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Conformation–activity correlations for chemotactic tripeptide analogs incorporating dialkyl residues with linear and cyclic alkyl sidechains at position 2

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Five stereochemically constrained analogs of the chemotactic tripeptide incorporating 1-aminocycloalkane-1-carboxylic acid (Ac_nc) and α,α-dialkylglycines (Deg, diethylglycine; Dpg, *n,n*-dipropylglycine and Dbg, *n,n*-dibutylglycine) at position 2 have been synthesized. NMR studies of peptides For-Met-Xxx-Phe-OMe (Xxx = Ac_7c, **I**; Ac_8c, **II**; Deg, **III**; Dpg, **IV** and Dbg, **V**; For, formyl) establish that peptides with cycloalkyl residues, **I** and **II**, adopt folded β-turn conformations in $CDCl_3$ and $(CD_3)_2SO$. In contrast, analogs with linear alkyl sidechains, **III–V**, favour fully extended (C_5) conformations in solution. Peptides **I–V** exhibit high activity in inducing β-glucosaminidase release from rabbit neutrophils, with ED_{50} values ranging from $1.4–8.0 \times 10^{-11}$ M. In human neutrophils the Dxg peptides **III–V** have ED_{50} values ranging from 2.3×10^{-8} to 5.9×10^{-10} M, with the activity order being **V** > **IV** > **III**. While peptides **I–IV** are less active than the parent, For-Met-Leu-Phe-OH, in stimulating histamine release from human basophils, the Dbg peptide **V** is appreciably more potent, suggesting its potential utility as a probe for formyl peptide receptors. © Munksgaard 1996.

Key words: chemotactic peptides; α,α-dialkylated residues; peptide conformation; β-turns

The ability of synthetic *N*-formyl tripeptides to induce neutrophil chemotaxis and activation (1–3) has stimulated a great deal of interest in the relationship between molecular conformation and biological activity in these sequences (3–6). Modifications of the parent sequence, For-Met-Leu-Phe-OH (FMLP), have provided clear evidence for the requirement of hydrophobic residues at all three positions (4–6). The Leu residue at position 2 has been replaced by several α,α-dialkylated residues with retention of biological activity (7, 8). There have been several attempts to delineate the 'biologically active backbone conformation' of the tripeptide chemotactic factors using conformationally constrained analogs as probes. α,α-Dialkylglycines have proved particularly useful as replacements for Leu (2), restricting significantly the available range of backbone conformations at the central position (9, 10). Using the prototype α,α-dialkyl residue, α-aminoisobutyric acid (Aib), which has a marked preference for helical conformations (11–13), and the related 1-aminocycloalkane-1-carboxylic acids (Ac_nc, $n = 3$, 5, 6) (7, 14–16), it was concluded that the observations of high biological activity correlated with the stabilization of folded conformations. β-Turn conformations at the Met-Xxx segment have been experimentally established in some active analogs in solution (17, 18) and for related sequences in crystals (19). An investigation of the analog For-Met-Dpg-Phe-OMe (Dpg, α,α-di-*n*-propylglycine) revealed high activity in inducing granule enzyme release in human neutrophils, while conformational analysis in solution and in crystals established a fully extended conformation at position 2 (20). This study also suggested that differences exist in the relative biological potencies determined with rabbit and human neutrophils (20).

In order to clarify the effects of Leu replacement by α,α-dialkylamino acids on conformation and biological activity, we have examined peptides of the type For-Met-Xxx-Phe-OMe [Xxx = 1-aminocycloheptane-1-carboxylic acid (Ac_7c), **I**; 1-aminocyclooctane-1-carboxylic acid (Ac_8c), **II**; diethylglycine (Deg), **III**;, α,α-di-n-propylglycine (Dpg), **IV**; and α,α-di-n-butylglycine (Dbg), V;

Fig. 1]. In this report we compare solution conformations of the position 2 analogs, with linear and cycloalkyl sidechains, and their ability to induce granule enzyme release in human and rabbit neutrophils and histamine release in human basophils.

EXPERIMENTAL PROCEDURES

Synthesis of peptides

All the chemotactic tripeptides For-Met-Xxx-Phe-OMe (where Xxx = Ac_7c, Ac_8c, Deg, Dpg, Dbg) were synthesised by conventional solution-phase procedures. *tert*-Butyloxycarbonyl (Boc) and methyl ester (OMe) groups were used for amino and carboxyl protection, and dicyclohexylcarbodimide (DCC) or DCC-1-hydroxybenzotriazole (HOBT) as coupling agents. Methyl ester hydrochlorides of Phe, Ac_7c, Ac_8c were prepared by the thionyl chloride–methanol procedure (21, 22). The esterification of Dxg amino acids was effected by passing dry HCl gas (until saturation) into solutions of amino acids in dry methanol, followed by storage at 10 °C for 10 d and then refluxing for 6 h (21, 22). All the intermediates obtained were checked for purity by TLC on silica gel and characterised by ^{1}H NMR (80 MHz). All the final peptides were purified by HPLC on a Lichrosorb RP C-18 (10μm) column using MeOH–H_2O gradients.

