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Solid-phase Synthesis of Chemotactic Peptides Using α-Azido Acids

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Abstract: Four chemotactic peptides, For-Met-Xxx-Phe-OMe, with an α,α-disubstituted amino acid at position 2 have been synthesized by the azido acid method [Meldal M, Juliano MA, Jansson AM. 1997. Azido acids in a novel method of solid-phase peptide synthesis. *Tetrahedron Lett*. **38**: 2531–2534] on solid-phase, and were tested for biological activity. Dipropylglycine in the central position (Xxx) was found to be as active as the natural chemotactic peptide for chemotactic activity toward human neutrophils. Higher yields were obtained than previously reported solution-phase syntheses of chemotactic peptides, and EEDQ was used successfully for the difficult solid-phase formylation of amino groups. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: azido acid; chemotactic peptide; α,α-disubstituted; solid-phase; fMLP; neutrophil

INTRODUCTION

The neutrophil cell surface receptors are the first line of defense against bacterial infections, and they are activated by binding of chemoattractants. Then the neutrophils move toward the bacteria from which the chemoattractants originate [1]. This is also called chemotaxis, the directed migration of phagocytes to inflammatory sites triggered by chemotactic factors. The infecting organism is hunted down and killed by phagocytosis, concurrent lysosomal enzyme release and activation of the NADPH oxidase to produce toxic oxygen metabolites, such as the superoxide anion, hydrogen peroxide and hypochlorous acid [2].

One of the early recognized chemotactic factors, or chemoattractants, was the hydrophobic peptide *N*-formyl-methionyl-leucyl-phenylalanine (For-Met-Leu-Phe-OH), which was produced by *Escherichia coli* amongst others, and became the model peptide for measuring chemotaxis or lysosomal enzyme release.

It interacts with a neutrophil receptor [3] with a specificity for hydrophobic *N*-formylated peptides, and two reports [1,4] have proposed a hydrophobic pocket in the receptor where leucine fits. Indications and conformational analyses have revealed that it is a rather large hydrophobic pocket, which fits from four [1] up to seven [4] carbon atoms. Full activity is reached only when the pocket is filled, so several analogs have been prepared, where leucine has been replaced by, for example, α,α-disubstituted amino acids. This makes the peptide less

Abbreviations: Ac_nc, 1-amino-cycloalkane-1-carboxylic acid, where n = size of the cycloalkane; Aib, 2-aminoisobutyric acid; Dbg, dibutylglycine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, dicyclohexylcarbodiimide; Deg, diethylglycine; DhbtOH, 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine; DIPEA, *N*,*N*-diisopropylethylamine; Dpg, dipropylglycine; Dph, diphenylglycine; DTT, dithiothreitol; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; For, formyl; HATU, *N*-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HMBA, 4-hydroxymethyl-benzoic acid; HOBt, 1-hydroxybenzotriazole; MSNT, mesitylenesulfon-1-yl-3-nitro-1,2,4-triazole; NEM, *N*-ethylmorpholine; PEGA, bis-aminopropyl ethylene glycol polyacrylamide copolymer; Pfp, pentafluorophenyl; TBDMS, *tert*-butyldimethylsilyl; TBTU, *N*-[(1H-benzotriazol-1-yl)-(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide.

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susceptible to enzymatic degradation; it limits the dihedral angles ϕ and ψ, and hence the conformation of the peptide. Furthermore, it increases the hydrophobicity because an extra alkyl or aryl side chain has been added.

The α,α-disubstituted amino acids used previously for replacing leucine had linear (Aib, Deg, Dpg, and Dbg) [5] or cyclic (Ac_3c, Ac_5c, Ac_6c, Ac_7c) [4] side chains, with activities ranging around that of the natural peptide, For-Met-Leu-Phe-OH (Figure 1).

The peptides with Dpg and Dbg replacing Leu were more active than the parent peptide, which supports the model proposing a hydrophobic pocket and the effect of filling it, because Dpg and Dbg are rather large amino acids [5]. In that report all the peptides were synthesized in solution with low yields using DCC and HOBt (around 25% crude material for the peptides containing Deg, Dpg and Dbg before HPLC purification) [5].

