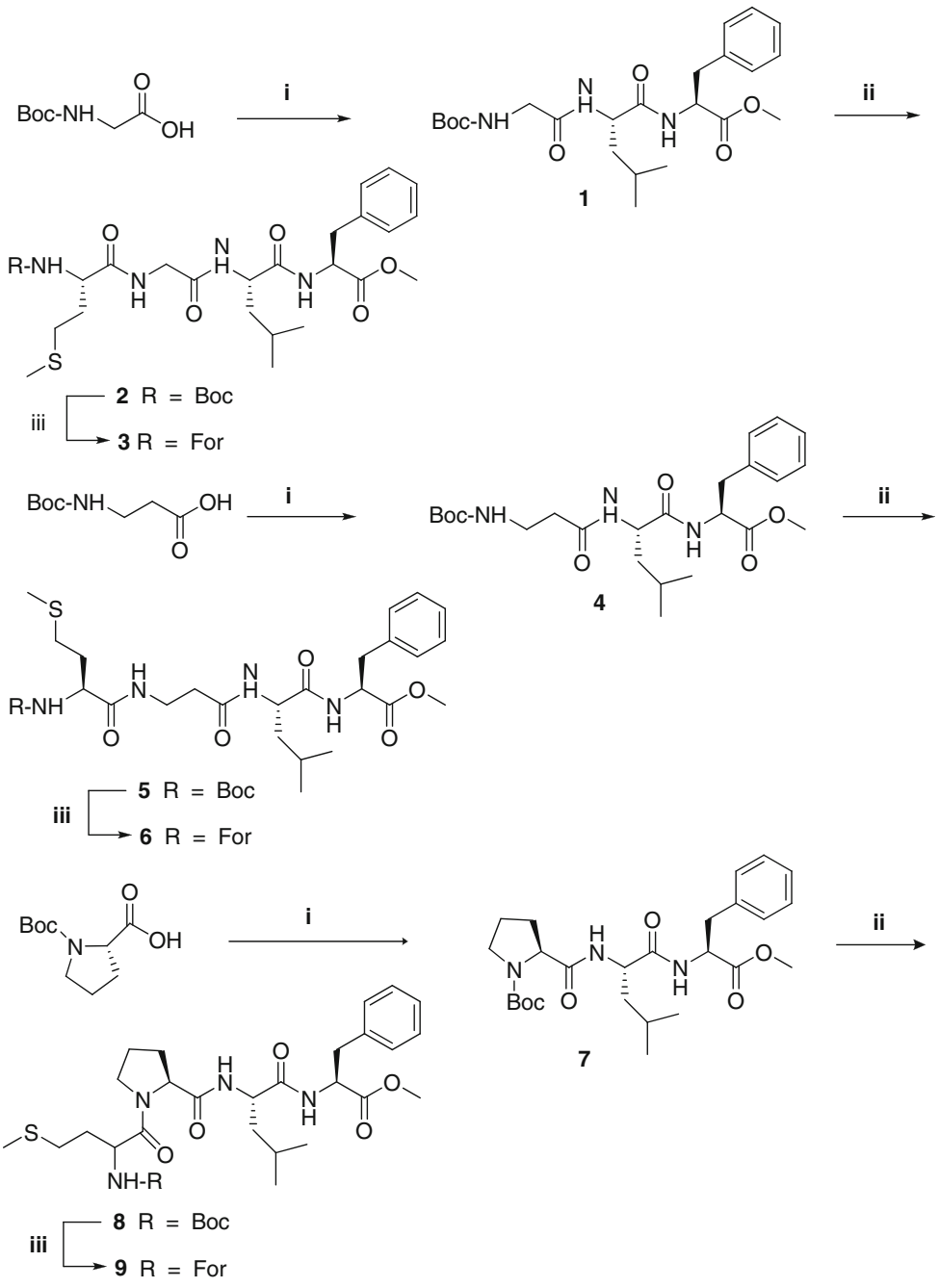
The synthesis of the *N*-Boc-tetrapeptides and the corresponding *N*-formyl derivatives was performed in solution according to the strategy reported in Schemes 1 and 2. Analogues of the Xaa^2 type were obtained by coupling the *N*-Boc-Xaa-OH derivatives with the *N*-terminal dipeptide fragment thus giving the three tripeptides Boc-Xaa-Leu-Phe-OMe (**1**, **4** and **7**). These were then deprotected and coupled with Boc-Met-OH to give the three tetrapeptides Boc-Met-Xaa-Leu-Phe-OMe **2**, **5** and **8**. A different strategy was adopted for the synthesis of the Xaa^3 models (see Scheme 2) where the three dipeptide derivatives Boc-Xaa-Phe-OMe were firstly synthesized; *N*-deprotection to **10**, **13** and **16** was followed by coupling with the common dipeptide fragment Boc-Met-Leu-OH to give the tetrapeptides *N*-Boc-Met Leu-Xaa-Phe-OMe **11**, **14** and **17**. Direct transformation of all the *N*-Boc derivatives