For-Met-Ac_7c-Phe-OMe (**1**)

Boc-Met-Ac_7c-OMe (**1**). 1.0 g (4 mmol) of Boc-Met-OH was dissolved in CH_2Cl_2 (10 mL) and cooled in an ice bath. H-Ac_7c-OMe, extracted from 1.45 g (7 mmol) of its hydrochloride after neutralizing with saturated sodium carbonate solution, was added followed by DCC (0.8 g, 4 mmol). The reaction mixture was stirred at room temperature for 48 h. The precipitated dicyclohexylurea (DCU) was filtered, and the filtrate was diluted with ethyl acetate (100 mL) and washed with 1 M $NaHCO_3$ (3 × 30 mL), 1 N HCl (3 × 30 mL) and an excess of water. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The peptide **1** was obtained as a gum. Yield 1.2 g (75%) ^{1}H NMR ($CDCl_3$, δ) 1.45, 9H, s, ($BocCH_3s$); 1.6, 1.65, 1.9, 2.0, 14H (Ac_7c ring-CH_2-protons, Met $C^\beta H_2$); 2.1, 3H, s (Met-SCH_3); 2.5, 2H, m (Met $C^\gamma H_2$); 3.65, 3H, s(-$COOCH_3$); 4.2, 1H, m (Met $C^\alpha H$); 5.10, 1H, d (Met NH); 6.58, 1H, s (Ac_7c NH).

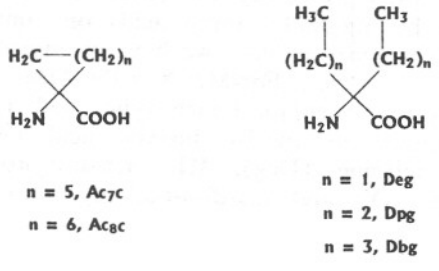

FIGURE 1

Structures of the α,α-dialkylated amino acid residues used at position 2 of the chemotactic tripeptide sequence.

Boc-Met-Ac_7c-OH (**2**). 1.0 g (2.48 mmol) of **1** was dissolved in MeOH (5 mL) and 2 N NaOH (5 mL) was added. The reaction mixture was stirred at room temperature for 40 h. After evaporation of methanol, the residue was diluted with water and washed with ether. The aqueous layer was cooled, neutralized by 2 N HCl and extracted with ethyl acetate. The solvent was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give peptide **2** as a gum. Yield 0.75 g (78%).

Boc-Met-Ac_7c-Phe-OMe (**3**)(**7**). 0.7 g (1.8 mmol) of **2** was dissolved in DMF (5 mL) and cooled in an ice bath. H-Phe-OMe obtained from 0.65 (3 mmol) of its hydrochloride was added, followed by DCC (0.36 g, 1.8 mmol) and HOBT (0.24 g, 1.8 mmol). The reaction mixture was stirred for 48 h. The residue was diluted with ethyl acetate and DCU was filtered. The organic layer was washed with excess water, 1 N HCl (3 × 30 mL), 1 M Na_2CO_3 (3 × 30 mL) and again with water. The solvent was dried over anhydrous Na_2SO_4 and then evaporated *in vacuo* to give compound **3.** Yield 0.65 g (66%). ^{1}H NMR ($CDCl_3$, δ) 1.25, 1.4, 1.6, 2.05, 2.1, 14H (Ac_7c ring CH_2-protons, Met $C^\beta H_2$); 1.45, 9H, s (Boc-CH_3s); 2.15, 3H, s (Met-S-CH_3) 2.55, 2H, m(Met $C^\gamma H_2$); 3.10, 2H m (Phe $C^\beta H_2$); 3.65, 3H, s(-$COOCH_3$); 4.15, 1H, m (Met $C^\alpha H$); 4.8, 1H, m (Phe$C^\alpha H$); 5.15, 1H, d(Met NH); 6.55, 1H, s (Ac_7c NH); 7.25, 6H (Phe ring protons, Phe NH).

For-Met-Ac_7c-Phe-OMe (**1**)(**7**). 0.6 g (1.09 mmol) of **3** was dissolved in 98% formic acid (4 mL) stoppered tightly and kept at room temperature. Progress of the reaction was followed by TLC. After completion of the reaction, formic acid was evaporated and the residue was washed with ether. The residue, i.e. formate salt, was dissolved in DMF (5 mL), 0.4 g of DCC was added, and the mixture was stirred for ca. 40 h. The conversion of formyl peptide was monitored by TLC. After completion of the reaction acetic acid was added to convert the excess of DCC into DCU. The precipitated DCU was filtered. The filtrate was diluted with ethyl acetate, washed with 1 M $NaHCO_3$ (3 × 30 mL) and water. The organic layer was dried over anhydrous Na_2SO_4 and solvent evaporated *in vacuo*. Yield 0.4 g (77%); m.p 98 °C. It was further purified by HPLC using MeOH/H_2O gradient elution (60–85% in 25 min; RT = 12 min) on a Lichrosorb RP-C8 column.

^{1}H NMR ($CDCl_3$, δ) 1.5, 1.65, 1.9, 1.98, 14H (Ac_7c ring CH_2 protons, Met $C^\beta H_2$); 2.08, 3H, s (Met-S-CH_3); 2.5, 2H, m (Met $C^\gamma H_2$) 3.07, 2H, m (Phe $C^\beta H_2$); 3.65, 3H, s(-$COOCH_3$); 4.5, 4.75, 2H,

m (Met $C^{\alpha}H$, Phe $C^{\alpha}H$); 6.2, 1H, d (Met NH); 6.5, 1H, s (Ac_7c NH); 6.68, 1H, d (Phe NH); 7.01, 7.15, 7.2, 5H m (Phe ring protons); 8.0, 1H, s (formyl proton).

