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# Modified chemotactic peptides: synthesis and activity of an azaTic-containing fMLP-OMe analogue

**G. Pagani Zecchini**[1], **I. Torrini**[1], **M. Paglialunga Paradisi**[1], **G. Lucente**[1], **G. Mastropietro**[1], and **S. Spisani**[2]

[1] Dipartimento di Studi Farmaceutici and Centro di Studio per la Chimica del Farmaco del CNR, Università "La Sapienza", Roma, and
[2] Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, Italy

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**Summary.** The synthesis and the biological activity of a pseudopeptide analogue of the chemotactic *N*-formyltripeptide fMLP-OMe, containing the azaTic (3,4-dihydro-2(*1H*)-phthalazinecarboxylic acid) residue replacing the native phenylalanine, is described. Whereas pseudopeptides containing linear $\alpha$-azaamino acids are currently studied, data on the new group of analogues containing cyclic $\alpha$-aza residues capable of limiting the rotameric distribution of the side chains (topological control) are just emerging in the literature. At our best knowledge, the here described [azaTic$^3$]fMLP-OMe represents the first example of the introduction of this new type of $\alpha$-aza residue into a natural bioactive peptide.

**Keywords:** Amino acids – Azapeptides – AzaTic – Chemotaxis – 3,4-Dihydro-2(*1H*)-phthalazinecarboxylic acid – Formylpeptides

## Introduction

Several and different strategies are adopted for the modification of bioactive peptides in order to generate synthetic analogues useful for studying structure-activity relationships and obtaining therapeutic agents. Relevant approaches rely upon the introduction of conformational constraints to reduce the backbone flexibility (global constraint) or to limit the rotameric distribution of the amino acid side chains (local constraint) (Hruby et al., 1990; Hruby and Bonner, 1994). This latter approach leads to topographically constrained analogues which are valuable tools to get information on how the spatial arrangement of the side chains (i.e. the topological arrangement) modulates the biological activity. A well established example of synthetic amino acids with biased rotamers is the tetrahydroisoquinolinecarboxylic acid (Tic) which can be considered a topographically constrained analogue of phenylalanine.

Due to the cyclic structure the Tic benzylic side chain cannot adopt the *trans* rotameric orientation and only $g^-$ and $g^+$ rotamers are found, depending upon the position that the Tic residue occupies in the peptide backbone (Hruby and Bonner, 1994).

By taking into account the influence that the replacement of the $\alpha$-carbon with a nitrogen atom can exert on the orientation of the $\alpha$-amino acid side chains in derived azapeptides (Spatola, 1983; Gante, 1989) and as a prosecution of our research in the field of linear and cyclic $\alpha$-azaamino acids (Pinnen et al., 1993; Pinnen et al., 1994; Pagani Zecchini et al., 1996), it seemed interesting to examine synthesis and properties of $\alpha$-azaTic-containing peptides. Although several papers have been dedicated to esters of tetrahydroderivative of 2-phthalazinecarboxylic acid (Grobelny, 1993; Grobelny, 1995; Russell et al., 1992) and 2,3-phthalazinedicarboxylic acid (Russell et al., 1992; Carpino, 1963; Rutjies et al., 1988), only very few attention has been paid to $\alpha$-azaTic-containing peptides and related structures. Recently two pseudopeptides incorporating the tetrahydrophthalazine nucleus, a constrained aza analogue of phenylalanine, has been described by T. Kline and coworkers (1996); no data are at the present available on azaTic-containing bioactive peptides.

Here we report on the introduction of the 3,4-dihydro-2(*1H*)-phthalazinecarboxylic acid (azaTic) residue, in place of the native phenylalanine, into the chemotactic tripeptide HCO-Met-Leu-Phe-OMe (fMLP-OMe).