(Schemes 1, 2) into the corresponding *N*-formyl analogues **3**, **6**, **9**, **12**, **15** and **18** was performed by treatment of each *N*-Boc derivative with formic acid and then with EEDQ (Lajoie and Kraus 1984).

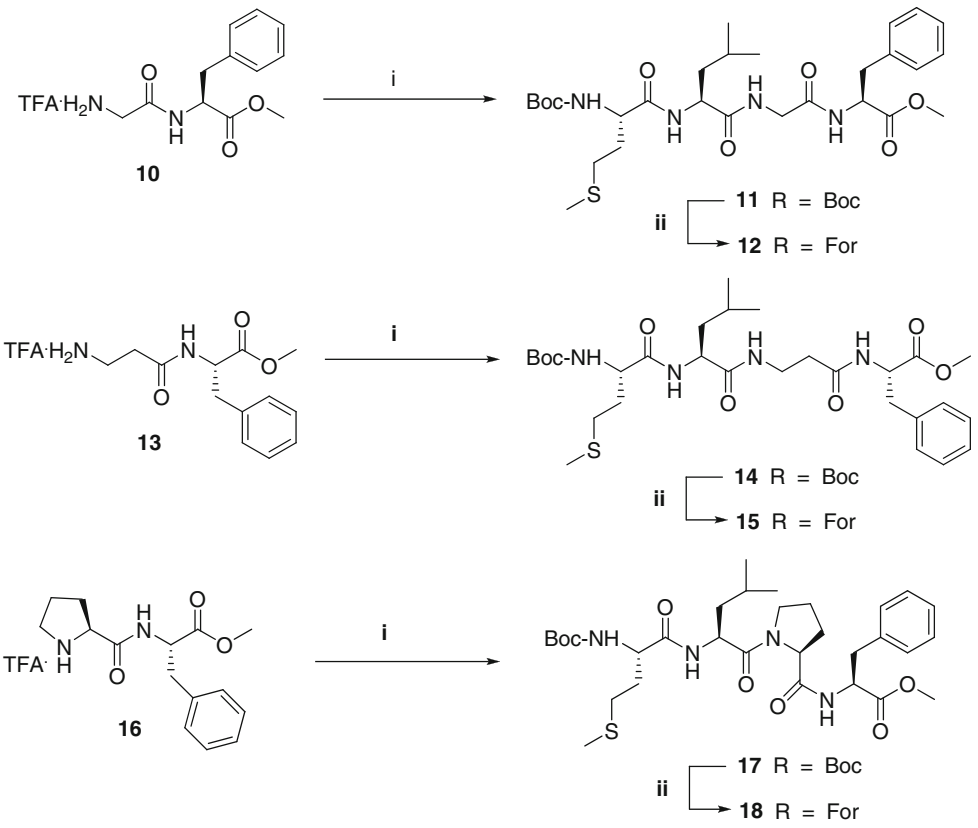
Biological results

The activity of the *N*-For and *N*-Boc tetrapeptide derivatives reported in Schemes 1 and 2 has been determined on human neutrophils. Directed migration (chemotaxis), superoxide anion production and lysozyme release have been measured. In the first set of experiments (see Figs. 2, 3) the agonistic activity was measured and compared to that of the standard tripeptide fMLF-OMe. In the second set (see Figs. 4, 5) the antagonistic activity of the *N*-Boc tetetrapeptides, which have been found inactive as agonists, have been examined as antagonists by measuring their ability to inhibit the effect stimulated by an optimal dose of fMLF-OMe.