For-Met-Ac_8c-Phe-OMe (**II**)

Boc-Met-Ac_8c-OMe (**4**). 1.25 g (5 mmol) of Boc-Met-OH was coupled to H-Ac_8c-OMe, isolated from 1.8 g (8 mmol) of its hydrochloride in CH_2Cl_2 (10 mL) using DCC as described in the case of **1**. Yield 1.1 g, white solid (52%), m.p 125–127 °C. ^1H NMR ($CDCl_3$, δ): 1.43, 9H, s (Boc CH_3 s); 1.4, 1.5, 1.8, 1.96, 16H (Ac_8c ring-CH_2-protons, Met $C^{\beta}H_2$); 2.06, 3H, s (Met-S-CH_3); 2.47, 2H m (Met $C^{\gamma}H_2$); 3.61, 3H, s (-$COOCH_3$); 4.13, 1H, m (Met$C^{\alpha}H$); 5.15, 1H. d (Met NH); 6.47, 1H, s (Ac_8cNH).

Boc-Met-Ac_8c-OH (**5**). 0.8 g of **4** was saponified using MeOH (15 mL) and 4 N NaOH (5 mL) as described in the case of **2**. The peptide was obtained as a gum. Yield 0.4 g (50%).

Boc-Met-Ac_8c-Phe-OMe (**6**). 0.44 g (1.09 mmol) of **5** was coupled to H-Phe-OMe isolated from 0.45 g (2 mmol) of its hydrochloride in DMF (5 mL), using DCC (0.2 g, 1.0 mmol) and HOBT (0.14 g, 1 mmol) as described in the case of **3**. Yield 0.4 g, white solid (71%) m.p 128–130 °C. ^1H NMR ($CDCl_3$, δ): 1.23, 1.4, 1.53, 2.06, 2.1,16H (Ac_8c ring-CH_2-protons, Met $C^{\beta}H_2$): 1.46, 9H, s (Boc-CH_3s); 2.12, 3H, s (Met-S-CH_3); 2.53, 2H, m(Met $C^{\gamma}H_2$); 3.1, 2H, m (Phe $C^{\beta}H_2$); 3.66, 3H, s (-$COOCH_3$); 4.13, 1H, m (Met $C^{\alpha}H$); 4.83, 1H, m (Phe $C^{\alpha}H$); 5.16, 1H, d (Met NH); 6.46, 1H, s (Ac_8cNH); 7.2, 6H (Phe ring protons, Phe NH).

For-Met-Ac_8c-Phe-OMe (**II**). 0.4 g of **6** was dissolved in 98% formic acid (4 mL) and after deprotection it was further treated with DCC (0.3 g) in DMF (3 mL), as in the case of **1**. Yield 0.3g (85%); m.p 127 °C. The peptide was purified by HPLC using MeOH/H_2O gradient elution on a Lichrosorb RP-C8 column (70–95% MeOH in 25 min, RT = 11.5 min). ^1H NMR ($CDCl_3$,δ): 1.25, 1.52, 1.75, 1.85, 1.96, 2.02, 16H (Ac_8c ring CH_2 protons, Met $C^{\beta}H_2$); 2.11, 3H, s (Met-S-CH_3); 2.56, 2H, m (Met $C^{\gamma}H_2$); 3.10, 2H, m (Phe $C^{\beta}H_2$); 3.69, 3H, s (-$COOCH_3$); 4.58, 1H m (Met $C^{\alpha}H$); 4.83; 1H, m (Phe $C^{\alpha}H$); 6.35,1H, d (Met NH); 6.52, 1H, s (Ac_8c NH); 6.81, 1H, d (Phe NH) 7.12, 7.25, 5H (Phe ring protons); 8.07, 1H (formyl proton).

For-Met-Deg-Phe-OMe (**III**)

Boc-Met-Deg-OMe (**7**). 1.0 g (4 mmol) of Boc-Met-OH was dissolved in DMF (5 mL) and cooled in ice. 0.56 g (4 mmol) of H-Deg-OMe obtained from its hydrochloride was added followed by DCC (0.8 g, 4 mmol) and HOBT (0.54 g, 4 mmol). The reaction mixture was stirred for 3 d. The work up of the reaction was done as in the case of **1**. Yield 0.72 g (47%) (gum). ^1H NMR ($CDCl_3$, δ) 0.77, 1.27, 6H, t (Deg $C^{\gamma}H_3$); 1.45, 9H, s (Boc-CH_3s); 1.81, 2.05, 2.36, 2.59, 8H (Deg $C^{\beta}H_2$, Met $C^{\beta}H_2$, Met$C^{\gamma}H_2$); 2.11 3H, s (Met-S-CH_3); 3.81, 3H, s (-$COOCH_3$); 5.36, 1H, d (Met NH); 7.0, 1H, s (Deg NH).

Boc-Met-Deg-OH (**8**). 0.7 g (1.86 mmol) of **7** was saponified using 4 N NaOH (4 mL) and methanol (10 mL) as in the case of **2**. Yield 0.6 g (83%) (gum).

Boc-Met-Deg-Phe-OMe (**9**). 0.5 g (1.38 mmol) of **8** was dissolved in DMF (3 mL) H-Phe-OMe obtained from its hydrochloride (0.61 g, 3 mmol) was added followed by 0.28 g (1.4 mmol) DCC and HOBT (0.2 g). The reaction mixture was stirred at room temperature for 5 d and worked up as in the case of **3**. Yield 0.6 g (83%) (gum). ^1HNMR ($CDCl_3$, δ) 0.55, 0.77, 6H, m (Deg $C^{\gamma}H_3$s); 1.45, 9H, s (Boc CH_3s); 1.27, 1.68, 1.95, 6H, m (Deg $C^{\beta}H_2$, Met $C^{\beta}H_2$); 2.09, 3H, s (Met-S-CH_3); 2.55, 2H, m (Met $C^{\gamma}H_3$); 3.17, 2H, m (Phe $C^{\beta}H_2$); 3.77, 3H, s (-$COOCH_3$); 4.18, 1H, m (Met $C^{\alpha}H$); 4.86, 1H, m (Phe $C^{\alpha}H$); 5.37, 1H, d (Phe NH); 6.63, 1H, d (Met NH); 7.22, 6H, m (Phe ring protons, Deg NH).

