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SYNTHESES OF AND CHEMOTACTIC RESPONSES ELICITED BY fMET-LEU-PHE ANALOGS CONTAINING DIFLUORO- AND TRIFLUOROMETHIONINE

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Abstract: N-Formylmethionylleucylphenylalanine tripeptides containing either *L*-difluoro- or *L*-trifluoromethionine as replacements for methionine were synthesized by solution methods. The fluorinated peptides were found to have potent chemoattractant activities on human neutrophils. Ab initio calculations were utilized to further understand the changes in electronic properties of the methionine side chain upon fluorination.

The electronic and conformational properties of the side chain of the amino acid methionine have been shown to make significant contributions to molecular recognition phenomena in protein–peptide and protein–protein interactions in biochemical systems.[1,2] In order to improve our understanding of these interactions and to develop novel ^{19}F NMR probes to investigate these events, we have recently reported the successful bioincorporation of a fluorinated analog, trifluoromethionine, into a recombinant phage muramidase.[3] Our studies indicated that the enzymatic activities of the fluorinated protein were unaltered and that ^{19}F NMR could be utilized to probe the ligand binding properties of this enzyme. To expand our knowledge of this and other analogs in molecular recognition phenomena that depend on methionine, a simpler molecular system that required this amino acid as a key structure for its biological properties was required. The N-formylated bacterial chemotactic tripeptide fMet-Leu-Phe appeared ideal in this regard.

Chemotaxis is defined as the directed migration of cells towards an increasing concentration gradient of chemical signals (chemoattractants).[4] In prokaryotes this serves as a means of locating essential nutrients, whereas in more complex systems it serves as a means by which polymorphonuclear neutrophils (PMN) and monocytes become localized at sites of inflammation caused by an infectious agent or an allergic stimulus. A number of substances are chemotactic including peptides containing N-formylmethionine, which are structurally similar to N-formylmethionine peptides generated from bacterial sources. Intense interest is being shown in this G-protein coupled formylpeptide receptor interaction for the development of diagnostic imaging agents to locate sites of inflammation and infection, as well as to develop therapeutic agents to treat inflammation.[5]

The chemotactic response of neutrophils to the N-formylated tripeptide fMet-Leu-Phe has been studied by a number of investigators.[4,6] Structure–activity studies have demonstrated that the fMet at position 1 is critical for optimal chemotactic activity.[7] The N-formyl group is important for good activity since N-acetylation or entire removal of the formyl group results in a 2000- to 7000-fold loss in chemotactic response. The methionine side chain also appears optimal as replacement by related analogs such as norleucine, ethionine, S-methylcysteine, S-

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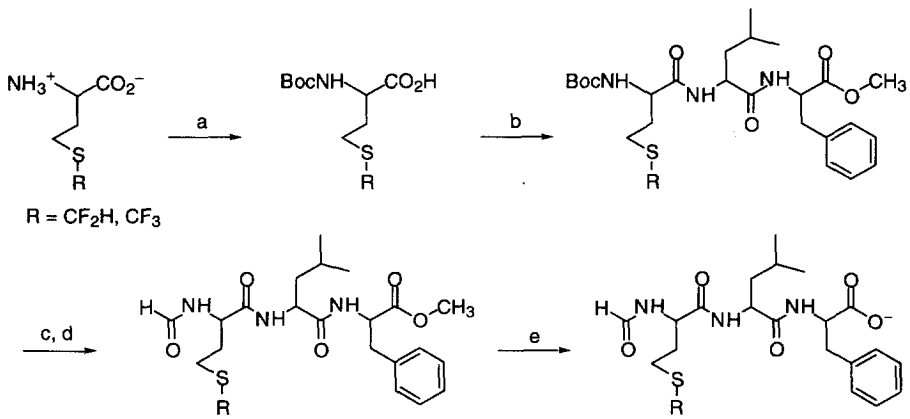
ethylcysteine or 2-aminoheptanoic acid results in a 3.6- to 264-fold decrease in chemotactic activity in rabbit and human neutrophil systems.[7] It has also been shown that the sulfoxide and sulfone of fMet-Leu-Phe are ineffective as chemoattractants for human neutrophils, whereas both derivatives stimulate monocyte chemotaxis but at concentrations 10- to 100-fold higher than the parent peptide.[8]