As shown in Figs. 2a and 3a all the *N*-For tetrapeptide analogues maintain moderate to high capacity to induce



Scheme 1 Synthesis of *N*-protected tetrapeptides of the Xaa[2] type (**2–3**: Xaa = Gly; **5–6**: Xaa = Ala; **8–9**: Xaa = Pro): *i* TFA · H_2N-Leu-Phe-OMe, TEA, HOBT, EDCI, dry EtOAc; *ii* a: TFA, CH_2Cl_2; **b**: Boc-Met-OH, HOBT, TEA, EDCI, dry EtOAc; *iii* HCOOH, EEDQ



Scheme 2 Synthesis of *N*-protected tetrapeptides of the Xaa^3 type (**11–12**: Xaa = Gly; **14–15**: Xaa = Ala; **17–18**: Xaa = Pro): *i* Boc-Met-Leu-OH, HOBT, TEA, EDCI, dry EtOAc; *ii* HCOOH, EEDQ

chemotaxis with dose-response curves showing an optimal concentration value around 10^{-9} M. The three models of Xaa^2 type Gly^2 (**3**), βAla^2 (**6**) and Pro^2 (**9**) (Fig. 2a) exhibit moderate activity and very similar behaviour with the Pro^2 model **9** with about the same potency but with lower efficiency (peptide concentration at which maximal activity is observed) than the βAla^2 (**6**) and Gly^2 (**3**). The activity of the Xaa^3 type analogues, on the other hand, is greatly influenced by the nature of the spacer residue (Fig. 3a). Thus, while the tetrapeptide **15**, containing βAla as spacer, is a very potent chemotactic agent—nearly as active as the reference molecule—compound **12**, containing Gly as spacer, is only moderately active with a maximum value comparable to those shown by the models of the Xaa^2 type.

Figures 2b and 3b illustrate the ability of the Xaa^2 and Xaa^3 models, respectively, to trigger superoxide anion production. Also in this case all tetrapeptides maintain moderate to good activity with values of 50 and 53 nmoles at 10^{-6} M for βAla containing models **6** (Fig. 2b) and **15** (Fig. 3b), respectively. Notable is also the dose-response curve exhibited by the Pro^3 tetrapeptide **18** (Fig. 2b) with a peak value of 56 nmoles at concentration 10^{-5} M. This result is opposite to that observed in the Xaa^2 series where the Pro^2 incorporation produces the least active model (compound **9** in Fig. 2b).

As for the enzyme secretagogue activity (Figs. 2c, 3c) all the tetrapeptides show dose-response curves with the same maximum value centred at 10^{-5} M and lysozyme release moderately higher in the Xaa^3 series, with values ranging between 46–49% and 34–42% for the Xaa^3 and Xaa^2 models, respectively.

As shown in Figs. 2 and 3 the agonistic activity values shown by the *N*-Boc derivatives, regardless the nature of the spacer residue and its location on the backbone, are not statistically significant for all the three biological functions tested. These compounds have been examined as antagonists by measuring their ability to inhibit the effect stimulated by an optimal dose of the reference peptide fMLF-OMe. The influence of increasing concentrations of *N*-Boc tetrapeptides of Xaa^2 type (Gly^2: **2**; βAla^2: **5**; Pro^2: **8**) and Xaa^3 type (Gly^3: **11**; βAla^3: **14**; Pro^3: **17**) on the three examined functional acivities are reported in Figs. 4 and 5, respectively. It can be seen that the *N*-Boc tetrapeptides of both the series exert significant dose-dependent inhibition (50% ca. at 10^{-9} M) of the CI with a similar profile of activity (Figs. 4a, 5a). Conversely, no significant inhibition is observed for the *N*-Boc tetrapeptides on both the superoxide anion production (Figs. 4b, 5b) and lysozyme release (Fig. 5c), except a very weak effect shown by the Xaa^2 tetrapeptides on lysozyme release