For-Met-Deg-Phe-OMe (**III**). 0.53 g (1 mmol) of **9** was converted into its formyl derivative using 98% formic acid (2 mL) and 0.3 g of DCC as in the case of **1**. Yield 0.35 g (77%). The peptide was purified by HPLC on a reversed phase Lichrosorb C18 column using MeOH–H_2O as eluent (60–80% in 20 min; RT = 6.8 min) ^1H NMR ($CDCl_3$, δ): 0.57, 0.72, 6H, t (Deg $C^{\gamma}H_3$s);1.48, 1.54, 1.62, 1.96, 6H, m (Deg $C^{\beta}H_2$, Met $C^{\beta}H_2$); 2.10, 3H, s (Met-S-CH_3); 2.55, 2H, m (Met $C^{\gamma}H_2$); 3.14, 2H, m (Phe $C^{\beta}H_2$); 3.75, 3H, s (-$COOCH_3$); 4.67, 4.91, 2H, m (Met $C^{\alpha}H$, Phe $C^{\alpha}H$); 6.15, 1H, d (Phe NH); 6.36, 1H, d (Met NH); 7.13, 7.27, 7.30, 6H (Phe ring protons, Deg NH); 8.19, 1H, s (formyl proton).

For-Met-Dpg-Phe-OMe (**IV**)

Boc-Met-Dpg-OMe (**10**). 1.0 g (4 mmol) of Boc-Met-OH was coupled to 0.6 g (3.46 mmol) of H-Dpg-OMe obtained from its hydrochloride in DMF (5 mL) using DCC (0.8 g) and HOBT (0.5 g) as described in case of **1**. The product was obtained as a gum. Yield 0.6 g (37%). ^1H NMR ($CDCl_3$, δ) 0.74, 0.93, 1.0, 1.1, 14H (Dpg $C^{\beta}H_2$, $C^{\gamma}H_2$, $C^{\delta}H_3$) 1.3, 9H, s (Boc CH_3s); 1.63, 1.84, 2H m (Met $C^{\beta}H_2$); 1.99, 3H, s (Met-S-CH_3) 2.73, 2H, m (Met $C^{\gamma}H_2$); 3.57, 3H, s (-$COOCH_3$) 4.06, 1H, m (Met $C^{\alpha}H$); 5.6, 1H, d (Met NH); 7.08, 1H, s (Dpg NH).

Boc-Met-Dpg-OH (**11**). 0.6 g of **10** was saponified using 4 N NaOH (5 mL) and methanol (5 mL) as described in the case of **2**. Yield 0.5g (86%) (gum).

Boc-Met-Dpg-Phe-OMe (**12**). 0.5 g (1.28 mmol) of **11** was dissolved in DMF (5 mL) and coupled to H-Phe-OMe isolated from 0.61 g (3 mmol) and HOBT (0.2 g) as described in case of **3**. Yield 0.6 g (85%) (gum). ^{1}H NMR ($CDCl_3$, δ): 0.75, 0.94, 1.0, 1.1, 14H (Dpg side chain protons); 1.41, 9H, s (Boc-CH_3s); 1.65, 1.85, 2H, m (Met$C^{\beta}H_2$); 2.15, 3H, s (Met-S-CH_3); 2.52, 2H, m (Met$C^{\gamma}H_2$) 3.12, 2H, m (Phe $C^{\beta}H_2$); 3.70, 3H, s (-$COOCH_3$); 4.16, 4.81, 2H, m (Met C^{α}H, Phe C^{α}H); 5.06, 1H, d (Met NH); 6.6, 1H, d (Phe NH); 7.21, 7.23,6H (Phe ring protons, DpgNH).

For-Met-Dpg-Phe-OMe (**IV**). 0.6 g (1.08 mmol) of **12** was formylated to **IV** using 98% formic acid (3 mL) and 0.3 g of DCC. The peptide **IV** was obtained as a solid. Yield 0.45 g (86%), m.p 138–140 °C. The peptide was further purified by HPLC on a reversed phase Lichrosorb C-18 column using MeOH/H_2O gradient elution. (60–80% in 20 min RT = 9.5 min). ^{1}H NMR ($CDCl_3$, δ): 0.8, 0.9, 1.12, 1.95, 2.1, 2.25, 2.48, 14 H (Dpg side chain protons, Met C^{β} H_2); 2.16, 3H, s (Met-S-CH_3); 2.53, 2H, m (Met $C^{\gamma}H_2$); 3.1, 2H, m (Phe $C^{\beta}H_2$); 3.75, 3H, s(-$COOCH_3$); 4.66, 1H, m(Met C^{α}H); 4.96, 1H, m (Phe C^{α}H); 6.13, 1H, d (Phe NH); 6.43, 1H, d (Met NH); 7.12, 7.26, 7.3, 5H (Phe ring protons); 7.2, 1H, s (Dpg NH); 8.22, s (formyl proton).

