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Chemotactic tripeptides incorporating at position 2 α-aminoacid residues with unsaturated side chains

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Summary. New *N*-For-Met-Leu-Phe-OMe (fMLF-OMe) analogues incorporating three different γ-δ-didehydro-α-aminoacid residues (namely: Alg = (S)-Allylglycine; Dag = Diallylglycine; Cpg = 1-Aminocyclopent-3-ene-1-carboxylic acid) replacing the native (S)-Leucine have been synthesized and their activity towards human neutrophils has been evaluated in comparison with that shown by the reference tripeptide fMLF-OMe. Chemotaxis, lysozyme release and superoxide anion production have been measured. ^{1}H NMR titration experiments and NOESY spectrum of the Cpg containing model **10** have been discussed in order to ascertain the preferred solution conformations. A fully extended (C_5) conformation at position 2 and a folded conformation with two consecutive γ-turns (C_7 structure) have been proposed for the Dag and Cpg containing tripeptides, respectively.

Keywords: Aminoacids with unsaturated side chains – Chemotactic peptides – Cyclopentenyl aminoacids – Conformation – Grubbs reaction – Human neutrophils

Abbreviations: Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DMF, dimethylformamide; EDC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; EEDQ, ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate; HOBT, 1-hydroxybenzotriazole; KRPG, Krebs-Ringer phosphate containing 0.1% w/v D-glucose; RCM, ring-closing metathesis; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

Introduction

Directed migration of neutrophils along an increasing chemical gradient (chemotaxis) from blood to inflammatory sites is the first line of defence against bacterial infections. Chemotaxis depends upon the interaction of a variety of extracellular messengers with specific receptors located on neutrophil plasma membrane. Several chemically unrelated substances, such as arachidonic acid metabolites, complement fragment C5a, bacterial or mitochondrial protein-derived *N*-formyl peptides and a number of chemokines have the ability to initiate the migratory response (Harvath, 1991). Binding of these ligands with neutrophil receptors gives rise, in addition to chemotaxis, to a series of connected biochemical events consisting primarily in particle phagocytosis, superoxide anion production and release of proteolytic enzymes. Among chemotactic agents, highly specific for human neutrophils, *N*-For-Met-Leu-Phe-OH (fMLF) and its methyl ester (fMLF-OMe) are recognized as the reference molecules for studies aimed at elucidating the structure-activity relationships. These tripeptide ligands bind to at least two G-protein coupled receptors, the high-affinity FPR and its low-affinity variant FPRL1 (Le et al., 2004). It has been shown that complex intracellular signal transduction systems exist in human neutrophils (Fabbri et al., 1997; Selvatici et al., 2006). However, although several studies deal with this topic, the relationships between intracellular signalling and the different biological activities are still not well established. Thus, design and synthesis of fMLF analogues capable to exhibit selective behaviour (Miyazaki et al., 1995), represent very useful tools for studying signal transduction systems.

The effects of native amino acid substitutions on fMLF sequence, as well as the biological consequences of the backbone conformation modifications, have been extensively studied (Rathore, 2005). While the sulphur-containing *N*-terminal For-Met-moiety and the aromatic side chain at the *C*-terminal position are crucial for both binding and receptor activation, the hydrophobic Leu res-

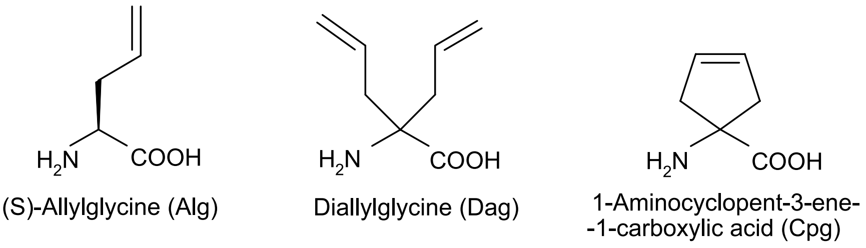


Fig. 1. Synthetic γ-δ-didehydro-α-aminoacid residues replacing native (S)-Leucine in the here reported fMLF-OMe analogues

idue at the position 2, although critical for backbone conformation, results to be the most tolerant towards replacements and modifications (Mills et al., 1998). In accordance with this observation, several studies on position 2 fMLF analogues have been performed with particular attention focused on: i) the control of the backbone conformations and ii) the production of ligands capable to exhibit selective behaviour towards the other cellular functions associated with the chemotaxis. Well known examples of the conformational control are the position 2 analogues containing $C^{\alpha\alpha}$disubstituted residues with linear or cyclic side chains, characterized by extended structure (Dentino et al., 1991; Torrini et al., 1997) and β-turn folding (Toniolo et al., 1989), respectively. Representative of a selectively acting analogue is the tripeptide $[\Delta^{z}\text{Leu}]^{2}$fMLF-OMe containing an α,β-didehydro residue replacing the native Leu; this compound is practically inactive as chemoattractant and highly active in the superoxide anion generation and lysozyme release (Pagani-Zecchini et al., 1993).

Based on the above observations indicating that peptide-receptor interactions in the specific area involving the position 2 of the *N*-formyl-tripeptides are important modulators of the biological effects and as prosecution of our previous research in this field (Mollica et al., 2006), we report here on a series of new fMLP-OMe analogues characterized by the presence at position 2 of three different types of synthetic α-aminoacids (Fig. 1), all containing γ-δ-didehydro side chains. (Alg = (S)-allylglycine; Dag = diallylglycine; Cpg = 1-aminocyclopent-3-ene-1-carboxylic acid). It can be noted that whereas C^{α}-allylglycine has been frequently used in peptide chemistry as starting residue for ring-closing metathesis (RCM) reactions, no data seem at the present available on biological activity of peptides incorporating the Dag or the Cpg residue. Model peptides containing a Boc protected *N*-terminal Cpg residue have been recently described (Kotha et al., 2001).

Materials and methods

General

Cpg-OEt has been synthesized starting from cis-1,4-dichloro-2-butene and the Schiff base obtained by reacting ethyl glycinate with 4-bromobenzaldehyde (Park et al., 1998). Dag-OEt was synthesized starting from ethyl isocyanoacetate and allyl bromide under phase transfer catalysis conditions (Kotha et al., 1998). All other starting materials and reagents were obtained commercially and used without further purification. Melting points were determined with a Büchi B 540 apparatus and are uncorrected. Optical rotations were taken at 20 °C with a Schmidt-Haensch Polartronic D polarimeter (1 dm cell, *c* 1.0 in $CHCl_3$, unless otherwise specified). IR spectra were recorded in 1% $CHCl_3$ solution (unless otherwise specified) employing a Perkin-Elmer FT-IR Spectrum 1000 spectrometer. ^{1}H NMR spectra were determined in $CDCl_3$ solution (unless otherwise specified) with a Bruker AM 400 spectrometer and chemical shifts were indirectly referred to TMS. NOESY spectra were recorded in $CDCl_3$ on Avance 400 Bruker console with a 800-msec mixing time and displayed in the phase-sensitive mode. Usually 256 × 1024 data points were collected and for each block 64 transients were collected for two-dimensional experiments. The data sets were linearly predicted to 512 × 1024 data points. A Gaussian window was applied in both dimensions. Zero filling was used for a final spectrum of 1024 × 1024 data points. 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.