α-Azido acids are versatile precursors for natural and non-natural amino acids and can be incorporated into peptides [6]. The azido group offers excellent protection of the amino group so the carboxyl group of a sterically crowded building block, e.g. an α,α-disubstituted amino acid, can be highly activated as the acid chloride and reacted rapidly and efficiently with amino groups on solid-phase and in solution. This solves one of the remaining obstacles in solid-phase peptide synthesis, namely the preparation of peptide sequences difficult to synthesize due to sterical crowding in the growing peptide chain [7]. The α-azido acids can be prepared from simple starting compounds, such as α-bromo carboxylic acids, incorporated into peptides and reduced on the solid-phase [6] (Figure 2).

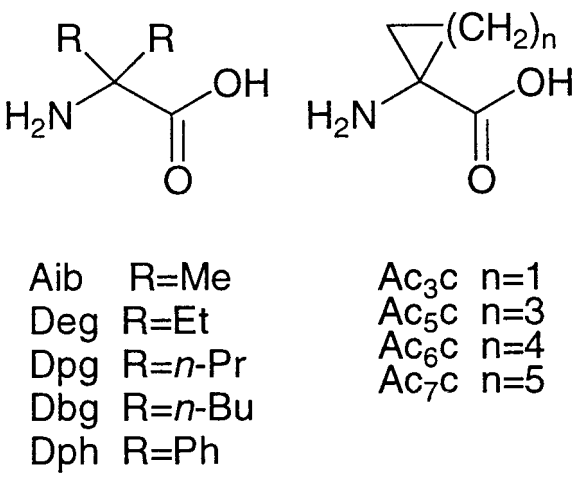

Figure 1 Linear and cyclic α,α-disubstituted amino acids used for incorporation in position 2 of the chemotactic peptide, For-Met-Xxx-Phe-OR.

MATERIALS AND METHODS

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DRX250 (250 MHz) and DRX600 (600 MHz). Electrospray mass spectrometry was performed in the positive mode on a Fisons VG Quattro instrument. Fluorometry was performed at 340 ± 5 nm excitation and 500 ± 10 nm emission wavelength in a Hitachi F-4000 fluorometer equipped with a magnetic stirrer and a thermostated cuvette. Analytical and preparative reverse-phase HPLC separations were performed on a Waters HPLC system using analytical RCM (8×100 mm) and Delta PAK (47×300 mm) C_{18} columns with a flow rate of 1 $cm^3\,min^{-1}$ and 20 $cm^3\,min^{-1}$, respectively. Detection was at 215 nm on a multiwavelength detector (Waters 490E) for analytical purposes and a photodiode array detector (Waters M991) was used for preparative separations. A solvent system consisting of A: 0.1% TFA in water and B: 0.1% TFA in 90% acetonitrile–10% water, was used, except for the

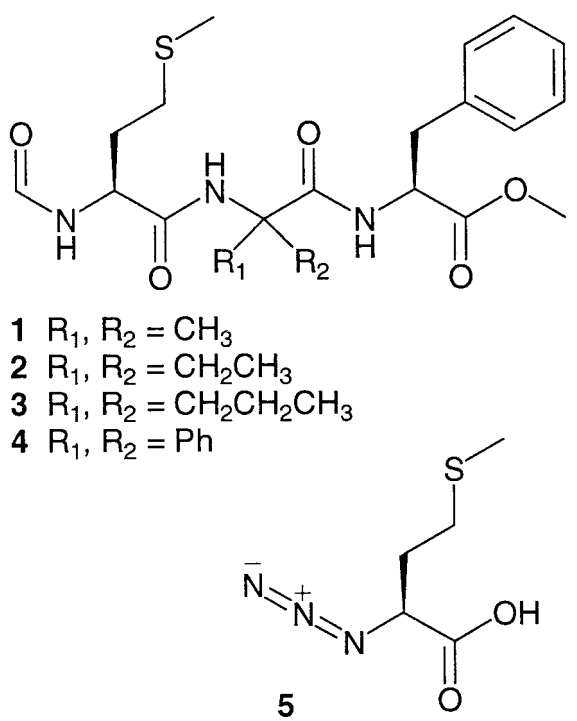

Figure 2 The prepared chemotactic peptides and the azido acid analog of methionine (**5**).

purification of **4**, where no TFA was used in the buffers.