For-Met-Dbg-Phe-OMe (**V**)

Boc-Met-Dbg-OMe (**13**). 0.75 g (3 mmol) of Boc-Met-OH was coupled to 0.61 g (3 mmol) of H-Dbg-OMe, obtained from its hydrochloride, using DCC (0.6 g, 3 mmol) and HOBT (0.4 g) as described in the case of **1**. Yield 0.65 g (50%)(gum). ^{1}H NMR ($CDCl_3$, δ) 0.8, 1.15, 1.45, 18H (Dbg side chain protons); 1.35, 9H, s (Boc CH_3s); 1.64, 1.85, 2H, m (Met $C^{\beta}H_2$); 1.96, 3H, s (Met-S-CH_3); 2.4, 2H, m (Met $C^{\gamma}H_2$); 3.6, 3H, s (-$COOCH_3$); 4.08, 1H, m (Met C^{α}H); 5.06, 1H, d (Met NH); 7.09, 1H, s (Dbg NH).

Boc-Met-Dbg-OH (**14**). 0.65 g of **13** was saponified using 4 N NaOH (6 mL) and methanol (8 mL) as described in the case of **2**. Yield 0.52 g (82%) (gum).

Boc-Met-Dbg-Phe-OMe (**15**). 0.52 g (1.24 mmol) of **14** was dissolved in DMF (5 mL) and coupled to H-Phe-OMe, obtained from 0.61 g (3 mmol) of its hydrochloride, using DCC (0.3 g, 1.5 mmol) and HOBT (0.2 g) as described in the case of **3**. Yield 0.61 g (84%) (gum). ^{1}H NMR ($CDCl_3$, δ): 0.75, 1,15, 1.45, 18H (Dbg side chain protons); 1.36, 9H,s (Boc CH_3s); 1.64, 1.84, 2H, m (Met $C^{\beta}H_2$); 2.01, 3H, s (Met-S-CH_3); 2.41, 2H, m (Met $C^{\gamma}H_2$); 3.08, 2H, m (Phe $C^{\beta}H_2$); 3.69, 3H, s (-$COOCH_3$); 4.10, 4.8, 2H, m (Met C^{α}H, Phe C^{α}H); 5.0, 1H, d (Met NH); 6.15, 1H, d (Phe NH); 7.05, 7.1, 7.15, 6H (Phe ring protons, Dbg NH).

For-Met-Dbg-Phe-OMe (**V**). 0.61 g (1.05 mmol) of **15** was converted into its formyl derivative **V** using 98% formic (3 mL) and 0.3 g of DCC as described in the case of **3**. Yield 0.41 g (75%). m.p 110 °C. Further purification was by HPLC on a reversed phase Lichrosorb C-18 using MeOH/H_2O gradient elution (60–80% in 20 min; RT 13.3 min). ^{1}H NMR ($CDCl_3$, δ); 0.75, 1.15, 1.25, 1.65, 1.8 (Dbg $C^{\delta}H_3$s,Dbg $C^{\gamma}H_2$s, Dbg $C^{\beta}H_2$s); 1.95, 2H, m (Met $C^{\beta}H_2$s); 2.1, 3H, s (Met-S-CH_3); 2.48, 2H, m (Met $C^{\gamma}H_2$); 3.1, 2H, m (Phe $C^{\beta}H_2$); 3.7, 3H, s (-$COOCH_3$); 4.6, 1H, m (Met C^{α}H); 4.8, 1H, m (Phe C^{α}H); 6.2, 1H, d (Phe NH); 6.49, 1H, d (Met NH); 7.1, 7.15, 7.3, 6H (Phe ring protons, Dbg NH); 8.15, s (formyl proton).

Biological assays

β-Glucosaminidase release from both rabbit and human neutrophils was assayed as described previously (7). Histamine release from human basophils was monitored as described earlier (32, 33).

RESULTS AND DISCUSSION

Solution conformations

The residue specific assignment of the NH and C^{α}H resonances in the peptides **I–V** was achieved using decoupling and difference NOE experiments. The chemical shifts for the backbone protons in $CDCl_3$ and $(CD_3)_2SO$ are summarised in Table 1. The involvement of NH groups in intramolecular hydrogen bonding was probed using solvent titration experiments in $CDCl_3$–$(CD_3)_2SO$ mixtures and temperature coefficients of chemical shifts ($d\delta/dt$) in $(CD_3)_2SO$ (23, 24). The solvent titration curves were monotonic over the entire range of solvent composition suggesting an absence of any dramatic solvent induced conformational change. The absence of NH–NH NOEs and lack of a C^{α} hydrogen at residue 2 rendered NOE experiments uninformative in analysing conformations of peptides **I–V**. The solvent shift ($\Delta\delta$) values listed in Table 1 are clearly indicative of solvent shielded Phe NH groups in Ac_nc containing peptides **I** and **II**. The Phe NH groups in these two peptides also exhibit low $d\delta/dt$ as compared to the Met and Ac_nc NH groups. In sharp contrast in Dxg peptides **III–V** the Dxg NH proton exhibits very low $\Delta\delta$ (0.15–0.20 ppm) and $d\delta/dt$ [$(0.9–1.0) \times 10^{-3}$ ppm/K] values. The NMR data thus establish that in the Ac_nc peptides, **I** and **II**, conformations involving the Phe NH in an intramolecular hydrogen bond are preferred. β-Turn conformations (Fig. 2a), involving 4→1 hydrogen bonding between formyl CO and the Phe NH groups, are consistent with the experimental observations and the known tendency of Ac_nc residues to promote peptide chain folding. In difference NOE experiments, a strong NOE was observed between Met C^{α}H and