With the availability of *L*-difluoromethionine (DFM) and *L*-trifluoromethionine (TFM) from our previous synthetic studies,[9] we undertook the synthesis of several fluorinated analogs of the fMet-Leu-Phe chemotactic peptide and explored their chemotactic activities for human neutrophils. Although Russian researchers[10] have reported the synthesis and antihypertensive activity of the trifluoromethionine analog of fMet-Leu-Phe, no investigations into its chemotactic effects have ever been reported. Boc-*L*-difluoromethionine and Boc-*L*-trifluoromethionine were prepared in approximately 85% yields by reaction of the amino acid[9] and di-*t*-butyldicarbonate in chilled THF:H_2O (1:1) with slow addition of triethylamine at 0 °C followed by stirring at 23 °C (Scheme 1). Purification was effected by silica gel chromatography ($CHCl_3$:MeOH, 6:1). The fMet-Leu-Phe peptide analogs were prepared by DCC/HOBT coupling. To the Boc-protected fluorinated methionine, HOBt and DCC dissolved in CH_2Cl_2:DMF (3:1) at 0 °C was added Leu-Phe-OMe[11] followed by stirring at 0 °C for 1 h and then at 23 °C for 12 h. Recrystallization from EtOAc/hexanes afforded the fluorinated tripeptides in approximately 50–55% yields. Reaction of the tripeptide with 98% HCO_2H (23 °C/3 h) followed by evaporation produced the intermediate formate salt of the tripeptide which was not isolated but was further reacted with N-ethoxycarbonyl-2-ethoxy-1,2 dihydroquinoline (EEDQ) in $CHCl_3$ at 23 °C for 3 h.[12] Mild acid workup followed by recrystallization from MeOH/H_2O yielded the formylated methyl ester of each tripeptide in approximately 86% yields. Mild hydrolysis of the methyl ester was accomplished with NaOH (1 equiv. 0.1 M aqueous NaOH in dioxane; 0 °C→23 °C) and produced the formylated tripeptides in greater than 95% yields following HPLC purification.[13]



Scheme 1. (a) di-*t*-butyldicarbonate/THF/H_2O/NEt_3/0 °C (~85%); (b) DCC/HOBT/CH_2Cl_2-DMF/Leu-Phe(OMe) (~55%); (c) formic acid; (d) EEDQ (~86% for HCO_2H/EEDQ); (e) NaOH/dioxane (~95%).

Chemotaxis assays were performed in protein-free media (Gey's balanced salt solution) according to the method of Falk and coworkers.[14] A 48-well chamber was used with a 10 micron thick polycarbonate membrane separating the cell suspension in the upper wells and chemoattractant in the lower wells. The chamber was incubated for 40 min at 37 °C. Non-migrating neutrophils were wiped from the upper surface of the membrane before it was fixed and stained. Neutrophils were counted with an image analyzer (Optomax System 40-10) and results are reported as the number of migrated neutrophils per mm^2 filter surface. Figure 1 summarizes the chemotaxis experiments in which human blood neutrophils were examined for their directed migration responses to various concentrations of analogs and parent compound fMet-Leu-Phe. In preliminary experiments, fluorination appeared to enhance the chemotactic activity of fMet-Leu-Phe by tenfold. The analogs fDFM-Leu-Phe, fTFM-Leu-Phe-OMe, and fTFM-Leu-Phe elicited maximal neutrophil responses at 10^{-8} M, whereas the nonfluorinated parent peptide elicited a maximal response at 10^{-7} M. Consistent with our observations for the fTFM-Met-Leu-Phe and its methyl ester is the finding by Dentino and coworkers[6b] that the methyl ester of fMet-Leu-Phe is somewhat less active than fMet-Leu-Phe itself in eliciting a chemotactic response from human neutrophils.