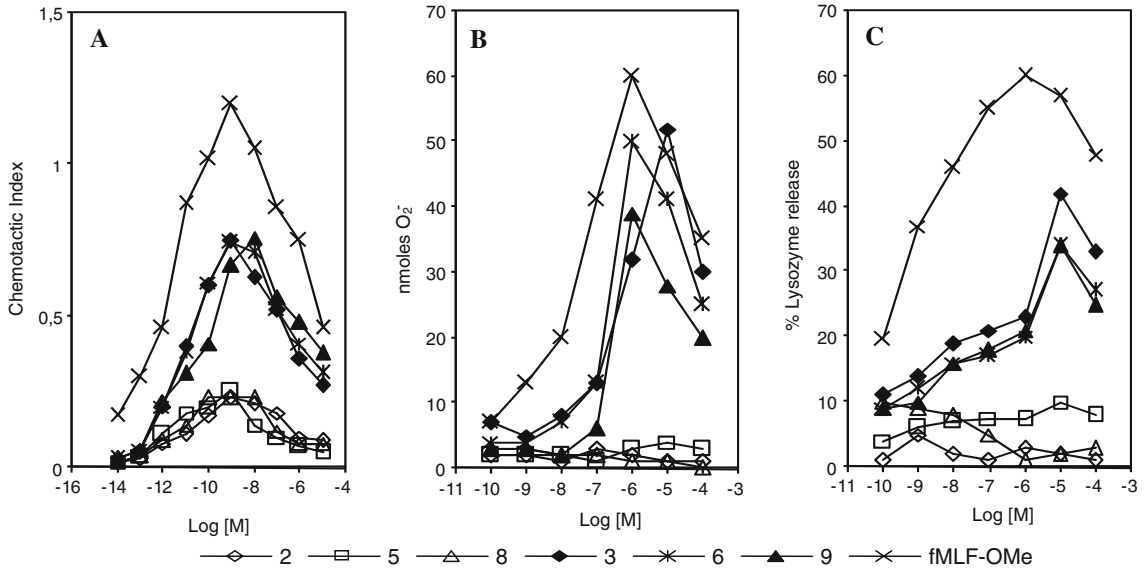


Fig. 2 Biological activity towards human neutrophils of *N*-For (**3**, **6** and **9**) and *N*-Boc (**2**, **5** and **8**) tetrapeptide analogues of Xaa^2 type (see Scheme 1) compared with the reference ligand fMLF-OMe. **a** Chemotactic index; **b** superoxide anion production; **c** release of granule enzymes evaluated by determining lysozyme release. Points are single representative experiments carried in duplicate

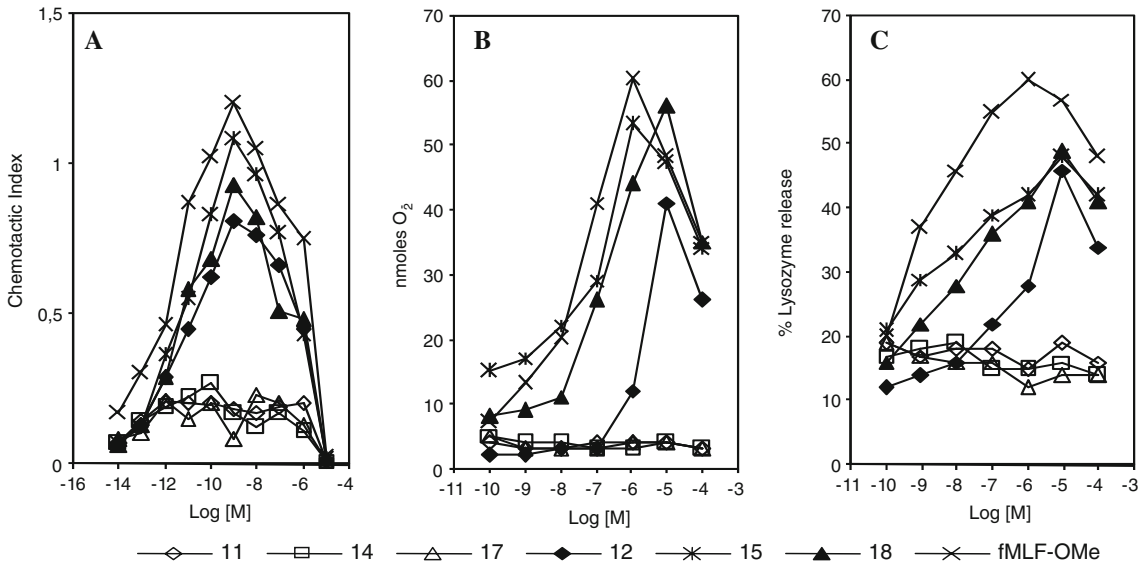


Fig. 3 Biological activity towards human neutrophils of *N*-For (**12**, **15** and **18**) and *N*-Boc (**11**, **14** and **17**) tetrapeptide analogues of Xaa^3 type (see Scheme 2) compared with the reference ligand fMLF-OMe. **a** Chemotactic index; **b** superoxide anion production; **c** release of granule enzymes evaluated by determining lysozyme release. Points are single representative experiments carried in duplicate

(Fig. 4c) where a 50% inhibition is reached only at high concentrations (10^{-6} to 10^{-5} M).