General carbodiimide coupling procedure

To an ice-cooled mixture containing the required *N*-Boc- or *N*-Fmoc-amino acid (1.0 mmol), the amino-derivative · HCl (1.0 mmol), HOBT (1.2 mmol), and TEA (2.2 mmol) in anhydrous EtOAc (6 mL), EDC (1.2 mmol) was added and the reaction mixture was allowed to warm to room temperature. After 12 h the reaction mixture was diluted with EtOAc (20 mL) and washed with 1 M $KHSO_4$ (2 × 15 mL), saturated aqueous $NaHCO_3$ (2 × 15 mL) and brine (15 mL). The organic phase was dried and evaporated under reduced pressure.

Fmoc-Alg-Phe-OMe (**1**)

Fmoc-Alg-OH (0.5 g, 1.48 mmol) was treated with Phe-OMe · HCl (0.320 g, 1.48 mmol) according to the carbodiimide coupling general procedure. Crystallization from EtOAc/Hexane gave the title product as a white solid (0.670 g, 90%).

Mp 150–153 °C; $[\alpha]_D$ +14° (1% $CHCl_3$); IR ν: 3421, 1739, 1680 cm^{-1}. ^{1}H NMR δ: 2.3 (2H, br, CH_2–CH=CH_2), 2.74–3.0 (2H, m, Phe β-CH_2), 3.5 (3H, s, $COOCH_3$), 3.88–4.30 (4H, m, CH–CH_2 and Alg α-CH), 4.5 (1H, m, Phe α-CH), 4.7–4.9 (2H, m, CH_2–CH=CH_2), 5.3–5.5 (1H, m, CH_2–*CH*=CH_2), 6.63 (1H, br, Phe NH), 6.80 (1H, broad d, Alg NH), 7–7.5 (13H, m, aromatic). Anal. Calcd for $C_{30}H_{34}N_2O_5$: C, 71.69; H, 6.82; N, 5.57. Found: C, 71.58; H, 6.77; N, 5.58.

H_2N-Alg-Phe-OMe (**2**)

Fmoc-Alg-Phe-OMe (0.670 g, 1.34 mmol) in CH_2Cl_2 (4 mL) was *N*-deprotected by treatment with piperidine (4 mL) for 1/2 h. The reaction solution was diluted with CH_2Cl_2 (2 × 15 mL) and washed with 1 N HCl (2 × 15 mL). The aqueous phase was basified with $NaHCO_3$ and the product extracted with CH_2Cl_2. Resulting H_2N-Alg-Phe-OMe (**2**) was used without further purification.

Boc-Met-Alg-Phe-OMe (**3**)

The above prepared H_2N-Alg-Phe-OMe (**2**) was acylated with Boc-Met-OH (0.284 g, 1.14 mmol) according to the carbodiimide coupling general procedure. Silica gel flash chromatography (CH_2Cl_2/MeOH 98:2) gave a pure product as a white solid (0.430 g, 64%).

$[\alpha]_D$ +2° (0.5% $CHCl_3$). IR ν: 3414, 1741, 1670 cm^{-1}. ^1H NMR δ: 1.45 [9H, s, $C(CH_3)_3$], 1.85–2.11 [5H, m, Met β-CH_2 and S–CH_3 (s at 2.10)], 2.45–2.57 (4H, m, Met γ-CH_2 and $\mathit{CH_2}$–CH=CH_2), 3.05 and 3.17 (2H, A and B of an ABX, J = 5.6, 10.4 and 6 Hz, Phe β-CH_2), 3.72 (3H, s, $COOCH_3$), 4.23 (1H, m, Met α-CH), 4.4 (1H, m, Alg α-CH), 4.83 (1H, m, Phe α-CH), 5.07–5.18 (3H, m, CH_2–CH=$\mathit{CH_2}$ and Met NH), 5.61–5.68 (1H, m, CH_2–CH=CH_2), 6.46 (1H, br, Phe NH), 6.7 (1H, d, J = 7.2, Alg NH) 7.01–7.5 (5H, m, aromatic). Anal. Calcd for $C_{25}H_{37}N_3O_6S$: C, 59.15; H, 7.35; N, 8.28. Found: C, 59.13; H, 7.31; N, 8.09.

Boc-Met-Dag-OEt (**5**)

To a solution of Boc-Met-OH (0.476 g, 0.19 mmol) in anhydrous EtOAc (20 mL), BOP (0.955 g, 0.216 mmol), HOBT (0.357 g, 0.264 mmol) and few drops of DMF were added. The solution was then ice cooled for few minutes and a suspension of Dag-OEt · HCl (0.500 g, 2.16 mmol) and NMM (0.7 mL, 6.48 mmol) in EtOAc (2 mL) was added. After 12 h at room temperature the reaction mixture was diluted with EtOAc (20 mL) and washed with 1 M $KHSO_4$ (2 × 15 mL), saturated aqueous $NaHCO_3$ (2 × 15 mL) and brine (15 mL). The organic phase was dried and evaporated under reduced pressure. Silica gel chromatography ($CHCl_3$) gave the pure product as pale yellow oil (0.7 g, 86%).

$[\alpha]_D$ −15° (1% $CHCl_3$). IR ν: 3423, 1799, 1677 cm^{-1}. ^1H NMR δ: 1.25 (3H, m, $COOCH_2\mathit{CH_3}$), 1.43 [9H, s, $C(CH_3)_3$], 1.72–2.11 [5H, m, Met β-CH_2 and SCH_3 (s, at 2.09)], 2.48–2.65 (4H, m, Met γ-CH_2 and $\mathit{CH_2}$–CH=CH_2), 2.99–3.25 (2H, m, $\mathit{CH_2}$–CH=CH_2), 4.10–4.22 (3H, m, $COO\mathit{CH_2}CH_3$ and Met α-CH), 4.99–5.30 (5H, m, Met NH and two CH_2–CH=$\mathit{CH_2}$), 5.49–5.73 (2H, m, two CH_2–CH=CH_2), 6.95 (1H, s, Dag NH). Anal. Calcd for $C_{20}H_{34}N_2O_5S$: C, 57; H, 8.27; N, 6.76. Found: C, 57.03; H, 8.28; N, 6.57.

Boc-Met-Dag-OH

Boc-Met-Dag-OEt (0.7 g, 1.64 mmol) was treated with 2N LiOH (2 mL) and MeOH (7 mL) and left overnight at room temperature. MeOH was removed at reduced pressure, water (10 mL) was added and the solution extracted with Et_2O (2 × 25 mL). The aqueous phase was then acidified with 1N $KHSO_4$ and extracted with EtOAc (3 × 20 mL). The organic phases were washed with brine, pooled, dried and evaporated. A white foam (0.406 g, 60%), pure on TLC was obtained and used without further purification.