TABLE 1

^1H NMR parameters for NH resonances in peptides For-Met-Xxx-Phe-OMe[a]

For-Met-Xxx-Phe-OMe	Chemical shift δ (ppm)						$\Delta\delta$NH (ppm) $(CD_3)_2SO$-$CDCL_3$			$d\delta/dT \times 10^{-3}$ ppm/K $(CD_3)_2SO$		
	$(CDCl_3)$			$(CD_3)_2SO$								
Xxx	**Met**	**Xxx**	**Phe**	**Met**	**Xxx**	**Phe**	**Met**	**Xxx**	**Phe**	**Met**	**Xxx**	**Phe**
Ac7c	6.20	6.50	6.68	8.30	7.85	7.15	2.10	1.35	0.47	5.0	5.1	2.1
Ac8c	6.35	6.52	6.81	8.31	7.90	7.48	1.96	0.98	0.67	4.9	5.4	3.8
Deg	6.36	7.30	6.15	8.37	7.45	8.15	2.01	0.15	2.00	3.8	0.9	4.3
Dpg	6.43	7.20	6.13	8.40	7.40	8.22	1.97	0.20	2.09	3.3	1.0	4.3
Dbg	6.49	7.30	6.20	8.35	7.48	8.20	1.86	0.18	2.00	3.2	0.9	3.8

[a]Peptide concentration 10 mM.

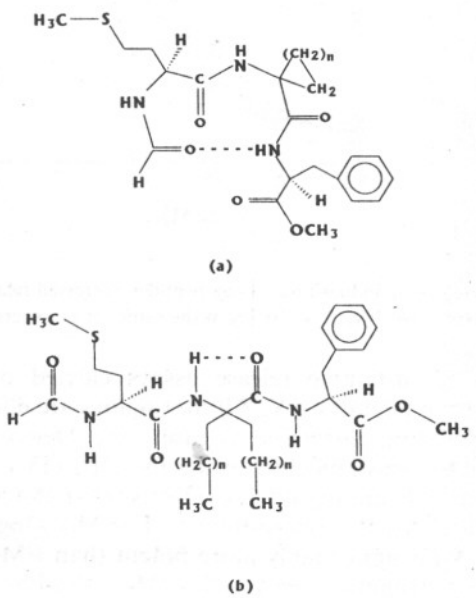

FIGURE 2

(a) Schematic β-turn conformation in $Ac_nc(2)$ analogs. (b) Extended conformations in Dxg (2) analogs. The C_{10} and C_5 interaction is indicated by a broken line.

Ac_nc NH protons, suggesting that type II β-Turn conformations are favoured (Met $\phi = -60\,^{\circ}C$, $\psi = 120\,^{\circ}$; Ac_nc, $\phi = 80\,^{\circ}$, $\psi = 0\,^{\circ}$). Similar conformations have been proposed for the Ac_6c analog (16). Type II β-turns have also been observed for the peptides Boc-Met-Aib-Phe-OMe (19) and Boc-Met-Ac_5c-Phe-OMe (7) in crystals and in solution. The NMR data for Dxg peptides **III**–**V** provide clear evidence for the absence of β-turn conformations described above for Ac_nc peptides. Theoretical calculations for Deg and Dpg residues reveal the presence of two comparable energy minima for these residues corresponding to fully extended ($\phi \approx \psi \approx 180\,^{\circ}$) and helical $\phi \approx \pm 60 \pm 20\,^{\circ}$, $\psi = \pm 30 \pm 20^{\circ}$) conformations (25, 26). The energy difference between these two minima appears to be a function of the bond angle at the C^{α} atom. Fully extended (C_5) conformations are preferred for $\tau \leqslant 108\,^{\circ}$ and helical structures for $\tau \geq 108\,^{\circ}$. Crystallographic studies on peptides containing Deg, Dpg and Dbg residues have provided examples of both extended and folded helical conformations. In crystals of short homo-oligopeptides of Deg and Dpg fully extended structures have been observed (25–27). An exception is the folded structure in a fully protected Dpg homotripeptide (28). In heteromeric sequences Dxg residues have been readily accomodated in β-turns (29) and peptide helices (30) in the solid state. In the tripeptide Boc-Leu-Dpg-Val-OMe both extended and folded conformations at the Dpg residue coexist in crystals (31). Evidence for a transition from a folded to an extended conformation at Dxg residues in tripeptides on dissolution of crystals has also been presented (22). The reported crystal structure of For-Met-Dpg-Phe-OMe reveals a fully extended conformation at Dpg (Dpg, $\phi = 173.1\,^{\circ}$, $\psi = 179.0\,^{\circ}$) (20). The NMR data presented above are consistent with a population of extended conformations at the central Dxg residues. The proximity of the Dxg NH to the Dxg CO in the extended C_5 conformation (Fig. 2b) probably accounts for the inaccessibility of this group to solvent. Solvation of the Dxg NH by $(CD_3)_2SO$ is likely to be impeded on the basis of both steric and electrostatic considerations.