Conclusion

The results reported here give for the first time information on the properties of a series of tetrapeptidic fMLF-OMe analogues obtained by performing incorporation of spacer residues in the molecule of the reference ligand and leaving at the same time intact the original stereochemistry and succession on the backbone of the three native amino acid residues of the reference ligand. This approach differs from previously reported studies in which one or more residues of fMLF have been replaced (Cavicchioni et al. 2006). As shown in Figs. 2a and 3a all *N*-For tetrapeptides maintain the ability to elicit high to moderate values of chemotaxis. However, when the chemotatic index of the two series of tetrapeptides is compared two points can be evidenced: (1) the incorporation of the Xaa^2 spacer between the Met and

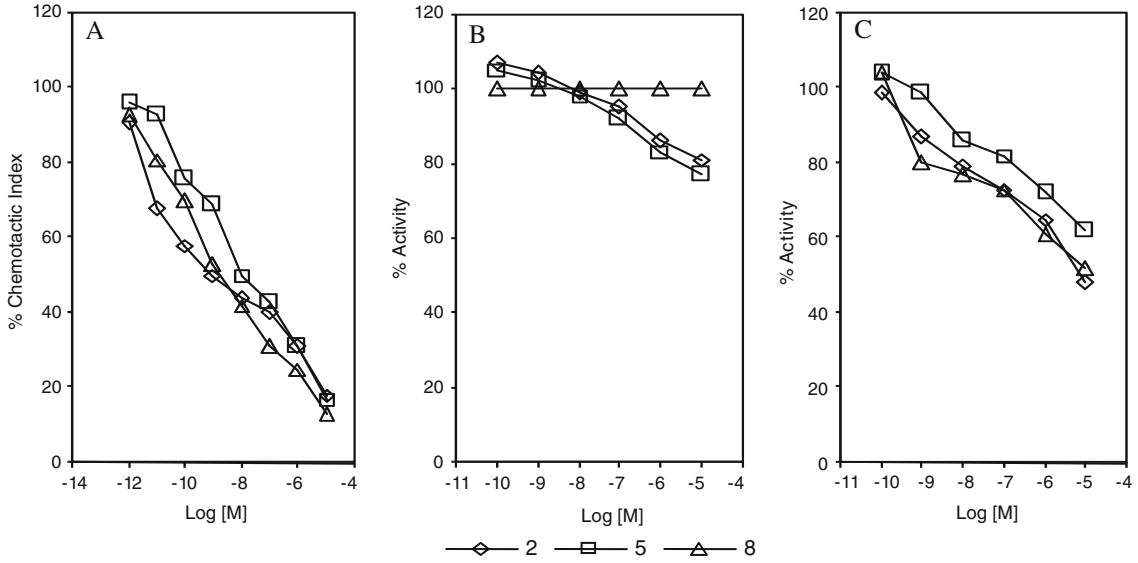


Fig. 4 Effect of *N*-Boc tetrapeptides of Xaa^2 type (Gly^2: **2**; βAla^2: **5**; Pro^2: **8**) on human neutrophil activities triggered by fMLF-OMe. **a** Chemotactic activity; **b** superoxide anion production; **c** release of granule enzymes