## Methods and materials

Mps were obtained using a Büchi oil bath apparatus and are uncorrected. Optical rotations were taken at 20°C with a Schmidt-Haensch Polartronic D polarimeter. IR spectra were recorded on a Perkin-Elmer 983 spectrophotometer. $^1$H NMR spectra were determined with a Varian EM-390 (90 MHz) and XL-300 (300 MHz) spectrometer using tetramethylsilane as internal standard. Column chromatographies were carried out using Merck silica gel 60 (230–400 mesh). TLC and PLC were performed on silica gel Merck 60 $F_{254}$ plates. The drying agent was sodium sulphate. All the reactions were carried out under nitrogen atmosphere.

## Experimental

### *tert-Butyl methyl 1,2-hydrazinedicarboxylate (**1**)*

Following a known procedure (Dutta and Morley, 1975) 95% methyl chloroformate (0.65 ml, 8 mmol) was added dropwise at 0°C to a stirred mixture of *tert*-butyl carbazate (1.057 g, 8 mmol), chloroform (12 ml), sodium hydroxide (0.36 g, 9 mmol), and water (4 ml). After stirring at room temperature for 24 h, ethyl acetate was added in excess. The organic layer was washed with water, 20% aq. citric acid and water, dried and evaporated to give pure compound **1** (1.52 g, 100%), mp 101–101.5°C (99–101°C, Sheppard and Korczykowski, 1969); $\nu_{max}$ (KBr): 3297, 3261, 1753, 1693, 1513 and 1253 cm$^{-1}$; $\delta$(90 MHz; $CDCl_3$): 1.43 [9 H, s, $C(CH_3)_3$], 3.73 (3 H, s, $COOCH_3$), 6.77 and 7.03 (2 H, two s, NH-NH).

*tert-Butyl methyl 1,4-dihydro-2,3-phthalazinedicarboxylate (**2**)*

Sodium hydride (80% dispersion, 0.18 g, 6 mmol) was suspended in dry dimethylformamide (DMF) (2.7 ml). After cooling at 0°C, a solution of Boc-NH-NH-COOMe (**1**) (0.571 g, 3 mmol) in DMF (4.6 ml) was added slowly. After stirring at room temperature for 30 min, the mixture was cooled at 0°C, and a solution of 96% $\alpha,\alpha'$-dibromo-*o*-xylene (0.825 g, 3 mmol) in dry DMF (1.5 ml) was then added dropwise. Stirring was continued at 0°C for 5 min and at room temperature for 24 h. The mixture was cooled at 0°C and the excess of the hydride was decomposed with the minimum amount of ethyl acetate. The solvent was removed *in vacuo* and the residue was taken up with ethyl acetate and washed with water, 20% aq. citric acid, saturated aq. $NaHCO_3$ and brine. The organic solution was dried and evaporated to give an oil which was purified by chromatography on a silica gel column (1:30), eluting with $CH_2Cl_2$, and by PLC ($CHCl_3$-*n*-hexane, 99:1) to obtain pure oily compound **2** (0.647 g, 74%); $\nu_{max}$ ($CHCl_3$): 1703, 1453, 1392, 1369 and 1156 $cm^{-1}$; $\delta$(90 MHz; $CDCl_3$): 1.43 [9 H, s, $C(CH_3)_3$], 3.76 (3 H, s, $COOCH_3$), 4.40 and 5.01 (4 H, A and B of an AB, $J$ = 16 Hz, two $CH_2$-N), 7.01–7.26 (4 H, m, ArH).

*Methyl 3,4-dihydro-2(1H)-phthalazinecarboxylate (**3**)*

*Method a.* Thionyl chloride (0.16 ml, 2.2 mmol) was added dropwise to a solution of the derivative **2** (0.585 g, 2 mmol) in dry methanol (2 ml), cooled at −15°C. After stirring at −15°C for 30 min and at 45°C for 2 h, the solution was evaporated under vacuum to give methyl 3,4-dihydro-2(*1H*)-phthalazinecarboxylate hydrochloride as a foam (0.457 g, 100%). This salt was partitioned between ethyl acetate and saturated aq. $NaHCO_3$. The organic phase was washed with water, dried, and evaporated to afford pure methyl ester **3** (0.299 g, 78%), mp 99–100°C (from ethyl acetate-*n*-hexane); $\nu_{max}$ (KBr): 3422, 3220, 1712, 1442 and 1235 $cm^{-1}$; $\delta$(300 MHz; $CDCl_3$): 3.80 (3 H, s, $COOCH_3$), 4.10 (2 H, s, $CH_2$-NH), 4.72 (2 H, s, $CH_2$-N-COO), 7.05–7.37 (4 H, m, ArH). *Anal.* Calc. for $C_{10}H_{12}N_2O_2$: C 62.5, H 6.3, N 14.6%. Found: C 62.1, H 6.3, N 14.4%.