Biological activity

The ability of peptides **I**–**V** to induce β-glucosaminidase release from rabbit neutrophils is summarised in Table 2. Peptides **III**–**V** have also been tested on human neutrophils. All five analogs are significantly more active than the parent peptide FMLP in stimulating enzyme release in rabbit neutrophils. Indeed, the activity of the Ac_7c analog (**I**) has been reported earlier (7). In the case of human neutrophils the Dxg peptides **III**–**V** show appreciable activity, with a dramatic increase being observed on lengthening the alkyl chain at position 2. Indeed, peptide **V**, For-Met-Dbg-Phe-OMe, appears to be a particularly potent activator of human neutrophils. In contrast, although all the peptides stimulate rabbit neutrophils

TABLE 2

β-Glucosaminidase release in neutrophils by formyl-methionyl tripeptides

Compounds	$ED_{50} \pm$S.E. (M)[a]	Maximum (%)[b]
Rabbit neutrophils		
CHO-Met-Leu-Phe-OH	$(7.4 \pm 1.2) \times 10^{-11}$	35 ± 0.1
CHO-Met-Ac_7c-Phe-Me	$(1.4 \pm 1.2) \times 10^{-11}$	35 ± 0.1
CHO-Met-Ac_8c-Phe-Me	$(1.4 \pm 1.3) \times 10^{-11}$	36 ± 0.5
CHO-Met-Deg-Phe-OMe	8.0×10^{-11}	27 ± 1.9
CHO-Met-Dpg-Phe-OMe	3.6×10^{-11}	32 ± 4.0
CHO-Met-Dbg-Phe-OMe	4.8×10^{-11}	30 ± 3.5
Human neutrophils		
CHO-Met-Deg-Phe-OMe	2.31×10^{-8}	33 ± 1.7
CHO-Met-Dpg-Phe-OMe	1.84×10^{-9}	32 ± 1.2
CHO-Met-Dbg-Phe-OMe	5.92×10^{-10}	34 ± 2.8

[a]ED_{50} is the concentration of the agonist inducing one half of the maximal release.

[b]Maximum release is calculated as percentage of total enzyme present.

at appreciably lower concentrations, a marked difference is not observed on increasing the size of the alkyl sidechains at position 2 in both Ac_nc and Dxg series. The biological activity determined in human neutrophils for For-Met-Dpg-Phe-OMe (**IV**) is in good agreement with the value reported by Dentino *et al.* (20). The significance of the differences in the behaviour of the peptides towards human and rabbit neutrophils is unclear.

FMLP has been shown to possess secretagogue activity and stimulates histamine release in human basophils (29, 30). Figures 3 and 4 summarize the

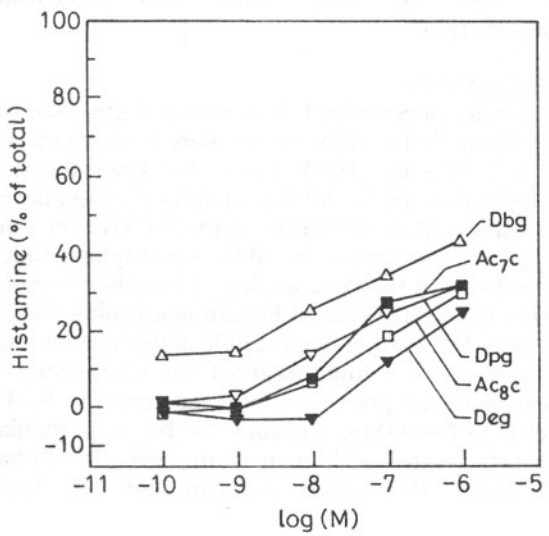

FIGURE 3

Basophil histamine release expressed as percentage of total histamine determined for FMLP analogs. Average values determined in six experiments or more.

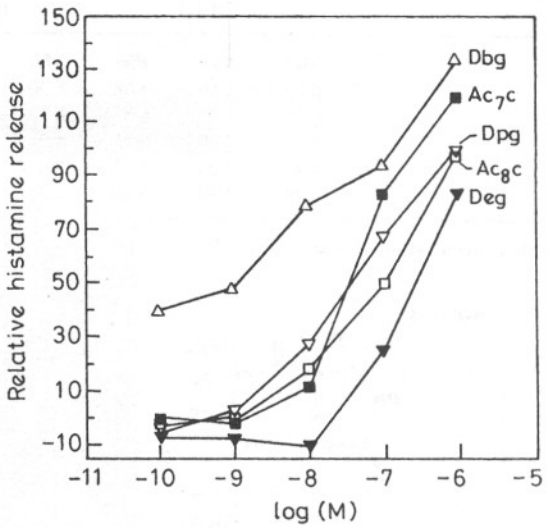

FIGURE 4

Histamine release induced by analog peptides expressed relative to that observed for FMLP at 10^{-6}M in the same set of experiments.

results of histamine release assays carried out in presence of peptides **I**–**V**. All the analogs are effective in stimulating histamine release. The Deg analog (**III**) is the least effective, while the Dpg (**IV**), Ac_7c (**I**), Ac_8c (**II**) are slightly less effective than the parent peptide FMLP. Interestingly, For-Met-Dbg-Phe-OMe (**V**) is appreciably more potent than FMLP in inducing histamine release. For-Met-Dbg-Phe-OMe thus appears to be a potentially important ligand for probing target receptor sites on both human neutrophils and human basophils.

CONCLUSION

The results of the present study establish that conformationally constrained analogs of the chemotactic tripeptide FMLP, which favour widely different solution conformations, exhibit high levels of biological activity as manifested in studies of granule enzyme and histamine release in neutrophils and basophils, respectively. In the case of neutrophil receptors, heterogeneity and differences between chemotactic peptide receptors of rabbit and human neutrophils need to be considered (34). Two possible explanations for the high biological activity of peptides with contrasting solution conformations must be considered:

1. Changes in the conformation of the peptides on receptor binding. This induced-fit explanation may be relevant for Dxg analogs, since these residues have been shown to adopt two distinct conformations, folded and extended, in structures of model peptides (20, 22, 25–31). Indeed recent crystal structures of model peptides reveals coexistence of both conformations at a central Dpg residue in crystals (31), suggesting that energy differences between the

two states may be easily compensated by local environmental interactions.