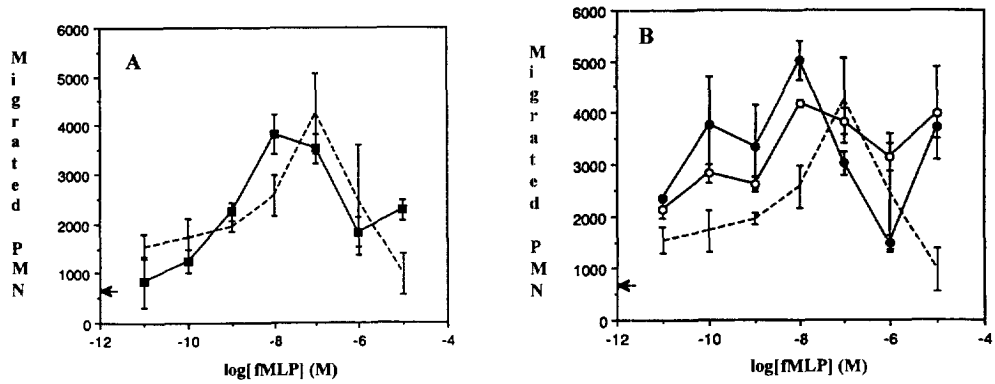


Figure 1. Number of migrated human polymorphonuclear neutrophils (PMN) vs. log of the concentration (M) of the nonfluorinated or fluorinated formylated peptide. (A) fMLP peptide reference (dotted line) and difluoromethionine fMLP analog (filled squares). (B) fMLP peptide reference (dotted line), trifluoromethionine fMLP analog (filled circles) and trifluoromethionine methyl ester (open circles). Black arrows in A and B indicate random migration of neutrophils in the absence of peptide. Data are presented as the mean ± 1SD of migrated PMN per square mm of filter surface of triplicate assays performed with PMN from an individual PMN donor.

Although the chemotactic receptor is known to exhibit demanding selectivity towards other methionine analogs at the N-terminal position in fMet-Leu-Phe, our results indicate that the fluorinated peptides are extremely active in the PMN chemotaxis assays. A consideration of the effects of fluorination upon the molecular properties of the thiomethyl group appeared warranted. Fluorination would be expected to increase the overall hydrophobicity at position 1 in the tripeptide ($logP_{CH_3SCH_3}$ = 0.46; $logP_{CH_3SCHF_2}$ = 1.77; $logP_{CH_3SCF_3}$ = 2.39).[15] It is thought that the side chain of methionine occupies a hydrophobic pocket when bound to the receptor and the