*Method b.* Following a known procedure (Russell et al., 1992) 95% methyl chloroformate (0.18 ml, 2.2 mmol) and sodium cyanoborohydride (0.251 g, 4 mmol) were added to a solution of phthalazine (0.26 g, 2 mmol) in methanol (12 ml) and the mixture was stirred at room temperature for 2 h. The excess of the hydride was carefully hydrolized with water, the solvent was removed under vacuum, and the residue was partitioned between ethyl acetate and brine. The organic phase was dried and evaporated to give a residue which was purified by chromatography on a silica gel column (1:40), eluting with $CH_2Cl_2$-EtOAc (1:1) to obtain homogeneous compound **3** (0.181 g, 47%), identical to the sample prepared following the method a.

*Methyl 3-[tert-butoxycarbonyl-(S)-leucyl]-3,4-dihydro-2(1H)-phthalazinecarboxylate (**4**)*

Isobutyl chloroformate (95%, 0.1 ml, 0.75 mmol) was added at −15°C to a stirred solution of Boc-Leu-OH·$H_2O$ (0.187 g, 0.75 mmol) and *N*-methylmorpholine (NMM) (0.1 ml, 0.9 mmol) in dry dichloromethane (3.6 ml). The temperature was kept at −15°C for 10 min and a solution of derivative **3** (0.144 g, 0.75 mmol) in dry dichloromethane (2.7 ml) was then added. The mixture was stirred at −15°C for 15 min and at room temperature for 24 h. Ethyl acetate was added in excess and the organic phase, washed with 5% aq. $KHSO_4$, water, saturated aq. $NaHCO_3$, and brine was dried and evaporated to give an oily residue. PLC purification ($CH_2Cl_2$-EtOAc, 8:2) afforded pure compound **4** as an oil (0.167 g, 55%); $[\alpha]_D$ −7° (*c* 1.0 in $CHCl_3$); $\nu_{max}$ ($CHCl_3$): 3437, 2956, 1721, 1709, 1677, 1500, 1368 and 1164 $cm^{-1}$; $\delta$ (300 MHz; DMSO-$d_6$): 0.85 [6 H, m, $CH(CH_3)_2$], 1.33 [9 H, s, $C(CH_3)_3$], 1.40–1.75 [3 H, m, $CH_2$-$CH$-$(CH_3)_2$], 3.71 (3 H, s, $COOCH_3$), 4.20 and 5.23 (2 H, A and X of an AX, $J$ = 16.5 Hz, $CH_2$-N-COO), 4.53 (1 H, m, Leu $\alpha$-CH), 4.62 and 5.08 (2 H, A and B of an AB, $J$ = 16.5 Hz, $CH_2$-N-CO-CH), 7.25 (5 H, apparent s, ArH and NH).

*Methyl 3-[tert-butoxycarbonyl-(S)-methionyl-(S)-leucyl]-3, 4-dihydro-2(1H)-phthalazinecarboxylate (**5**)*