CH_2Cl_2 was distilled from CaH_2, DMF was fractionally distilled *in vacuo* and all solvents were stored over molecular sieves (3 or 4 Å). Water was Milli-Q (Millipore). Deuterated solvents used for NMR were from Cambridge Isotope Laboratories and all other solvents were from Labscan. All amino acids were L-amino acids. The following commercially available chemicals were used: DIPEA (Sigma), DTT (Aldrich), EEDQ (Fluka), Fmoc-amino acids (Bachem or Novabiochem), HMBA (Bachem), MSNT (Novabiochem), *N*-ethylmorpholine (Merck), *N*-methylimidazole (Merck), piperidine (Aldrich), TBTU (Novabiochem) and thionyl chloride (Merck). The preparation of the α-azido acids will be reported elsewhere [Tornøe CW, Meldal M. In preparation].

N^{α}-Fmoc-deprotection was effected with 20% piperidine in DMF. Between acylations and deprotections, the resin was washed with six volumes of DMF, and when changing solvents from DMF to CH_2Cl_2, the resin was also washed with six volumes of CH_2Cl_2. When the resin was drained it acted as a hydrophilic sponge and the water retained acted as a competing nucleophile during the subsequent acylation. However, washing with excess CH_2Cl_2 and only draining the solvent to the surface of the resin bed allowed the exclusion of water and the efficient coupling of azido acid chlorides. All reactions took place in DMF unless otherwise stated. Azido acid chlorides were coupled with a five-fold excess in dichloromethane in the presence of a tertiary amine, *N*-ethylmorpholine (ten equivalents).

Synthesis of For-Met-Aib-Phe-OMe 1

The tripeptide **1** was synthesized manually by the syringe method [8] starting with a PEGA-resin [9] (0.492 g, 0.197 mmol) derivatized with 4-(*tert*-butyl-dimethyl-silanyloxymethyl)-benzoic acid (135.3 mg, 0.502 mmol) using TBTU [10] activation (149.9 mg, 0.468 mmol) for linker attachment. The TBDMS group was removed with tetrabutylammonium fluoride (ten equivalents) and acetic acid (four equivalents) in THF overnight. Fmoc-Phe-OH (229.7 mg, 0.593 mmol) was esterified onto the HMBA linker with MSNT [11] (179.5 mg, 0.605 mmol) and *N*-methylimidazole (31 µl, 0.390 mmol) in CH_2Cl_2. After removal of the Fmoc-group from Phe with 20% piperidine in DMF, 2-azido-2-methylpropanoic acid (94.5 mg, 0.732 mmol) was converted into the acid chloride using a binary mixture of dry CH_2Cl_2 (0.4 ml) and freshly redistilled $SOCl_2$ (0.4 ml) in a large excess by refluxing for 1 h under argon. The solvents were removed and the acid chloride was coupled onto the resin with *N*-ethylmorpholine (186 µl, 1.47 mmol) as a base in CH_2Cl_2. The azido group was reduced quantitatively with DTT (1 M) and DIPEA (0.5 M) in DMF at 50°C overnight and Fmoc-Met-OH (136.4 mg, 0.367 mmol) was coupled overnight by TBTU-activation. After Fmoc removal with 20% piperidine/DMF, formylation was accomplished with formic acid (17 µl, 0.451 mmol) and EEDQ [12] (113.6 mg, 0.464 mmol) in CH_2Cl_2 overnight. The peptide was cleaved off the resin with NaOMe (0.1 M) in MeOH. Reverse-phase HPLC purification afforded the tripeptide **1** (58.8 mg, 94%). ES-MS, m/z 424.4 ($M+H^+$). $C_{20}H_{29}O_5N_3S$ requires 423.5. 250 MHz ^{1}H-NMR in $CDCl_3$, δ ppm: 1.49 (s. 3H, Aib $C^{\beta}H_3$), 1.52 (s, 3H, Aib $C^{\beta'}H_3$), 2.01 (m, 2H, Met $C^{\beta}H_2$), 2.11 (s, 3H, Met-S-CH_3), 2.57 (m, 2H, Met $C^{\gamma}H_2$), 3.14 (dd, 2H, $J=6$ Hz, Phe $Cl^{\beta}H_2$), 3.74 (s, 3H, Phe-OMe), 4.17 (q, 1H, $J=7$ Hz, $J'=14$ Hz, Met $C^{\alpha}H$), 4.84 (q, 1H, $J=6$ Hz, $J'=13$ Hz, Phe $C^{\alpha}H$), 6.59 (br t, 2H, Phe NH, Met NH), 6.89 (s, 1H, Aib NH), 7.09, 7.12, 7.28, 7.29 (5H, Phe aromatic ring protons), 8.14 (s, 1H, formyl proton).