Boc-Met-Dag-Phe-OMe (**6**)

To a solution of Boc-Met-Dag-OH (0.406 g, 0.98 mmol) in anhydrous EtOAc (20 mL), BOP (0.490 g, 1.1 mmol), HOBT (0.185 g, 1.36 mmol) and few drops of DMF were added. The solution was cooled for few minutes and a suspension of Phe-OMe · HCl (0.240 g, 1.11 mmol) and NMM (0.32 mL, 2.94 mmol) in EtOAc (1 mL) was added. The reaction mixture was allowed to stand overnight at room temperature. EtOAc (20 mL) was then added and the solution washed with 1 M $KHSO_4$ (2 × 15 mL), saturated acqueous $NaHCO_3$ (2 × 15 mL) and brine (15 mL). The organic phase was dried and evaporated under reduced pressure. Silica gel chromatography (EtOAc/Hexane 3:7) and crystallization from EtOAc/Hexane gave the pure title product (**6**) as white solid (0.3 g, 53%).

IR ν: 3432, 1741, 1667 cm^{-1}. ^1H NMR δ: 1.44 [9H, s, $C(CH_3)_3$], 1.89–2.10 [5H, m, Met β-CH_2 and S–CH_3 (s, at 2.09)], 2.50–2.63 (4H, m, Met γ-CH_2 and $\mathit{CH_2}$–CH=CH_2), 2.76 (2H, m, $\mathit{CH_2}$–CH=CH_2), 3.05–3.14 (2H, m, Phe β-CH_2), 3.7 (3H, s, $COOCH_3$), 4.15 (1H, m, Met α-CH), 4.83 (1H, m, Phe α-CH), 5.00–5.08 (5H, m, two CH_2–CH=$\mathit{CH_2}$ and Met NH), 5.45–5.57 (2H, m, two CH_2–CH=CH_2), 6.81 (1H, br, Phe NH), 6.88 (1H, s, Dag NH), 7.0–7.5 (5H, m, aromatics). Anal. Calcd for $C_{28}H_{41}N_3O_6S$: C, 61.40; H, 7.55; N, 67.00. Found: C, 61.26; H, 7.77; N, 67.25.

Boc-Met-Cpg-OEt (**8**)

Boc-Met-OH (0.268 g, 1.07 mmol) was coupled with Cpg-OEt (0.152 g, 0.980 mmol) according to the carbodiimide coupling general procedure. The title compound was obtained as a foam, pure on TLC (0.221 g, 58%).

$[\alpha]_D$ +1.1° (1% $CHCl_3$). IR ν: 3422, 1739, 1660 cm^{-1}. ^1H NMR δ: 1.27 (3H, t, J = 7.6 Hz, $COOCH_2\mathit{CH_3}$), 1.46 [9H, s, $C(CH_3)_3$], 1.90–2.15 [5H, m, Met β-CH_2 and S–CH_3 (s at 2.13)], 2.58–2.69 (4H, m, Met γ-CH_2 and $\mathit{CH_2}$–CH=CH), 3.10 (2H, apparent t, $\mathit{CH_2}$–CH=CH), 4.21 (2H, q, $COO\mathit{CH_2}CH_3$), 4.27 (1H, m, Met α-CH), 5.18 (1H, br, Met NH), 5.68 (2H, sharp m, CH=CH), 6.89 (1H, s, Cpg NH). Anal. Calcd for $C_{18}H_{30}N_2O_5S$: C, 55.94; H, 7.82; N, 7.25. Found: C, 56.22; H, 7.77; N, 7.22.

Boc-Met-Cpg-OH

Boc-Met-Cpg-OEt (0.210 g, 0.54 mmol) was treated with 1 N NaOH (2.17 mL) and MeOH (10.0 mL) and left at room temperature overnight. The MeOH was evaporated under vacuum, water (10 mL) was added and the solution extracted with diethyl ether (2 × 25 mL). The aqueous phase was then acidified with 1N $KHSO_4$ and the product extracted with EtOAc (3 × 20 mL). The organic phases were washed with brine, pooled, dried and evaporated to give TLC pure title product as white foam (0.190 g, quantitative yield). Anal. Calcd for $C_{16}H_{26}N_2O_5S$: C, 53.61; H, 7.31; N, 8.2. Found: C, 53.5; H, 7.49; N, 8.38.

Boc-Met-Cpg-Phe-OMe (**9**)

Boc-Met-Cpg-OH (0.195 g, 0.54 mmol) was treated with Phe-OMe · HCl (0.117 g, 0.54 mmol) according to the carbodiimide coupling general procedure. Crystallization from EtOAc gave the title pure product as a colourless crystals (0.181 g, 64%).

Boc-Met-Cpg-Phe-OMe (**9**) was also prepared by using the ring-closing methatesis (RCM) reaction: (20 mg, 0.045 mmol) of Boc-Met-Dag-Phe-OMe (**6**) in DCM (2 mL) was treated with 2nd generation Grubbs catalyst (benzylidene [1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene]-dichloro-(phenylmethylene)-(tricyclohexylphosphine) ruthenium) (10% mol) and the reaction mixture was left at room temperature for 5 h. The solvent was evaporated and the crude product was purified by preparative TLC (eluant EtOAc/$CHCl_3$ 1:1) to give 11 mg (47%) of product which resulted to be identical to Boc-Met-Cpg-Phe-OMe obtained by coupling the above reported Boc-Met-Cpg-OH with Phe-OMe.

Mp 148.1–148.9 °C. IR ν: 3428, 1742, 1667 cm^{-1}. ^1H NMR δ: 1.46 [9H, s, $C(CH_3)_3$], 1.87–2.14 [5H, m, Met β-CH_2 and S–CH_3 (s at 2.11)], 2.47–2.62 (2H, m, Met γ-CH_2), 2.72–2.92 (4H, m, two $\mathit{CH_2}$–CH=CH), 3.08 and 3.18 (2H, A and B of an ABX, J = 5.6, 6.8 and 14 Hz, Phe β-CH_2) 3.74 (3H, s, OCH_3), 4.22 (1H, m, Met α-CH), 4.84 (1H, m, Phe α-CH), 5.16 (1H, br, Met NH), 5.71 (2H, m, CH=CH), 6.67 (1H, br, Phe NH), 7.16 (1H, s, Cpg NH), 7.09–7.33 (5H, m, aromatic). Anal. Calcd for $C_{26}H_{37}N_3O_6S$: C, 60.09; H, 7.18; N, 8.09. Found: C, 59.98; H, 7.11; N, 7.78.