The title azapeptide **5** was prepared following the mixed anhydride method described above, starting from Boc-Met-OH (0.181 g, 0.725 mmol) and a solution in dry $CH_2Cl_2$ (2.6 ml) of NMM (0.08 ml, 0.725 mmol) and HCl·H-Leu-azaTic-OMe, obtained by the usual deprotection of compound **4** (0.294 g, 0.725 mmol) with thionyl chloride-methanol. Ethyl acetate was added in excess and the organic phase, washed with 2M HCl, water, saturated aq. $NaHCO_3$, and water, was dried and evaporated. The residue was chromatographed on PLC ($CH_2Cl_2$-EtOAc, 8:2) to give pure compound **5** (0.297 g, 76%), mp 137–138°C; $[\alpha]_D$ −15° (*c* 1.0 in $CHCl_3$); $\nu_{max}$ (KBr): 3334, 2956, 1731, 1689, 1680, 1644, 1529, 1446, 1365 and 1232 $cm^{-1}$; $\delta$(300 MHz; DMSO-$d_6$): 0.81 and 0.89 [6 H, two d, $J$ = 6.5 Hz, $CH(CH_3)_2$], 1.33 [9 H, s, $C(CH_3)_3$], 1.40–1.90 [5 H, m, Met $\beta$-$CH_2$ and Leu $CH_2$-$CH$-$(CH_3)_2$], 1.96 (3 H, s, S-$CH_3$), 2.37 (2 H, t, $J$ = 7.5 Hz, $CH_2$-S), 3.73 (3 H, s, $COOCH_3$), 4.00 (1 H, m, Met $\alpha$-CH), 4.20 and 5.21 (2 H, A and X of an AX, $J$ = 16.5 Hz, $CH_2$-N-COO), 4.68–5.13 [3 H, m, Leu $\alpha$-CH (at 4.83) and $CH_2$-N-CO-CH), 6.88 (1H, d, $J$ = 8 Hz, Met NH), 7.24 (4 H, s, ArH), 8.13 (1H, d, $J$ = 7.2 Hz, Leu NH).

*Methyl 3-[formyl-(S)-methionyl-(S)-leucyl]-3,4-dihydro-2(1H)-phthalazinecarboxylate (**6**)*

The Boc-azapeptide **5** (0.183 g, 0.34 mmol) was dissolved in HCOOH (2 ml) and the solution was stirred at room temperature for 24 h. After removal of the excess of formic acid *in vacuo*, the residue was dissolved in dry $CHCl_3$ (2 ml) and 97% EEDQ (0.104 g, 0.41 mmol) was added. The solution was stirred at room temperature for 24 h. Evaporation under reduced pressure afforded a solid residue which was purified on PLC ($CH_2Cl_2$-EtOAc, 7:3) to give pure formyl derivative **6** (0.138 g, 87%), mp 95–98°C (from $CH_2Cl_2$-*n*-hexane); $[\alpha]_D$ +4° (*c* 1.0 in $CHCl_3$); $\nu_{max}$ (KBr): 3276, 2956, 1738, 1730, 1688, 1680, 1660, 1642, 1548, 1445, 1382 and 1233 $cm^{-1}$; $\delta$ (300 MHz; DMSO-$d_6$): 0.81 and 0.89 [6 H, two d, $J$ = 6.5 Hz, $CH(CH_3)_2$], 1.33–1.88 [5 H, m, Met $\beta$-$CH_2$ and Leu $CH_2$-$CH$-$(CH_3)_2$], 1.94 (3 H, s, S-$CH_3$), 2.35 (2 H, t, $J$-7.5 Hz, $CH_2$-S), 3.73 (3 H, s, $COOCH_3$), 4.20 and 5.22 (2H, A and X of an AX, $J$ = 16.5 Hz, $CH_2$-N-COO), 4.43 (1 H, m, Met α-CH), 4.74-5.13 [3H,m, Leu $\alpha$-CH (at 4.81) and $CH_2$-N-CO-CH), 7.25 (4 H, s, ArH), 7.97 (1 H, s, H-CO), 8.23 (1H, d, $J$ = 8 Hz, Met NH), 8.41 (1H, d, $J$ = 7 Hz, Leu NH). *Anal.* Calc. for $C_{22}H_{32}N_4O_5S$: C 56.9, H 7.0, N 12.0%. Found: C 56.7, H 7.3, N 11.7%.

