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The Importance of the Methionine Configuration in for-Met-Leu-Phe-OMe Biological Activities

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for-D-Met-L-Leu-L-Phe-OMe was synthesized in order to understand the importance of the methionine configuration on human neutrophil biological activity. Our results show that both chemotactic response and lysozyme release are lower than those of the parent fMLP-OMe, indicating that the specific receptor pocket possesses a well-located, restricted positive area that does not completely face the D-Met residue. The triggering of superoxide anion production does not seem to be sensitive to this variation of the configuration.

INTRODUCTION

N-formylpeptides are involved in the defense mechanisms against bacterial infections by binding to specific receptors (FPR), which involve heterotrimeric G-proteins in the transduction pathway. Occupancy of the FPR results in neutrophil chemotaxis, oxidase activation, or degranulation, depending on the peptide concentrations and experimental conditions (*1*).

In the field of the structure–activity relationships concerning chemotactic *N*-formyltripeptides, several studies have been made on the backbone modification of the prototypical chemotactic ligand *N*-for-Met-Leu-Phe-OH (fMLP) and its for-Met-Leu-Phe-OMe analogue (fMLP-OMe) (*2–4*) for their pharmaceutical relevance. Attention was recently focused by us, as well as by other groups, to ascertain the features of the receptor pocket where the first residue allocates. In particular, the substitutions at the Met residue can be split in two categories: the first has the aim of defining the pocket dimensions varying the side chain features (*5*), the second of defining the charge interaction conditions which influence both the intensity and position of the residue charge (*6,7*). It has been ascertained that the side chain of the residue at position 1 penetrates in a hydrophobic pocket of limited depth and capacity. A positively charged area has been proposed that surrounds the electron-rich sulphur atom, which in turn must be in a well-defined precise position (*5,8*).

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As a continuation of our studies, we thought it would be interesting to ascertain the influence of a configurational change in the Met residue on human biological responses. We therefore synthesized the tripeptide for-D-Met-L-Leu-L-Phe-OMe **1,** since only scarce, marginal information is available (*9*); we tested this peptide on neutrophil functions involved in the inflammatory response, such as directional locomotion (chemotaxis), superoxide anion (O_2^-) production, and lysozyme release. The biological data were compared with those of the parent compound fMLP-OMe

METHODS

Chemical Synthesis

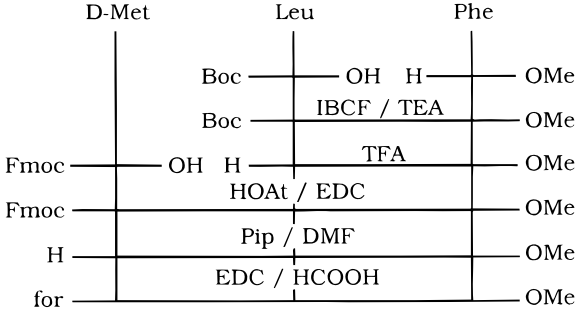
The formylpeptide **1** was synthetized following standard procedures in solution (Scheme 1), purified by HPLC, and characterized by TLC and ^{1}H-NMR.

Fmoc-D-Met, Boc-L-Leu, and L-Phe-OMe hydrochloride residues were purchased from Fluka. Removal of the *t*-Boc group was performed by treatment with a 1:1 mixture of TFA-$CHCl_3$. Peptide coupling was achieved (i) by the racemization-free mixed-anhydride method with isobutylchloroformate (IBCF) and triethylamine (TEA), and (ii) by the 1-hydroxy-7-azabenzotriazole (HOAt)-*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) method. Piperidine (Pip) and dimethylformamide (DMF) was used to remove the Fmoc group. The formyl group was introduced at the level of tripeptide using EDC and HCOOH.