General procedure for the preparation of the *N*-formyl derivatives

The *N*-Boc derivative (1.0 mmol) was dissolved in formic acid (3 mL) and the mixture was allowed to stand at room temperature overnight. After removal of the excess formic acid under vacuum, the residue was dissolved in dry CH_2Cl_2 (10 ml) or dry DMF (2 mL). EEDQ 97% (1.2 mmol) was added and the solution was stirred at room temperature for 24 h. Evaporation under reduced pressure afforded the crude *N*-formyl derivative (Lajoie and Kraus, 1984).

For-Met-Alg-Phe-OMe (**4**)

From Boc-Met-Alg-Phe-OMe (0.100 g, 0.19 mmol) in dry DMF according to the general procedure for preparation of the N-formyl derivatives. Silica gel flash chromatography (CH_2Cl_2/MeOH 98:2) gave the pure product as a white solid (0.07 g, 86%).

$[\alpha]_D$ +3° (1% $CHCl_3$). IR ν: 3414, 1741, 1670 cm^{-1}. ^1H NMR δ: 1.91–2.11 [5H, m, Met β-CH_2 and S–CH_3 (s, at 2.09)], 2.40–2.61 (4H, m, Met γ-CH_2 and CH_2–CH=CH_2), 3.09 and 3.14 (2H, A and B of an ABX, J = 5.6, 6 and 13.6 Hz, Phe β-CH_2), 3.7 (3H, s, $COOCH_3$), 4.54 (1H, m, Alg α-CH), 4.73 (1H, m, Met α-CH), 4.81 (1H, m, Phe α-CH), 5.10 (2H, m, CH_2–CH=CH_2), 5.6 (1H, m, CH_2–CH=CH_2), 6.72 (2H, apparent d, Phe NH and Met NH), 6.93 (1H, d, J = 7.2 Hz, Alg NH) 7.02–7.5 (5H, m, aromatic), 8.17 (1H, s, HCO). Anal. Calcd for $C_{21}H_{29}N_3O_5S$: C, 57.91; H, 6.71; N, 9.65. Found: C, 57.37; H, 6.76; N, 9.71.

For-Met-Dag-Phe-OMe (**7**)

From Boc-Met-Dag-Phe-OMe (0.100 g, 0.17 mmol) in dry DMF according to the general procedure for preparation of the N-formyl derivatives. Silica gel flash chromatography (CH_2Cl_2/MeOH 98:2) gave the pure product as a colourless oil (0.06 g, 70%).

IR ν: 3414, 1741, 1670 cm^{-1}. ^1H NMR δ: 1.87–2.11 [5H, m, Met β-CH_2 and S–CH_3 (s, at 2.09)], 2.48–2.65 (4H, m, Met γ-CH_2 and CH_2–CH=CH_2), 2.81–2.90 (2H, m, CH_2–CH=CH_2), 3.09 and 3.14 (2H, A and B of an ABX, J = 5.6, 7.2 and 14 Hz, Phe β-CH_2), 3.7 (3H, s, $COOCH_3$), 4.57 (1H, m, Met α-CH), 4.83 (1H, m, Phe α-CH), 4.97–5.10 (4H, m, two CH_2–CH=CH_2), 5.45–5.57 (2H, m, two CH_2–CH=CH_2), 6.37 (2H, d, J = 7 Hz, Met NH), 6.63 (1H, d, J = 7.2 Hz, Phe NH), 6.93 (1H, s, Dag NH), 7.1–7.3 (5H, m, aromatic), 8.13 (1H, s, HCO). Anal. Calcd for $C_{24}H_{33}N_3O_5S$: C, 60.61; H, 6.99; N, 8.84. Found: C, 60.64; H, 6.95; N, 8.65.

For-Met-Cpg-Phe-OMe (**10**)

From Boc-Met-Cpg-Phe-OMe (0.06 g, 0.11 mmol) in CH_2Cl_2 according to the general procedure for preparation of the N-formyl derivatives. Silica gel flash chromatography (EtOAc/$CHCl_3$ 7:3) gave the pure product as a colourless oil (0.05 g, 98%).

IR ν: 3411, 1742, 1670 cm^{-1}. ^1H NMR δ: 1.90–2.11 [5H, m, Met β-CH_2 and SCH_3 (s at 2.11)], 2.45–2.63 (2H, m, Met γ-CH_2), 2.71–2.98 (4H, m, two CH_2–CH=CH), 3.08 and 3.17 (2H, A and B of an ABX, J = 5.6, 6.8 and 14 Hz, Phe β-CH_2), 3.74 (3H, s, OCH_3), 4.64 (1H, m, Met α-CH), 4.83 (1H, m, Phe α-CH) 5.71 (2H, m, CH=CH), 6.57 (1H, d, J = 7.6 Hz, Met NH), 6.61 (1H, d, J = 7.6 Hz, Phe NH), 7.08–7.34 (6H, m, aromatic and Cpg NH), 8.13 (1H, s, HCO). Anal. Calcd for $C_{25}H_{29}N_3O_5S$: C, 59.34; H, 6.53; N, 9.39. Found: C, 59.01; H, 6.6; N, 9.5.

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

Chemotaxis

Each peptide was added to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution with KRPG containing 1 mg/ml of bovine serum albumin (BSA; Orha Behringwerke, Germany) and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (CI), which is the ratio (migration toward test attractant minus migration toward the buffer/migration toward the buffer); the values are the mean of six separate experiments performed in duplicate. Standard errors are in the 0.02–0.09 CI range.

Superoxide anion (O_2^-) production

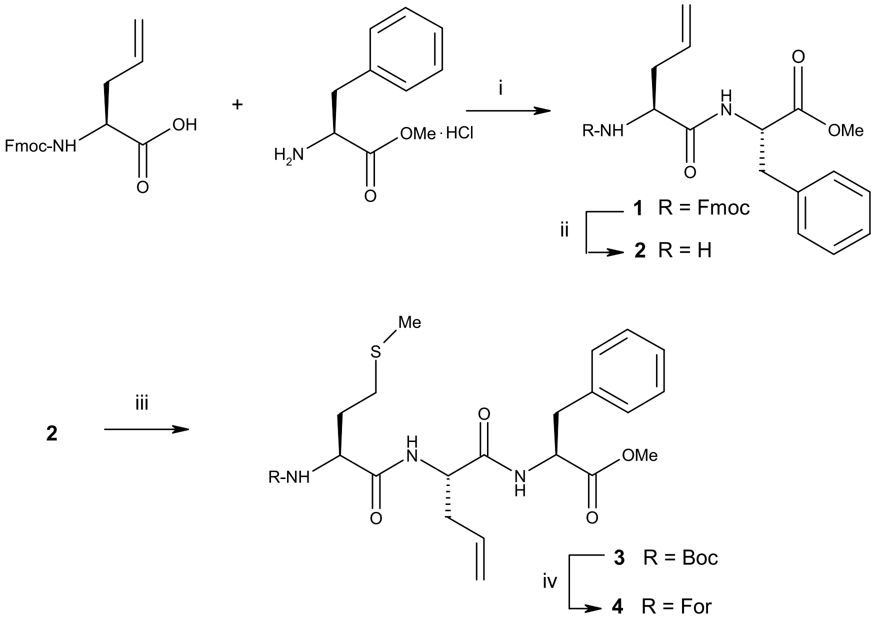
This anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sigma, USA) modified for microplate-based assays. Tests were carried out in a final volume of 200 µl containing 4×10^5 neutrophils, 100 nmoles cytochrome *c* and KRPG. At zero time different amounts (10^{-10}–8×10^{-5} M) of each peptide were added and the plates were incubated into a microplate reader (Ceres 900, Bio-TeK Instruments, Inc.) with the compartment temperature set at 37 °C. Absorbance was recorded at 550 and 468 nm. The difference in absorbance at the two wavelengths was used to calculate nmoles of O_2^- produced using an absorptivity for cytochrome *c* of 18.5 mM^{-1} cm^{-1}. Neutrophils were incubated with 5 µg/ml cytochalasin B (Sigma) for 5 min prior to activation by peptides. Results were expressed as net nmoles of O_2^- per 1×10^6 cells per 5 min and are the mean of six separate experiments performed in duplicate. Standard errors are in 0.1–4 nmoles O_2^- range.