*Biological assay*

Cells

Human peripheral blood neutrophils were purified employing the standard techniques of dextran (Pharmacia) sedimentation, centrifugation on Ficoll-Paque (Pharmacia), and hypotonic lysis of red cells. The cells were washed twice and resuspended in KRPG (Krebs-Ringer phosphate containing 0.1% w/v glucose, pH 7.4) at a concentration of 50 × $10^6$ cells/ml. Neutrophils were 98–100% pure.

*Random locomotion*

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Italy) and the migration into the filter was evaluated by the method of leading-front (Zigmond and Hirsch, 1973). The actual control random movement is 32 $\mu$m ± 3 SE of ten separate experiments done in duplicate.

*Chemotaxis*

In order to study the potential chemotactic activity, each peptide was added to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution ($10^{-2}$ M in DMSO) with KRPG containing 1 mg/ml of bovine serum albumin (Orha Behringwerke, FRG) and used at concentrations ranging from $10^{-11}$ M to $10^{-5}$ M. Data were expressed in terms of chemotactic index, 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 done in duplicate. Standard errors are in the 0.02–0.09 chemotactic index range.

*Superoxide anion ($O_2^-$) production*

$O_2^-$ release was monitored continuously in a thermostatted spectrophotometer as superoxide dismutase-inhibitable reduction of ferricytochrome c (Sigma, USA), as described elsewhere (Spisani et al., 1992). At zero time, different amounts ($10^{-8} - 2 \times 10^{-5}$ M) of each peptide were added and absorbance change accompanying cytochrome c reduction was monitored at 550 nm. Neutrophils were incubated with 5 $\mu$g/ml cytochalasin B (Sigma) for 5 min prior to activation by peptides. Results were expressed as net nmoles of $O_2^-/2 \times 10^6$ cells/5 min and are the mean of six separate experiments done in duplicate. Standard errors are in 0.1–4 nmoles $O_2^-$ range.

*Enzyme assay*

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity (Spisani et al., 1992); this was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus* (Sigma). Enzyme release was expressed as a net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 $\pm$ 1 $\mu$g/1 $\times$ $10^7$ cells/min. To study the degranulation-inducing activity of each peptide, neutrophils were first incubated with cytochalasin B for 15 min at 37°C and then in the presence of each peptide in a final concentration of $10^{-9} - 2 \times 10^{-5}$ M for a further 15 min. The values are the mean of five separate experiments done in duplicate. Standard errors are in 1–6% range.