Synthesis of For-Met-Deg-Phe-OMe 2

Peptide **2** was synthesized in the same way as **1** except that the non-silylated HMBA-linker was used and Fmoc-Phe-OH was coupled twice to ensure full acylation of the linker. The reduction of the 2-azido-2-ethylbutyric acid residue was accomplished with a DTT (2 M) and DIPEA (1 M) solution in DMF at 50°C overnight. The peptide was cleaved off the resin with NaOMe (0.1 M) in MeOH. Reverse-phase HPLC purification afforded the tripeptide **2** (31.5 mg, 45%). ES-MS, m/z 452.3 ($M+H^+$). $C_{22}H_{33}O_5N_3S$ requires 451.6. 250 MHz ^{1}H-NMR in $CDCl_3$, δ ppm: 0.58 (t, 3H, $J=7$ Hz, Deg $C^{\gamma}H_3$), 0.74 (t, 3H, $J=7$ Hz, Deg $C^{\gamma'}H_3$), 1.44 (dq, 1H, $J=7$ Hz, $J'=14$ Hz, Deg $C^{\beta}_{A}H_2$), 1.57 (dq, 1H, $J=7$ Hz, $J'=14$ Hz, Deg $C^{\beta'}_{A}H_2$), 2.01 (m, 2H, Met $C^{\beta}H_2$), 2.11 (s, 3H, Met-S-CH_3), 2.43 (m, 2H, Deg $C^{\beta}_{B}H_2$, Deg $C^{\beta'}_{B}H_2$), 2.56 (m, 2H, Met $C^{\gamma}H_2$), 3.07 (dd, 1H, $J=7$ Hz, $J'=14$ Hz, Phe $C^{\beta}H$,), 3.19 (dd, 1H, $J=5$ Hz, $J'=14$ Hz, Phe $C^{\beta'}H_2$), 3.76 (s, 3H, Phe-OMe), 4.71 (q, 1H, $J=7$ Hz, $J'=14$ Hz, Met $C^{\alpha}H$), 4.91 (q, 1H, $J=7$ Hz, $J'=13$ Hz, Phe $C^{\alpha}H$), 6.08 (d, 1H, $J=8$ Hz, Phe NH), 6.68 (d, 1H, $J=7$ Hz, Met NH), 7.10, 7.13, 7.28, 7.31 (5H, Phe aromatic ring protons), 7.22 (s, 1H, Deg NH), 8.22 (s, 1H, formyl proton).

Synthesis of For-Met-Dpg-Phe-OMe 3

The tripeptide **3** was synthesized in the same way as **2** except 2-azido-2-propylpentanoic acid was used to incorporate the dipropylglycine residue. Reverse-phase HPLC purification afforded the tripeptide **3** (40.2 mg, 39%). ES-MS, m/z 480.4 ($M + H^+$). $C_{24}H_{37}O_5N_3S$ requires 479.6. 250 MHz ^{1}H-NMR in $CDCl_3$, δ ppm: 0.78 (t, 3H, $J = 8$ Hz, Dpg $C^{\delta'}H_3$), 0.80 (m, 1H, Dpg $C^{\beta}_{B}H_2$), 0.84 (t, 3H, $J = 7$ Hz, Dpg $C^{\delta}H_3$), 1.07 (br m, 3H, Dpg $C^{\gamma}_{B}H_2$, $C^{\gamma'}_{B}H_2$, $C^{\beta'}_{B}H_2$), 1.30 (m, 1H, Dpg $C^{\beta}_{A}H_2$), 1.45 (m, 1H, Dpg $C^{\beta}_{A}H_2$), 2.01 (br t, 2H, Met $C^{\beta}H_2$), 2.11 (s, 3H, Met-S-CH_3), 2.40 (br m, 2H, Dpg $C^{\gamma}_{A}H_2$), 2.55 (m, 2H, Met $C^{\gamma}H_2$), 3.15 (dd, 2H, $J = 7$ Hz, $J' = 14$ Hz, Phe $C^{\alpha}H_2$), 3.77 (s, 3H, Phe-OMe), 4.70 (q, 1H, $J = 7$ Hz, $J' = 14$ Hz, Met $C^{\alpha}H$), 4.90 (q, 1H, $J = 8$ Hz, $J' = 13$ Hz, Phe $C^{\alpha}H$), 6.08 (d, 1H, $J = 8$ Hz, Phe NH), 6.77 (d, 1H, $J = 8$ Hz, Met NH), 7.09, 7.11, 7.28, 7.29 (5H, Phe aromatic ring protons), 7.28 (s, 1H, Dpg NH), 8.23 (br s, 1H, formyl proton).