Enzyme assay

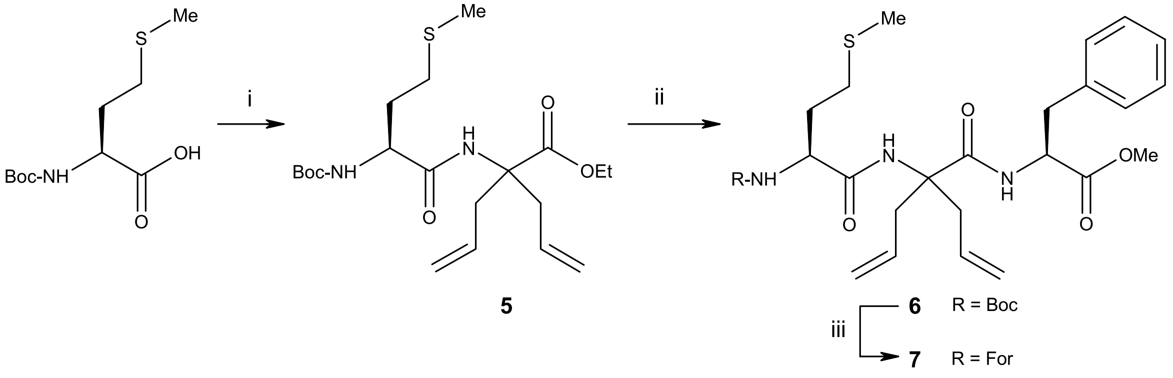
The release of neutrophil granule enzymes was evaluated by determination of lysozyme activity, modified for microplate-based assays. Cells, 3×10^6/well, were first incubated in triplicate wells of microplates with 5 µg/ml cytochalasin B at 37 °C for 15 min and then in the presence of each peptide at a final concentration of 10^{-10}–8×10^{-5} M for a further 15 min. The plates were then centrifuged at $400 \times g$ for 5 min and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus*. The reaction rate was measured using a microplate reader at 465 nm. Enzyme release was expressed as a net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 mg per 1×10^7 cells/min. The values are the mean of five separate experiments done in duplicate. Standard errors are in the range of 1–6%.

Synthesis

The synthesis of the Boc-peptides and the corresponding *N*-formyl analogues was performed according to Schemes 1–3. Fmoc-Alg-Phe-OMe (**1**) was obtained by coupling Fmoc-Alg-OH with Phe-OMe·HCl using EDC/



Scheme 1. Synthesis of (S)-Allylglycine (Alg) containing tripeptides **3** and **4**: i) TEA, HOBt, EDC, EtOAc; ii) piperidine, CH_2Cl_2, r.t.; iii) Boc-Met, TEA, HOBt, EDC, EtOAc; iv) Formic acid, EEDQ, dry $CHCl_3$



Scheme 2. Synthesis of Diallylglycine (Dag) containing tripeptides **6**–**7**: i) BOP, HOBT, Dag-OEt, dry EtOAc, DMF; ii) a: 2N LiOH, MeOH; b: BOP, HOBT, dry EtOAc, DMF; c: Phe-OMe · HCl, NMM, EtOAc; iii) Formic acid, EEDQ, dry DMF

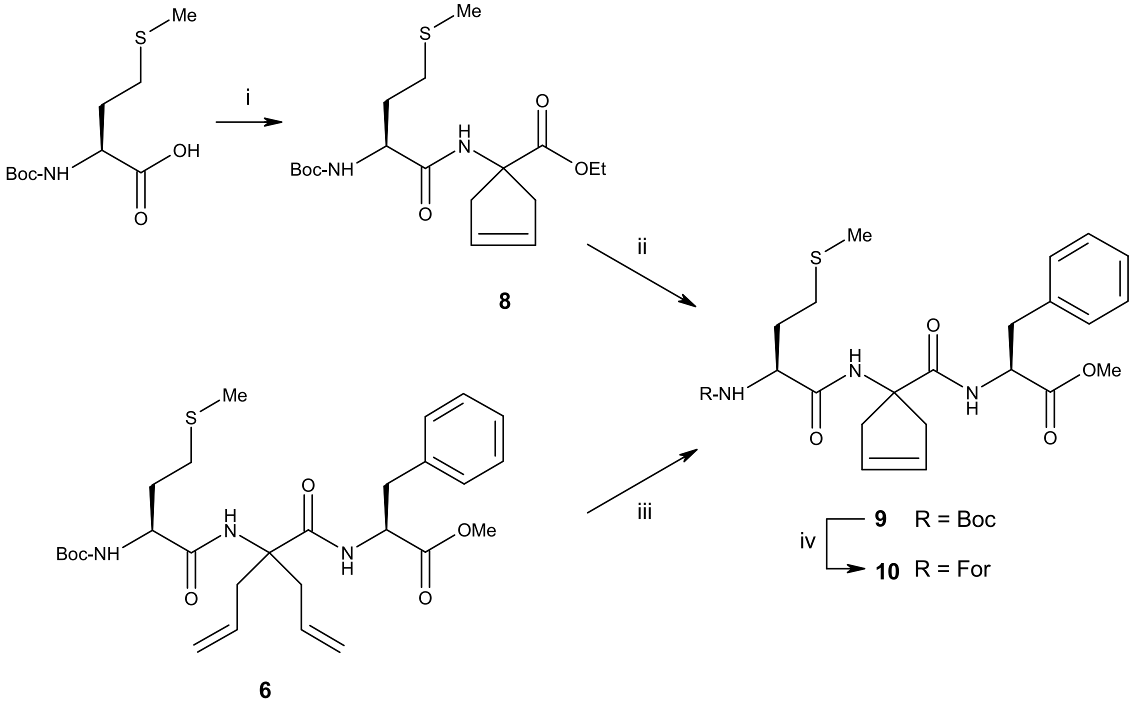
HOBT. After *N*-deprotection of **1** by pyperidine treatment, the dipeptide **2** was acylated (EDC/HOBT) by Boc-Met-OH to give Boc-Met-Alg-Phe-OMe (**3**). Coupling of Boc-Met-OH with Dag-OEt (BOP/HOBT) or Cpg-OEt (EDC/HOBT) afforded Boc-Met-Dag-OEt (**5**) and Boc-Met-Cpg-OEt (**8**), respectively. *C*-deprotection by hydrolysis of **5** and **8** gave the free acids which were coupled (EDC/HOBT) with Phe-OMe·HCl to give Boc-Met-Dag-Phe-OMe (**6**) and Boc-Met-Cpg-Phe-OMe (**9**), respectively. RCM reaction by using second generation Grubbs catalyst performed on the tripeptide **6** containing central Dag afforded in good yields the Cpg analogue **9**. Direct transformation of the *N*-Boc derivatives **3**, **6**, and **9** into the corresponding *N*-formyl analogs **4**, **7** and **10** was performed by treatment of the *N*-Boc derivatives with formic acid followed by EEDQ (Lajoie and Kraus, 1984).