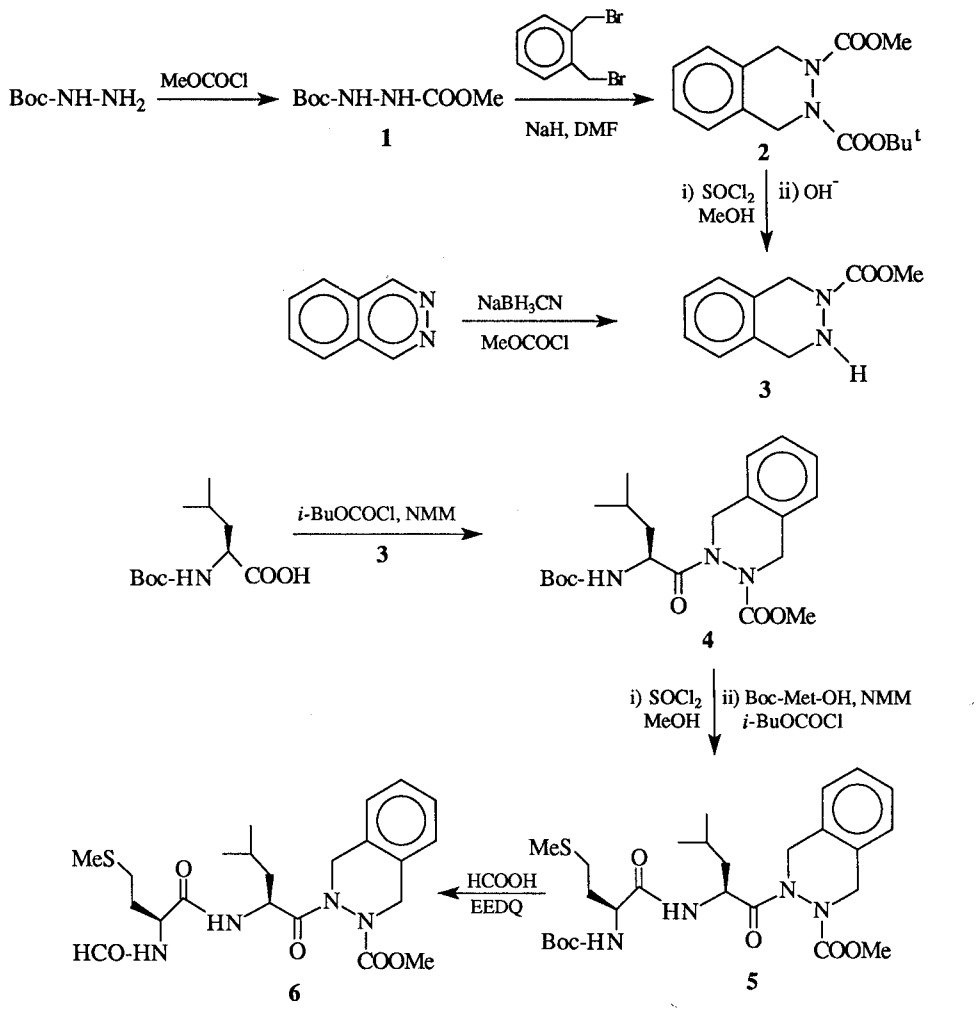
*Statistical analysis*

The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups.

## Results and discussion

The synthesis of the azaTic-containing fMLP-OMe analogue **6** is outlined in Scheme 1.

The key intermediate methyl 3,4-dihydro-2(*1H*)-phthalazinecarboxylate (azaTic-OMe) **3** has been obtained by following two different procedures: a) the bicyclic derivative **2**, obtained by reacting the protected hydrazine **1** with $\alpha,\alpha'$-dibromo-*o*-xylene (Carpino, 1963), was selectively deprotected with $SOCl_2$-MeOH to give **3** in 58% overall yield; b) the exposure of the commercially available phthalazine to methyl chloroformate and sodium cyanoborohydride afforded **3** in a single step and in a 47% yield (Russell et al., 1992). Although the new compound azaTic-OMe **3** is stable enough to be isolated and stored for months at 0°C, it tends to decompose on standing in air and in the presence of acids to give dehydrogenated products. The here evidenced tendency of azaTic-OMe to undergo dehydrogenation reactions and air oxidation has been previously observed on related tetrahydrophthalazine derivatives (Patel, 1973). The reactivity of **3** towards the introduction of a second acylating group is low. Thus, the synthesis of azapeptide **4** failed when carbodiimides were used as condensing agents; moderate yields (55%) can be




**Scheme 1**

obtained by using the mixed anhydride method with isobutyl chloroformate, as reported in Scheme 1. Deprotection of **4** with $SOCl_2$-MeOH, followed by coupling with Boc-Met-OH gave the Boc-azatripeptide **5**. Final treatment with ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate (EEDQ) (Lajoie and Kraus, 1984) afforded the formyl azapeptide **6**.

The biological activity of the formylpeptide **6** has been determined on human neutrophils and compared with that of the related tripeptide analogue HCO-Met-Leu-Ain-OMe previously studied by us (Torrini et al., 1991) and containing the 2-aminoindane-2-carboxylic acid (Ain) residue in place of the native phenylalanine. Directed migration (chemotaxis), superoxide anion production, and lysozyme release have been measured. As shown in Fig. 1A, the Ain-containing analogue shows a chemotactic activity very similar to that exhibited by the standard peptide fMLP-OMe and is also active as segretagogue agent (Fig. 1C) and activator of the superoxide anion production (Fig. 1B). In contrast, the azaTic-containing analogue **6** is unable to elicit all the tested biological responses.