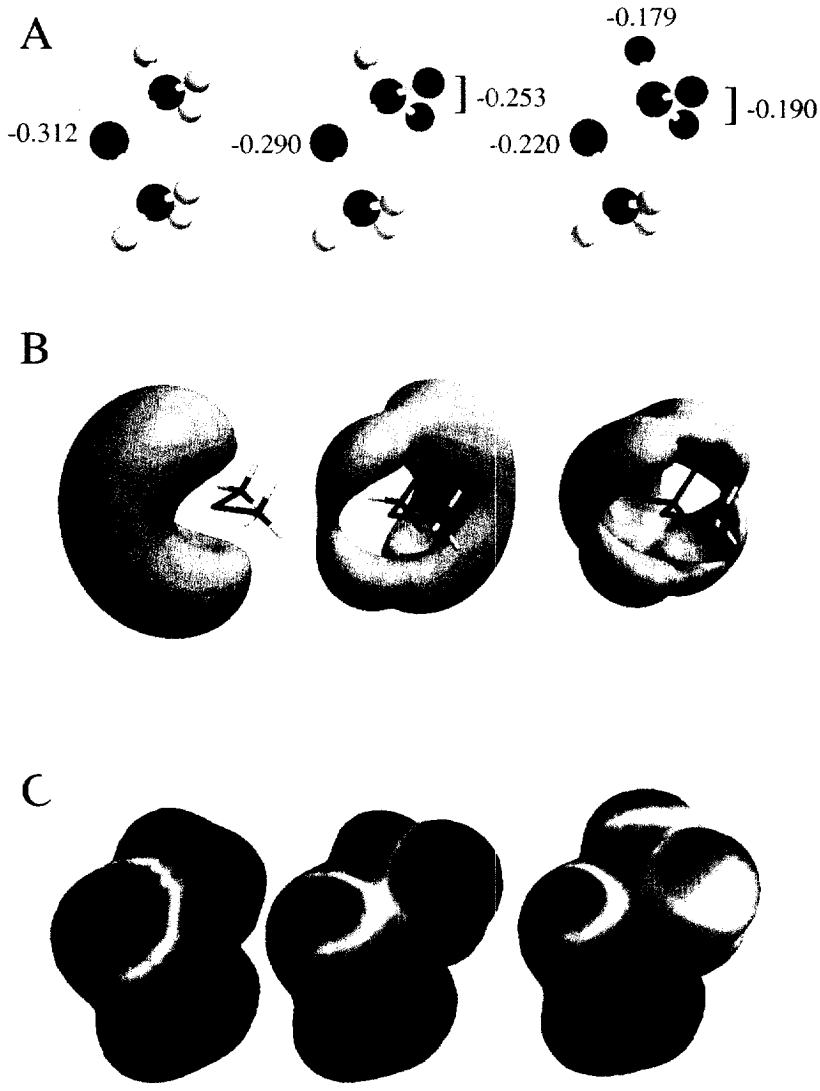


Figure 2. (A) Electrostatically fit charges (in electrons) for $(CH_3)_2S$, CH_3SCHF_2, and CH_3SCF_3 at the RHF/6-31G*//RHF/6-31G* level. (B) Electrostatic potential isosurface at −10 kcal/mol. (C) Electrostatic potentials (in kcal/mol using a range of −12 (red) to +12 (blue)) mapped onto the electron density isosurface (0.002 electrons/au^3) for $(CH_3)_2S$, CH_3SCHF_2, and CH_3SCF_3. Orientation as in A.

increased hydrophobicity of the fluorinated chemotactic peptides could well enhance binding by strengthening this interaction.[7c,16] Based on ab initio calculations[17] at the RHF/6-31G*//RHF/6-31G* level for dimethyl sulfide as a model, both the atomic charge on sulfur as well as the global electrostatic potential isosurface are seen to dramatically alter upon fluorination (Figure 2A, B). An indication of the steric changes that occur upon fluorination can be obtained by calculation of the volume enclosed by the electron density isosurface (Figure 2C) calculated at the 0.002 electrons/(atomic units)^3 level and this results in estimated volumes of 71.2 Å^3, 80.9 Å^3, and 84.4 Å^3 for $(CH_3)_2S$, CH_3SCHF_2, and CH_3SCF_3, respectively. The electrostatic potential mapped onto this electron density isosurface further indicates the electronic changes that occur upon fluorination (Figure 2C) and the resulting changes might serve to endow the tripeptide with a more complementary molecular structure to the receptor. Although conformational studies on the peptides are under investigation, energy versus torsion angle calculations at the B3LYP/6-31+G(d,p) level for CH_3-S-CH_2-CH_3 and CF_3-S-CH_2-CH_3 as models for the side chains of methionine and TFM respectively indicate that the barrier for rotation between *trans* and *gauche* conformations is approximately 1.5 kcal/mol greater for the fluorinated analog (data not shown) and hence subtle conformational effects may also contribute to enhanced binding.

The potent chemotactic activities observed for fMet-Leu-Phe peptides incorporating DFM and TFM suggest that these methionine analogs may prove beneficial in other peptide or peptidomimetic systems. The enhanced stability of the fluorinated thioalkyl moiety to oxidation[3] as well as the presence of an endogenous ^{19}F NMR probe in its structure are advantageous properties that could also be exploited.