Conformational studies

^{1}H NMR

Information on the conformational preferences of the tripeptides **7**, **9** and **10** containing the stereochemically constrained residues of Dag and Cpg has been obtained by examining the involvement of the NH groups in intramolecular H-bonds by using ^{1}H NMR titration experiments.

In Fig. 2 the chemical shift dependence of the shift values as a function of increasing DMSO-d_6 concentrations in $CDCl_3$ solution (10 mM) is reported. In Table 1 the solvent exposure of the NH groups is expressed as the difference (Δδ, ppm) between the chemical shift observed in neat $CDCl_3$ and in a $CDCl_3$ solution containing 10% DMSO-d_6. High values of Δδ correspond to efficient H-



Scheme 3. Synthesis of 1-Aminocyclopent-3-ene-1-carboxylic acid (Cpg) containing tripeptides **9** and **10**: i) HOBT, EDC, Cpg-OEt, EtOAc; ii) a: 1N NaOH, MeOH; b: Phe-OMe · HCl, HOBT, TEA, EDC, dry EtOAc; iii) second generation Grubbs reagent, CH_2Cl_2; iv): Formic acid, EEDQ, DCM, r.t.

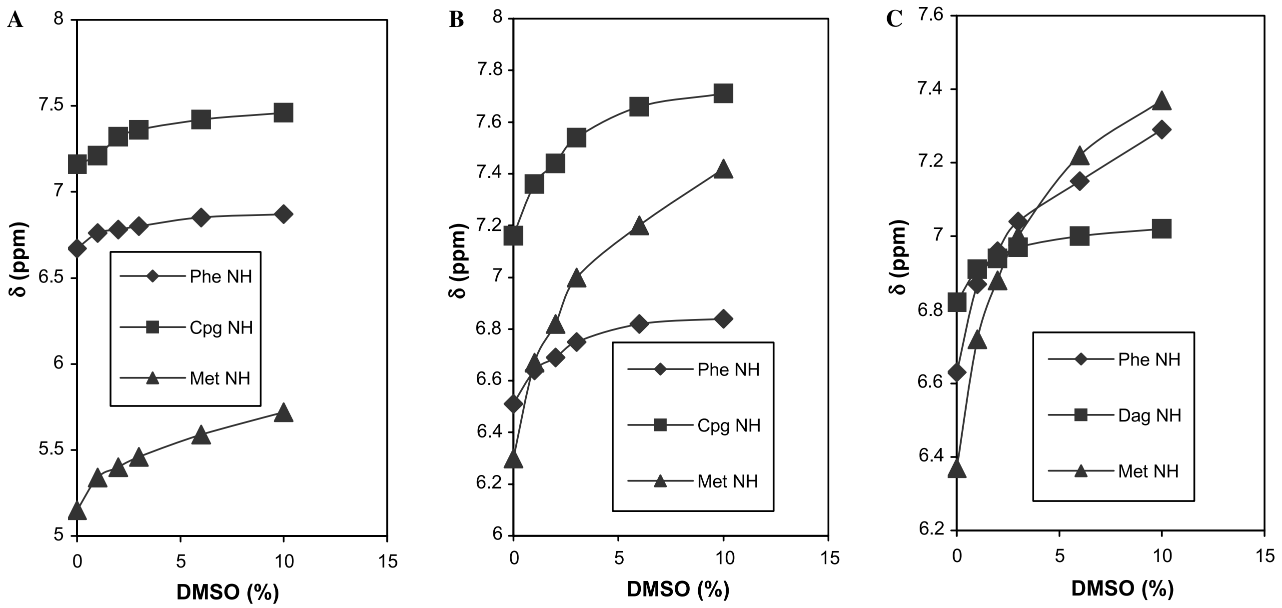


Fig. 2. Delineation of NH chemical shifts as a function of increasing percentage of DMSO-d_6 to the $CDCl_3$ solution (vol/vol) for (**A**): Boc-Met-Cpg-Phe-OMe (**9**); (**B**): For-Met-Cpg-Phe-OMe (**10**); (**C**): For-Met-Dag-Phe-OMe (**7**)

bonding interaction of the NH groups with the DMSO-d_6 while low values indicate scarce sensibility to solvent composition variation and presumable involvement in intramolecular H-bonds. The values of here studied peptides reported in Table 1 are compared with the data previously observed for the related models Boc-Met-Ac_5c-Phe-OMe (Toniolo et al., 1989) and HCO-Met-Dpg-Phe-OMe (Dentino et al., 1991) containing 1-amino-1-cyclopropane

Table 1. Solvent-composition effects on NH chemical shifts of peptides. Values reported are the differences ($\Delta\delta$, ppm) between NH proton chemical shifts observed in $CDCl_3$ solution containing 10% DMSO-d_6 and neat $CDCl_3$

Compound	Met NH	Central NH	Phe NH
HCO-Met-Dag-Phe-OMe (**7**)	1.00	0.20	0.66
HCO-Met-Cpg-Phe-OMe (**10**)	1.12	0.55	0.33
Boc-Met-Cpg-Phe-OMe (**9**)	0.57	0.30	0.20
Boc-Met-Ac_5c-Phe-OMe[a]	1.00	0.94	0.15
HCO-Met-Dpg-Phe-OMe[b]	1.40	0.25	1.30

[a] See reference: Toniolo et al. (1989)

[b] See reference: Dentino et al. (1991)

carboxylic acid (Ac_5c) residue and di-*n*-propylglycine (Dpg), respectively.