The present results indicate that the replacement of the Phe at position 3 by the azaTic residue in *N*-formyltripeptides causes the loss of the biological activities on human

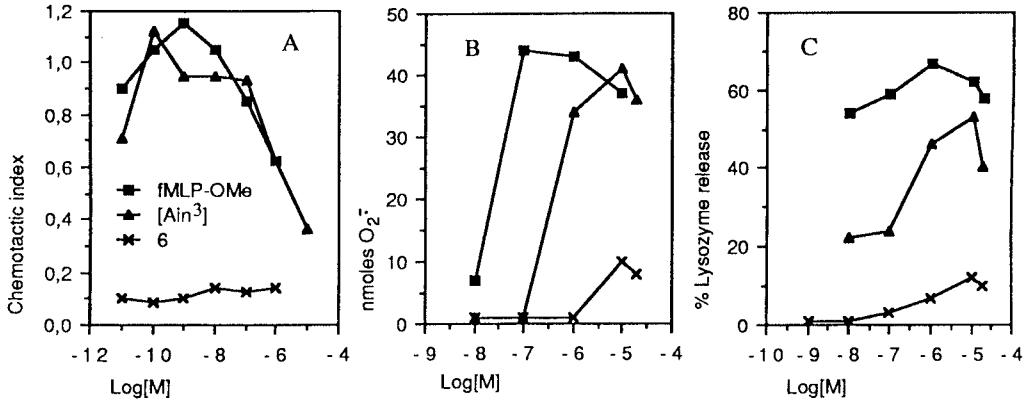



**Fig. 1.** Biological activities of **6**, [Ain$^3$]fMLP-OMe, and fMLP-OMe toward human neutrophils. **A** Chemotactic activity. **B** Superoxide anion production. **C** Release of neutrophil granule enzymes evaluated by determining lysozyme activity

neutrophils. This behavior differs from that shown by the above-mentioned analogue cointaining Ain, a different constrained mimic of the *C*-terminal Phe, and paralles that evidenced in the case of (*Z*)-2,3-didehydrophenylalanine ($\Delta^Z$Phe)-containing formyltripeptides. These data can be rationalized by considering the role exerted by the orientation of the aromatic ring, with respect to the backbone adjacent atoms, on the biological activity. We have previously discussed the activity of the [Thp$^1$, Ain$^3$] (Thp = 4-aminotetrahydrothiopyran-4-carboxylic acid), [Thp$^1$, $\Delta^Z$Phe$^3$], and [$\Delta^Z$Phe$^3$] fMLP-OMe analogues (Torrini et al., 1991; Torrini et al., 1994) on the basis of a different type of constraint introduced at the Phe side-chain level. In particular, the spatial orientation of the aromatic ring with respect to the backbone adjacent atoms is opposite in the two ligands: the active [Thp$^1$, Ain$^3$] analog presents the aromatic ring of the residue at position 3 practically perpendicular to the plane of the backbone adjacent atoms $N_3$, $C_3^\alpha$, $C_3'$, while in the inactive $\Delta^Z$Phe analogues the aromatic ring is nearly on the same plane of the backbone $N_3$, $C_3^\alpha$, $C_3'$ atoms. In accordance with these data, the biological inactivity of the azaTic derivative **6** under study can be related to the arrangement of the aromatic ring in the *C*-terminal residue. This, as can be clearly deduced by examining the azaTic molecular model, is very similar to that adopted by the $\Delta^Z$Phe containing tripeptides and different from that of the highly active [Ain$^3$] analogue. Thus, as previously observed (Torrini et al., 1994), the local constraint involving the hydrophobic *C*-terminal residue of the chemotactic *N*-formyltripeptides can profoundly influence the activity.

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**Authors' address:** Prof. Gino Lucente, Dipartimento di Studi Farmaceutici, Università "La Sapienza", Piazzale A. Moro 5, I-00185 Roma, Italy.

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