HCO-D-Met-L-Leu-L-Phe-OMe. M.p. 166–168∞C; *[α]* = –34.3°C, *c* = 1 in MeOH. ^{1}H-NMR ($CDCl_3$) δ 8.13 (s, 1H, HCO), 7.29 (s, 5H, phenyl), 7.11 (d, J = 7.58 Hz, 1H, NH), 6.55 (d, J = 7.35 Hz, 1H, NH), 6.40 (d, J = 8,01 Hz, 1H, NH), 4.83 (m, 1H, CH), 4.62 (m, 1H, CH), 4.38 (m, 1H, CH), 3.72 (s, 3H, CH_3), 3.05 and 3.17 (AB of ABX, 2dd, J_{AB} = 13.19 Hz, J_{AX} = 6.63 Hz, J_{BX} = 5.74 Hz, 2H, CH_2), 2.46 and 2.59 (AB of ABX, 2dd, J_{AB} = 13.19 Hz, J_{AX} = 6.56 Hz, J_{ABX} = 7.14, Hz, 1H, CH_2), 2.11 (s, 3H, CH_3), 1.42–1.62 (m, 5H, $2CH_2$ + CH), 0.89 and 0.92 (2d, J = 5.53 Hz, 6H, $2CH_3$).

BIOLOGICAL ASSAYS

Human neutrophils were purified employing the standard techniques of dextran sedimentation of heparinized blood, followed by centrifugation on Ficoll-Paque. The



SCHEME 1. Synthesis of compound **1.**

cells were washed twice and resuspended in Krebs–Ringer–Phosphate containing 0.1% w/v Glucose (KRPG), pH 7.4, at a concentration of 50×10^6 cells/ml.

Chemotaxis was performed with a 48-well microchemotaxis chamber, and the migration into the filter was evaluated by the leading-front method (*10*). Chemotaxis was studied by adding each peptide to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution (10^{-2} M in dimethylsulfoxide) with KRPG containing 1 mg/ml of bovine serum albumin and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (C.I.), which is the ratio: (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer). The actual control random movement is 32 μm $\pm$ 3 Standard Error (SE) of 10 separate experiments done in duplicate. The dose–response curves are typical of chemoattractants that rise to a peak and then decline to zero as the concentration of ligand is increased above its optimum value (*11*). A likely explanation for this decrease is that the chemical gradient was diminished from the increased rate of diffusion of that attractant, resulting in a greater degree of saturation of the cells in the upper compartment of the chemotactic chamber (*12*).

Superoxide anion production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c (*3*) modified for microplate based assays. The tests were carried out in a final volume of 200 µl containing 4×10^5 neutrophils, 100 nmol cytochrome c, and KRPG. At zero time, different amounts (10^{-8}–10^{-4} M) of each peptide were added and the plates were incubated into a microplate reader (Ceres 900, Bio-Tek instruments, INC) with the compartment T set at 37°C. Absorbance was recorded at wavelenghts of 550 and 468 nm. Differences in absorbance at the two wavelenghts were used to calculate nmoles of O_2^- produced, using molar extinction coefficient for cytochrome c of 15.5 mM^{-1} cm^{-1}. Data are expressed as net nanomoles of O_2-production/1×10^6 neutrophils/5 min. Neutrophils were preincubated with 5 μg/ml cytochalasin B for 5 min prior to activation by peptides.

Granule enzyme assay. Release of neutrophil granule enzymes was evaluated by determining lysozyme activity (*3*) modified for microplate-based assays. Cells were incubated in microplates wells in the presence of each peptide in a final concentration of 10^{-9}–10^{-4} M for 15 min at 37°C. The plates were then centrifuged for 5 min at 400 g and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus.* Neutrophils were preincubated with 5 μg/ml cytochalasin B for 15 min at 37°C prior to activation by peptides. Reaction rate was measured with a microplate reader at 465 nm. Enzyme was expressed as net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 mg/1×10^7 cells/min.

RESULTS AND DISCUSSION

As shown in Fig. 1, the introduction of the D-Met significantly influences the chemotactic response, which is strongly reduced to an amount varying from a half to a third of the prototype values at physiological concentrations (10^{-10}–10^{-8} M). It induces about 0.7 C.I. at 10^{-10} M, evidencing an efficacy 10 times higher than fMLP-OMe.

In Fig. 2 the superoxide anion production is reported. Compound **1** has a behavior similar to the parent fMLP-OMe only at high concentrations, showing its peak at 10^{-5} M. Its potency, however, is lower than fMLP-OMe at 10^{-6} M (23 and 44 nmol, respectively).