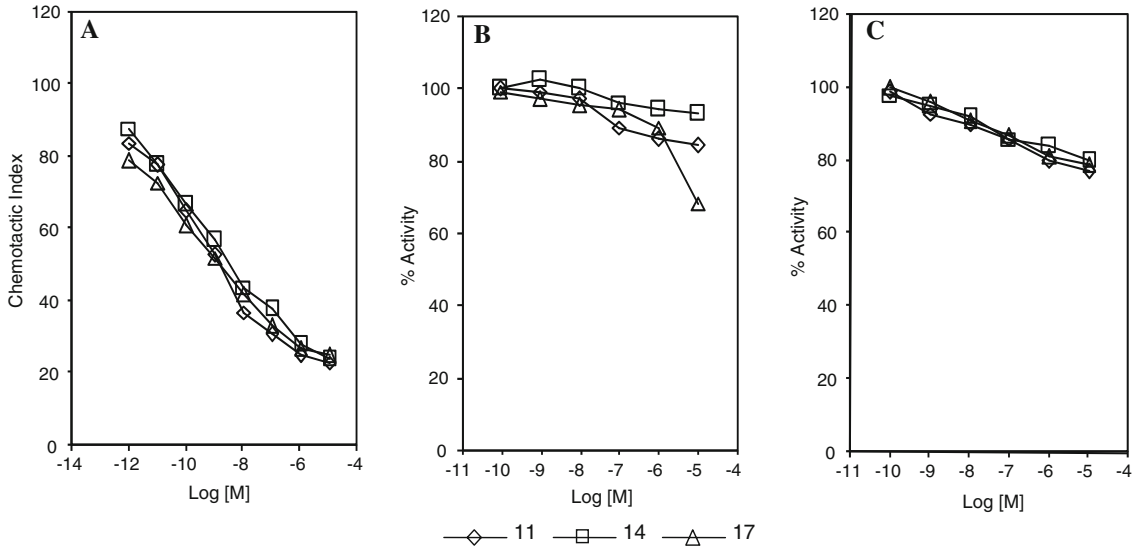


Fig. 5 Effect of *N*-Boc tetrapeptides of Xaa^3 type (Gly^3: **11**; βAla^3: **14**; Pro^3: **17**) on human neutrophil activities triggered by fMLF-OMe. **a** Chemotactic activity; **b** superoxide anion production; **c** release of granule enzymes

Leu (Fig. 2a) is generally more detrimental for the activity than the corresponding Xaa^3 modification involving Leu and Phe (Fig. 3a). Remarkable is the activity shown by the βAla^3 containing model **15** (Fig. 3a) which reaches an activity comparable to that of fMLF-OMe; (2) at variance with Xaa^3 series, the nature of the incorporated spacer does not affect appreciably the activity of Xaa^2 models which exhibit very close dose-response curves (Fig. 2a). Only the Pro^2 analogue **9** shows a slightly different behaviour with a peak of activity centred at higher concentration than the Gly^2 **3** and βAla^2 **6** models. This effect can be related to the well known influence of the Pro residue on hydrogen bonding and backbone conformation. On the basis of these findings it can be speculated that the Met and Leu binding pockets on the receptor are more critically related and probably more nearly located than those relating Leu and Phe. The sensibly higher chemotactic activity of the βAla^3 analogue **15**, as compared with the corresponding Gly^3 containing model **12** (Fig. 3a), seems in accordance with this preliminary interpretation.

With regard to the superoxide anion production (Figs. 2b, 3b) it can be noted the general good agonist activity shown by both the Xaa^2 and Xaa^3 series of analogues as compared with the only modest activity exhibited by the same models for the

lysozyme release (Figs. 2c, 3c). It can be observed here that the best results are obtained with the βAla containing models in both the Xaa^2 and Xaa^3 series (model **6** and **15** in Fig. 2b, 3b, respectively). Thus, the incorporation of the β-Ala residue at position 3 leads to tetrapeptide ligands with high activity for both chemotaxis and generation of reactive oxygen species. These findings stimulate further research on fMLF-OMe analogues obtained through incorporation of β-residues and in particular of achiral higher homologues of βAla. Models of this type, designed according the results reported in Figs. 2b and 3a, b, appear in fact well suited to give highly active new tetrapeptides as well as information on the interaction with receptor hydrophobic pockets.

As shown in Figs. 2 and 3 the presence of the bulky *N*-Boc protecting group does not modify the expected inactivity as agonists of the new analogues. These compounds have been then tested as antagonists. Figures 4 and 5 clearly show that the incorporation of a spacer gives rise, almost regardless the nature of the incorporated amino acid residue and its position on the backbone, to ligands characterized by relevant inhibition against migratory activity (Figs. 4a, 5a). A different result is observed in the case of the other two biological functions tested (Figs. 4b, c, 5b, c) where all the performed modifications do not produce appreciable inhibitory effects at physiological concentrations. Thus, the elongation of the fMLF-OMe backbone, through spacer incorporation at the two lateral positions, combined with the presence of a bulky *N*-protecting group, leads to effective antagonists which can be selective for the chemotactic function.

Taken together the here reported results indicate that the adopted approach of modification of the prototype of the family of chemotactic peptides gives rise to an articulate picture of data which represent a promising basis for the study of the FPR receptor and for the design of new ligands with both agonistic and selective antagonistic activity. Studies are in progress in our laboratories to further examine these points.

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