Synthesis of For-Met-Dph-Phe-OMe 4

The HMBA-linker (70.6 mg, 0.464 mmol) was coupled to a PEGA-resin (0.4386 g, 0.175 mmol) with TBTU and Fmoc-Phe-OH (208.5 mg, 0.538 mmol) was esterified onto the linker with MSNT. 2-Azido-2,2-diphenylacetic acid (221.8 mg, 0.876 mmol) was converted to the acid chloride using a binary mixture of CH_2Cl_2 (0.4 ml) and $SOCl_2$ (0.4 ml) for 2.5 h at reflux and coupled onto phenylalanine in the presence of *N*-ethylmorpholine (ten equivalents) in CH_2Cl_2 overnight. The reduction was accomplished overnight with DTT (2 M) and DIPEA (1 M) in DMF at 50°C. (*S*)-2-Azido-4-methylsulfanyl-butyric acid (**5**, 168.4 mg, 0.961 mmol) was converted into the acid chloride in 1 h in a similar way to the above (some darkening of the solution was observed), and coupled overnight with NEM (ten equivalents). Reduction to methionine was performed with DTT (0.25 M) and DBU (two equivalents) in DMF overnight at 20°C. Formylation was performed with formic acid (20 μl, 0.530 mmol) and EEDQ (132.0 mg, 0.534 mmol) in CH_2Cl_2 overnight. Compound **4** was cleaved off the resin with NaOMe (0.1 M) in MeOH and reverse-phase HPLC purification afforded the pure tripeptide (40.1 mg, 42%). ES-MS, m/z 548.3 ($M + H^+$). $C_{30}H_{33}O_5N_3S$ requires 547.7. 250 MHz ^{1}H-NMR in $CDCl_3$, δ ppm: 1.98 (s, 3H, Met-S-CH_3), 2.00 (m, 2H, Met $C^{\beta}H_2$), 2.42 (m, 2H, Met $C^{\gamma}H_2$), 3.03 (m, 2H, Phe $C^{\beta}H_2$), 3.67 and 3.69 (s, 3H, Phe-OMe), 4.76 (q, 1H, $J = 7$ Hz, $J' = 14$ Hz, Met $C^{\alpha}H$), 4.88 (m, 1H, Phe $C^{\alpha}H$), 6.13 (d, 1H, $J = 8$ Hz, Phe NH), 6.31 (t, 1H, $J = 7$ Hz, Met NH), 6.73 (dd, 1H, $J = 2$ Hz, $J' = 8$ Hz, Phe *ortho*-proton), 6.81 (dd, 1H, $J = 2$ Hz, $J' = 7$ Hz, Phe ortho-proton′), 7.34 (13H, Phe and Dph aromatic ring protons), 8.01 and 8.04 (s, 1H, Dph NH), 8.16 and 8.18 (s, 1H, formyl proton).

Doublets of the formyl, Dph NH, Met NH and OCH_3 resonances were observed, but the H^{α}s did not show any sign of racemization at 600 MHz (Figure 3). The doublings of the signals are most likely due to two conformations of the peptide because of slow *cis*/*trans* isomerization of an amide bond, or restricted rotation in the peptide because of the bulky geminal phenyl groups.