The solvent perturbation experiments performed on Cpg containing models **9** and **10** indicate that the Phe and the Cpg NH (Table 1) are scarcely affected by the change of the solvent composition ($\Delta\delta = 0.20$ and 0.30 ppm for **9** and 0.33 and 0.55 for **10**, respectively). The *N*-terminal Met NH shows, on the other hand, a value of 1.12 ppm for the HCO–NH of **10** and 0.57 for the Boc-NH of **9**. To properly evaluate the solvent shielding exhibited by the Met NH of **9** it should be recalled that the bulky *t*-butyloxycarbonyl group significantly hinders the interaction of the NH with the solvent (Aschi et al., 2003). Thus, the $\Delta\delta$ value found for **9**, lower than that expected for a free NH group, is only in part attributable to the participation to intramolecular H-bonding since the effect of the *N*-Boc must be considered. By taking into account this latter consideration it can be assumed that both **9** and **10** are characterized by a similar conformer population characterized by the central Cpg and the *N*-terminal Phe NH groups not freely interacting with the solvent and then presumably involved in intramolecular H-bonds.

It is interesting to note that the above reported **9** and **10** titration data differ significantly from those exhibited by the tripeptide Boc-Met-Ac_5c-Phe-OMe (Toniolo et al., 1989). In this case (Table 2) both the *C*-terminal Met and the central Ac_5c NH groups interact efficiently with the solvent ($\Delta\delta = 1.0$ and 0.94 ppm, respectively) while the Phe NH appears sensibly unaffected by the solvent composition ($\Delta\delta = 0.15$ ppm). Thus, the preferred solution conformation of the Ac_5c containing tripeptide has been described as a type-II β-turn (C_{10} ring) stabilized by a single intramolecular H-bond involving the Boc CO and the *C*-terminal Phe NH. In the case of the analogues **9** and **10**, containing the central Cpg residue, the picture is less clearly defined since both the Cpg NH and the *C*-terminal Phe NH, appear not completely free to interact with the solvent. Thus, a conformation which takes into account this situation should be considered.

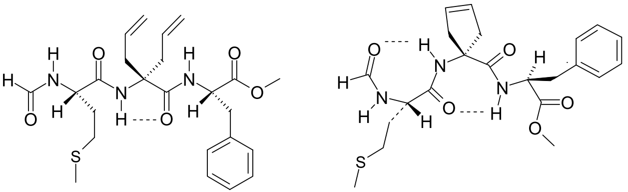
Table 2. Observed nuclear Overhauser effects (NOEs) in the Noesy spectrum of HCO-Met-Cpg-Phe-OMe (**10**)

HCO···Met NH	m	Cpg NH···Met $C^{\alpha}H$	s
Met NH···Met $C^{\alpha}H$	w	Cpg $C^{\gamma}H$···Cpg $C^{\beta}H_2$	s
Met $C^{\alpha}H$···$C^{\beta}H_2$	m	Cpg $C^{\delta}H$···Cpg $C^{\varepsilon}H_2$	s
Phe NH···Phe $C^{\alpha}H$	m	Cpg $C^{\beta}H_2$···Phe NH	m
Phe $C^{\alpha}H$···Phe $C^{\beta}H_2$	m	Cpg $C^{\varepsilon}H_2$···Phe NH	m

s strong; *m* medium; *w* weak

In order to get further information on the preferred conformation induced by the central Cpg residue, a two-dimensional NMR experiment was carried out on the tripeptide **10**. Detection of nuclear Overhauser effects (NOEs) in the NOESY spectra are related to the through-space proximity of the protons of the molecule. The estimates of the cross-peak relative intensities found for **10** are summarized in Table 2 on an arbitrary scale. The interresidue NOE HCO···Met NH is in accordance with the *cis* configuration of the two H atoms of the H–CO–NH group, as always found in the crystal structures of *N*-formylpeptides. Noteworthy is the absence of the NOE between the NH groups of the $(i+2)$ and $(i+3)$ residue (*i.e.* Cpg NH···Phe NH) which is diagnostic of a β-turn structure. Furthermore, the observation of NOEs between the Phe NH and the Cpg β- and ε-CH_2 groups, does not support the hypothesis of the C_{10} conformation and suggests that the Phe NH interacts with the Met-CO thus stabilizing a γ-turn centred at the Cpg residue. Concerning the Cpg NH···Met $C^{\alpha}H$ spatial connectivity it can be observed that this finding, taken together with the partial solvent inaccessibility shown by the Cpg NH ($\Delta\delta =$ 0.30 ppm), suggests the presence, although in limited extent, of a second γ-turn structure (Rao et al., 1983; Millet et al., 2001) centred at the Met residue and involving the formylic carbonyl and the Cpg NH (Fig. 3).

The titration experiments were then extended to the analogue HCO-Met-Dag-Phe-OMe (**7**) containing the dia-



7 **10**

Fig. 3. Preferred conformation adopted by *N*-formyl-tripeptides **7** and **10** in $CDCl_3$ solution. The intraresidue H-bond in the C_5 conformation of **7** and in the two consecutive γ-turns (C_7 structures) of **10** are indicated by dotted lines

llylglycine residue in place to the native (S)-leucine (Fig. 2C). In this case the NH of the central residue presents a pronounced solvent inaccessibility ($\Delta\delta = 0.20$, Table 1) while the Met and, in less extent, the Phe NH groups interact efficiently with the solvent. In accordance with literature these data suggest the presence in $CDCl_3$ solution of a significant population of the peptide molecules in an extended conformation with the Dag NH involved in a C_5 intraresidue H bond (Fig. 3). An analogue behaviour has been previously observed (Dentino et al., 1991) for the corresponding saturated formyl tripeptide HCO-Met-Dpg-Phe-OMe in which the NH of the central $C^{\alpha,\alpha}$-tetrasubstituted residue is strongly solvent shielded while the Met and Phe NH groups are completely free (see Table 1).

IR

The presence in the tripeptides under study of intramolecularly H bonded NH groups has also been studied by the examining the IR absorptions in the NH stretching region ($CHCl_3$, 5 mM). In the spectrum of *N*-Boc derivative **9** two bands are observed at 3428 and 3375 cm^{-1} corresponding to free and H bonded groups, respectively. An analogue outcome is shown by the corresponding formyl derivative **10** which exhibits a shoulder at 3378 cm^{-1} partially superimposed to the band at 3411 cm^{-1} due to the free NH stretching. In the spectrum of **7** two bands are observed at 3416 and 3368 cm^{-1}; the band at lower frequency can be assigned to the intraresidue H bonded Dag NH group. Finally a strong absorption band at 3416 cm^{-1}, attributable to free NH stretching, is exhibited by the *N*-formyltripeptide **4** which presents a weak large band centered at 3320 cm^{-1}. This suggests that, at variance with derivatives **7**, **9** and **10**, only weak intramolecular H-bonds occur in the $CHCl_3$ solution of **4**.