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13. **fDFM-Leu-Phe-OMe**: ^{1}H NMR (250 MHz, D_2O) δ 8.01 (s, 1H, HCONH), 7.18–7.09 (m, 5H, Ar), 6.94 (t, 1H, J_{HF} = 56.5 Hz, CF_2H), 4.57 (t, 1H, J = 6.6 Hz, CHα Phe), 4.46 (t, 1H, J = 6.3 Hz, CHα DFM), 4.34 (t, 1H, J = 7.5 Hz, CHα Leu), 3.59 (s, 3H, CO_2CH_3), 3.02 (m, 2H, CH_2β Phe), 2.70 (t, 2H, J = 7.3 Hz, CH_2γ DFM), 2.01-1.85 (m, 2H, CH_2β DFM), 1.62-1.41 (m, 3H, CH_2β, CHγ Leu), 0.82 (2d, 6H, J = 6.2, 6.3 Hz, $2CH_3$δ Leu); ^{13}C NMR (63 MHz, CD_3OD) δ 174.3, 173.2, 172.5, 163.6, 138.0, 130.2, 129.5, 127.9, 122.8 (t, J_{CF} = 270.3 Hz, CF_2H), 53.0, 52.7, 52.1, 51.1, 41.9, 38.3, 34.5, 24.6, 24.5, 23.3, 22.1; ^{19}F NMR (188.3 MHz, $CDCl_3$) δ -91.9.; MS (PDMS), *m/z* 489 (MH^+); **fTFM-Leu-Phe-OMe**: ^{1}H NMR (200 MHz, CD_3OD) δ 8.01 (s, 1H), 7.18–7.09 (m, 5H), 4.57 (t, 1H, J = 6.6 Hz), 4.51 (t, 1H, J = 6.3 Hz, CHα TFM), 4.34 (t, 1H, J = 7.5 Hz), 3.59 (s, 3H), 3.02 (m, 2H), 2.70 (t, 2H, J = 7.3 Hz, CH_2γ TFM), 2.01–1.85 (m, 2H, CH_2β TFM), 1.62–1.41 (m, 3H), 0.82 (2d, 6H, J = 6.2, 6.3 Hz); ^{13}C NMR (63 MHz, CD_3OD) δ 174.2, 173.2, 172.2, 163.6, 138.0, 133.5 (q, J_{CF} = 303.6 Hz, CF_3), 130.2, 129.4, 127.8, 53.0, 52.7, 51.9, 51.1, 41.9, 38.3, 34.0, 26.9, 25.8, 23.3, 22.1; ^{19}F NMR (188.3 MHz, $CDCl_3$) δ –40.3; MS (PDMS), *m/z* 505.5 (MH^+); **fDFM-Leu-Phe**: ^{1}H NMR (250 MHz, D_2O) δ 8.01 (s, 1H), 7.18–7.09 (m, 5H), 6.94 (t, 1H, J_{HF} = 56.5 Hz), 4.57 (t, 1H, J = 6.6 Hz), 4.46 (t, 1H, J = 6.3 Hz), 4.34 (t, 1H, J = 7.5 Hz), 3.02 (m, 2H), 2.70 (t, 2H, J = 7.3 Hz), 2.01–1.85 (m, 2H), 1.62–1.41 (m, 3H), 0.82 (2d, 6H, J = 6.2, 6.3 Hz); ^{13}C NMR (63 MHz, CD_3OD) δ 174.3, 173.2, 170.5, 163.6, 138.0, 130.2, 129.5, 127.9, 122.8 (t, J_{CF} = 270.3 Hz, CF_2H), 53.0, 52.7, 52.1, 41.9, 38.3, 34.5, 24.6, 24.5, 23.3, 22.1; MS (PDMS), *m/z* 496.6 (MNa^+); **fTFM-Leu-Phe**: ^{1}H NMR (200 MHz, CD_3OD) δ 8.01 (s, 1H), 7.18–7.09 (m, 5H), 4.57 (t, 1H, J = 6.6 Hz), 4.51 (t, 1H, J = 6.3 Hz), 4.34 (t, 1H, J = 7.5 Hz), 3.02 (m, 2H), 2.70 (t, 2H, J = 7.3 Hz), 2.01–1.85 (m, 2H), 1.62–1.41 (m, 3H), 0.82 (2d, 6H, J = 6.3, 6.3 Hz); ^{13}C NMR (63 MHz, CD_3OD) δ 174.2, 173.2, 170.2, 163.6, 138.0, 133.5 (q, J_{CF} = 303.6 Hz, CF_3), 130.2, 129.4, 127.8, 53.0, 52.7, 51.9, 41.9, 38.3, 34.0, 26.9, 25.8, 23.3, 22.1; MS (PDMS), *m/z* 514 (MNa^+). Final compounds were purified to homogeneity by HPLC (Vydac SB-C8; linear gradient: water/0.02% TFA to acetonitrile/0.02% TFA). All compounds were homogeneous under a variety of TLC and HPLC conditions and had spectroscopic and mass analysis in agreement with the assigned structures.

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