Screening of Chemotactic Peptides Against fMLP-receptor

Neutrophils were isolated from freshly prepared buffy coats as previously described [13] by dextran sedimentation of red blood cells and centrifugation of the leukocyte rich supernatant on Lymphoprep. Contaminating red blood cells were lysed by hypotonic shock. Neutrophils (1×10^7 cells ml^{-1}) were resuspended in buffer [NaCl (140 mM), KCl (5 mM), $MgCl_2$ (1 mM), glucose (5 mM), HEPES (10 mM), pH 7.4] and tumbled for 25 min at 25°C in the presence of 1 μM Fura-2/AM (Fura-2 acetoxymethylester) in the dark. The following operations were also carried out in the dark. Varidase (8 U ml^{-1}) was added to prevent cell aggregation. The cells were washed twice with the buffer, now including 1 mM Ca^{2+}, and were resuspended to 0.5×10^7 cells ml^{-1} and placed on a melting ice bath. Aliquots (2 ml) were used for fluorometry and they were heat-equilibrated for 3 min and then transferred to a cuvette in the fluorometer and kept at 37°C. Stock solutions (1 mM) of peptides **1**–**4** and For-Met-Leu-Phe-OH in ethanol were diluted with buffer to a concentration of 1 μM. A portion (20 μl) of this solution was added by syringe to the cuvette, so that the final concentration in the cuvette was 10 nM. The intracellular free calcium concentration was calculated using the fluorescence value from the emission spectrum of the calcium chelator Fura-2 as described earlier [14].

RESULTS

The synthesis of **1** containing an Aib residue was uncomplicated and gave a 94% yield of the expected product. In contrast, the yield of **2** and **3** were lower