Biological results

The biological activity of the *N*-formyl analogues **4**, **7**, and **10**, together with that of the corresponding *N*-Boc derivatives **3**, **6** and **9** has been determined on human neutrophils and compared with that of the reference ligand fMLF-OMe. Directed migration (chemotaxis), superoxide anion production and lysozyme release have been measured. The results of the agonistic chemotactic activity of the three *N*-formyl tripeptides, expressed as chemotactic index (C.I.), are summarized in Fig. 4A. It can be seen that the three formyl tripeptides **4**, **7**, and **10** are able to significantly stimulate neutrophil chemotaxis although with lower potency than the parent ligand fMLF-OMe. Notably, the Cpg containing analogue **10** reaches the same potency of the parent at a concentration value of 10^{-7} M. Both the Alg and Dag containing models **4** and **7**

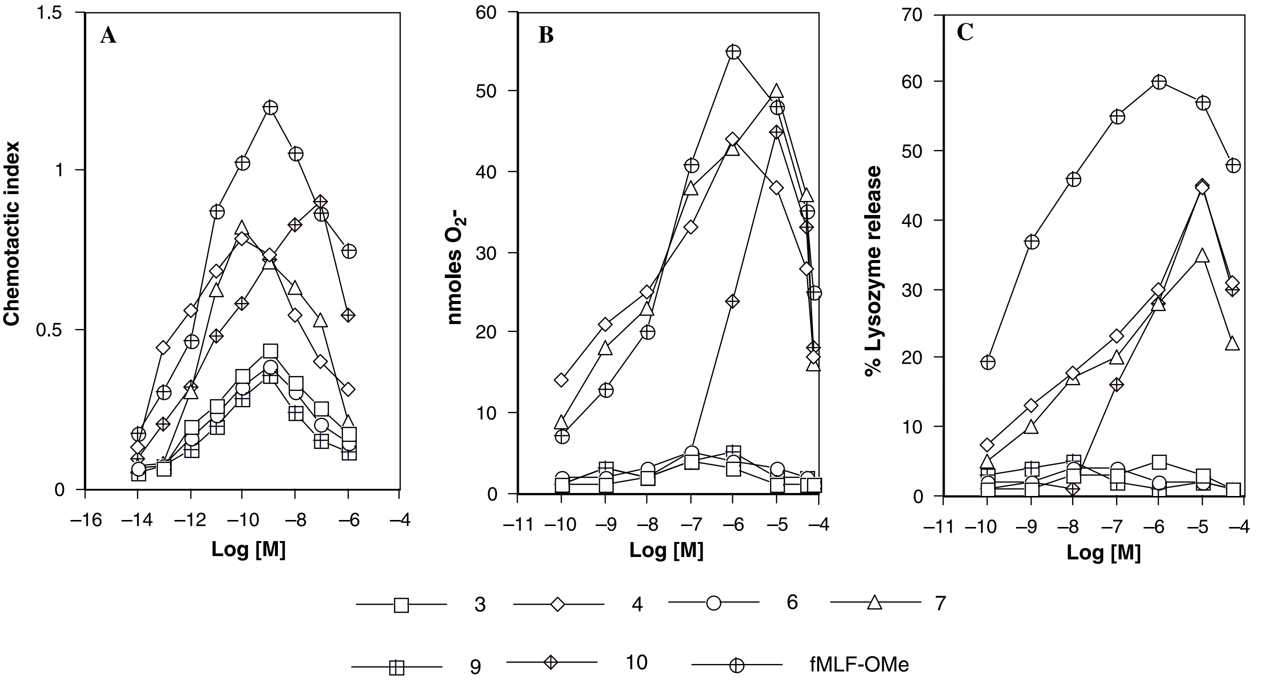


Fig. 4. Biological activity of centrally modified *N*-For and *N*-Boc fMLF-OMe analogues **3**, **4**, **6**, **7**, **9** and **10**. (**A**) Chemotactic index; (**B**) superoxide anion production and (**C**) release of neutrophil granule enzyme evaluated by determining lysozyme activity

show an activity peak (C.I. = 0.78 and 0.82, respectively) centred at a lower concentration (10^{-10} M) than that of the parent (10^{-9} M). Furthermore, at very low concentration (10^{-13} M) the Alg containing model **4** shows higher activity than the parent molecule (C.I. = 0.44 against 0.33), thus exhibiting higher efficacy. As also shown in Fig 4A all the *N*-Boc derivatives **3**, **6**, and **9** exhibit a very similar common behaviour with an activity peak centred at 10^{-9} M and a very modest activity.

As shown in Fig. 4B the three formylpeptides **4**, **7**, and **10** are highly active in stimulating superoxide anion production with the Alg containing analogue **4** showing the activity peak at the same concentration of the parent although with lower activity. Both the other two analogues **7** and **10** induce the same maximal anion production at concentration 10^{-5} M with a value corresponding to that of the f MLF-OMe. Figure 4C reports the results concerning the lysozyme release. Here all the three *N*-formyl derivatives exhibit a similar profile of activity with a maximum centred at concentration 10^{-5} M and a rank of potency: **10** = **4** >**7**.

As generally found and as shown in Figs. 4B and C the three *N*-Boc derivatives **3**, **6** and **9**, at variance with the corresponding *N*-formyl analogues, are completely unable to elicit superoxide anion and lysozyme release.

Conclusion

In summary we have described synthesis, conformational preferences and activity of a new group of fMLF-OMe analogues structurally related by the common presence at position 2 of residues bearing unsaturated side chains. As reported in Fig. 4 all the three *N*-For analogues **4**, **7** and **10** are capable to elicit high to moderate values of chemotaxis as well as of superoxide anion production and lysozyme release. These findings confirm the versatility of the fMLF-OMe to accept structural modifications at the central position.

The preferred conformations of the models incorporating the $C^{\alpha,\alpha}$-disubstituted residues Dag and Cpg (Fig. 3) indicate that an analogous effect is induced by the Dag and the corresponding saturated residue. It is found in fact that the tripeptide **7**, containing two linear allylic side chains at position 2, adopts the expected C_5 extended conformation also found in the case of the di-*n*-propylglycine (Dpg) containing tripeptide (Dentino et al., 1991). On the other hand, the conformation of the tripeptide **10**, incorporating the Cpg residue differs from the β-turn structure found in the case of the related model containing the cyclopentane ring (Toniolo et al., 1989).

The last reported finding underlines the interest to further explore the ability of Cpg to induce specific conformations into peptide backbones in which this residue is incorporated. In addition to this, the chemical reactivity of the unsaturated 5-membered ring must be considered. This property appears particularly interesting in the light of recent transformation reactions performed on *N*-protected Cpg esters such as hydroboration-oxidation reactions (Hodgson et al., 1999) and 1,3-dipolar additions (Conti et al., 2003). These data and the concomitant lack of information on the biochemical consequences of the incorporation of the Cpg residue into native peptides, stimulate further studies on this unsaturated cyclic $C^{\alpha,\alpha}$-disubstituted aminoacid and on peptides containing residues with cyclopentenyl side chains.

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