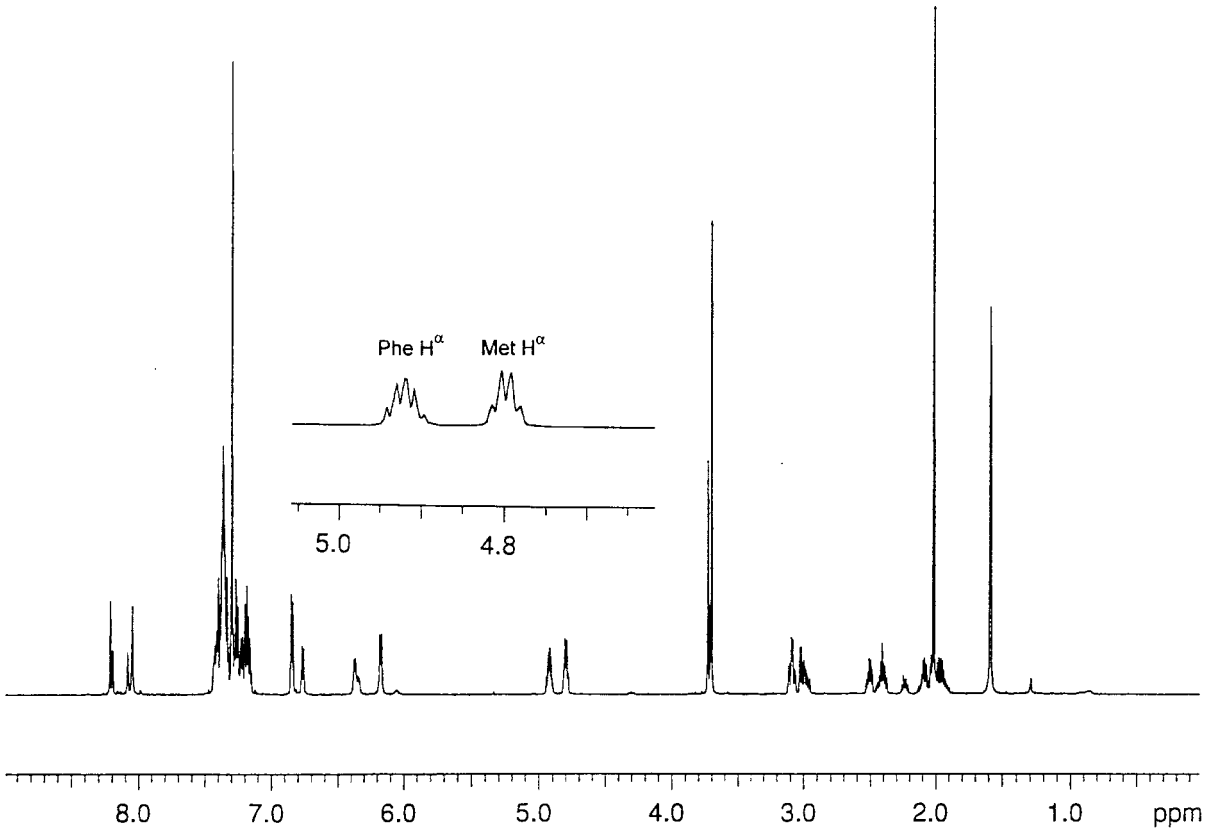

Figure 3 ^{1}H-NMR spectrum (600 MHz) of **4** in $CDCl_3$ at 300 K.

and by-products could be observed in the HPLC. The main by-products in the synthesis of **2** and **3** where the deletion peptides with methionine missing because of incomplete acylation of the sterically hindered Deg and Dpg residue. Furthermore, TBTU or HATU [15] activation of Fmoc-Met-OH did not give any of the expected product (**4**) when acylating the diphenylglycine, but instead the formylated deletion peptide, For-Dph-Phe-OMe had been formed. The reactive mixed carbonic anhydride formed from formic acid and EEDQ [12] was small enough to formylate the amino group, however, reagents of increasing size like TBTU-activated Fmoc-amino acids were too bulky and not sufficiently reactive. Therefore, it was decided to use the less hindered azido acid analog of L-methionine and convert it into the α-azido acid chloride. This was reacted with the resin-bound Dph-Phe dipeptide, reduced, formylated, cleaved and purified to give **4** in a modest yield of 42%.

Solid-phase peptide synthesis of chemotactic peptides is faster and higher yielding than solution-phase synthesis, and in this case the yields of **2** and **3** were improved to approximately 40% purified product compared with 25% crude material for solution-phase [5] and the yields can be enhanced further by use of the azido acid chloride analog of methionine.

Not much is known about the neutrophil receptor but structure–activity relationship studies have revealed a preference for large hydrophobic side chains at position 2 of the chemotactic peptide For-Met-Xxx-Phe-OMe and analogs with increasing side chains show higher biological activity (Table 1).

Table 1 The Calculated Values of Cytosylic [Ca^{2+}] Release Measured by Stimulating the fMLP Receptor with Different Peptides

Peptide	Cytosylic [Ca^{2+}] (nM)
For-Met-Leu-Phe-OH	310
For-Met-Aib-Phe-OMe	1169
For-Met-Deg-Phe-OMe	2257
For-Met-Dpg-Phe-OMe	3307
For-Met-Dph-Phe-OMe	4234

Binding assays to the fMLP-receptor showed that peptide **4** was 25% less active than the natural peptide, For-Met-Leu-Phe-OH, and similar to that of **2** containing a Deg residue. The known peptides **1**–**3** showed comparable activities with those previously measured [5,16]. The two phenyl rings in the Dph residue in **4** can prevent the peptide from binding properly, hence the lower activity. The proposed hydrophobic pocket must then be limited to alkyl side chains and not the larger aromatic rings, since **4** (cytosylic Ca^{2+} release of 234 nM) was less active than the natural peptide, For-Met-Leu-Phe-OH (Ca^{2+} release of 310 nM). The background level of intracellular [Ca^{2+}] was 100 nM.

DISCUSSION

The synthesis of four chemotactic peptides on solid-phase by the azido acid methodology has been demonstrated and a new analog, For-Met-Dph-Phe-OMe has been prepared. The four peptides were screened against human neutrophil receptors.

The azido acid protocol [6] was validated and it showed great promise concerning incorporation of sterically hindered unnatural amino acids into peptides, especially α,α-disubstituted amino acids. They can be difficult to acylate because of sterical hindrance, however, the protection of the amino group as the azide allowed high activation of the carboxyl group as the acid chloride and gave high yields. Azido acids with a variety of different side chains may be incorporated into position 2 of the chemotactic peptide to increase the range of analogs with agonist or antagonist activity.

The four chemotactic peptides were investigated in a binding assay to the fMLP receptor to compare their activites and the new chemotactic peptide, For-Met-Dph-Phe-OMe **4**, was shown not to be as active as the natural ligand, For-Met-Leu-Phe-OH.

Acknowledgements

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