2. Heterogeneity of receptor sites with some sites recognizing peptides as folded structures, while others have an affinity for extended structures. While clear evidence for multiplicity of receptors recognizing distinct conformational species is still awaited, the experience with receptors for other biologically active peptides such as opioids (35) suggests that this explanation cannot be dismissed without further evidence.

The availability of high affinity peptide ligands with well defined and distinct conformations should prove useful in further probing structural requirements of formyl peptide receptors in diverse cell types.

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REFERENCES

1. Schiffmann, E., Corcoran, B.A. & Wahl, S.M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1059–1062
2. Showell, H.J. Freer, R.J. Zigmond, S.H., Schiffmann, E., Aswanikumar, S., Corcoran, B. and Becker, E.L. (1976). *J. Exp. Med.* **143**, 1154–1169
3. Becker, E.L. (1987) *Am. J. Pathol.* **129**, 16–24
4. Freer, R.J., Day, A.R., Muthukumaraswamy, N., Pinon, D., Wu, A., Showell, H.J. & Becker, E.L. (1982) *Biochemistry* **21**, 257–263
5. Freer, R.J., Day, A.R., Radding, J.A., Schiffmann, E., Aswanikumar, S., Showell, H.J. & Becker, E.L. (1980) *Biochemistry* **19**, 2404–2410
6. Becker, E.L., Freer, R.J., Toniolo, C. & Balaram, P. (1985) in Czech, M.P. & Kahn, C.R., eds. *Membrane Receptors and Cellular Regulation* A.R. Liss, New York, pp. 129–134
7. Toniolo, C., Crisma, M., Valle, G., Bonora, G.M., Polinelli, S., Becker, E.L., Freer, R.J., Sudhanand, Balaji Rao., R., Balaram, P. & Sukumar, M. (1989) *Peptide Res.* **2**, 275–281
8. Formaggio, F., Pantano, M., Crisma, M., Toniolo, C., Boesten, W.H.J., Schoemaker, H.E., Kamphuis, J. & Becker, E.L. (1993) *Bioorg. Med. Chem. Lett.* **3**, 953–956
9. Toniolo, C. (1989) *Biopolymers*, **28**, 247–257
10. Balaram, P. (1992) *Curr. Opin. Struct. Biol.* **2**, 845–851
11. Prasad, B.V.V. & Balaram, P. (1984) *CRC. Crit. Rev. Biochem.* **16**, 307–347
12. Karle, I.L. & Balaram, P. (1990) *Biochemistry* **29**, 6747–6756
13. Toniolo, C. & Benedetti, E. (1991) *Macromolecules* **24**, 4004–4009
14. Bardi, R., Piazzesi, A.M., Toniolo, C., Sukumar, M., Raj, P.A. & Balaram, P. (1985) *Int. J. Peptide Protein Res.* **25**, 628–639
15. Paul, P.K.C., Sukumar, M., Bardi, R., Piazzesi, A.M., Valle, G., Toniolo, C. & Balaram, P. (1986) *J. Am. Chem. Soc.* **108**, 6363–6370
16. Valle, G., Crisma, M., Toniolo, C., Sudhanand, Balaji Rao, R., Sukumar, M. & Balaram, P. (1991) *Int. J. Peptide Protein Res.* **38**, 511–518
17. Iqbal, M., Balaram, P., Showell, H.J., Freer. R.J. & Becker, E.L. (1984) *FEBS Lett.* **165**, 171–174
18. Sukumar, M., Raj, P.A., Balaram, P. & Becker, E.L. (1985) *Biochem. Biophys. Res. Commun.* **128**, 339–344
19. Bardi, R., Piazzesi, A.M., Toniolo, C., Raj, P.A., Raghothama, S. & Balaram, P. (1986) *Int. J. Peptide Protein Res.* **27**, 229–238
20. Dentino, A.R., Raj, P.A., Bhandary, K.K., Wilson, M.E. & Levine, M.J. (1991) *J. Biol. Chem.* **266**, 18460–18468
21. Prasad, S. (1992) *Ph.D. Thesis* Banaras Hindu University, Varanasi, India
22. Prasad, S., Balaji Rao, R. & Balaram, P. (1994). *Biopolymers* **35**, 11–20
23. Kessler, H. (1982) *Angew. Chem., Int. Ed. Engl.* **21**, 512–523
24. Balaram, P. (1985) *Proc. Ind. Acad. Sci. Chem. Sci.* **95**, 21–38
25. Benedetti, E., Toniolo, C., Hardy, P., Barone, V., Bavoso, A., Di Blasio, B., Grimaldi, P., Lelj, F., Pavone, V., Pedone, C., Bonora, G.M. & Lingham, I. (1984) *J. Am. Chem. Soc.* **106**, 8146–8152
26. Benedetti, E., Barone, V., Bavoso, A., DiBlasio, B., Lelj. F., Pavone, V., Pedone, C., Bonora, G.M., Toniolo, C., Leplawy, M.T., Kazmarek, K &. Redlinski, A. (1988) *Biopolymers* **27**, 357–371
27. Toniolo, C. & Benedetti, E. (1991) in Balaram, P. & Ramaseshan, S. eds. *Molecular Conformation and Biological Interactions.* Indian Academy of Sciences, Bangalore, pp. 511–521
28. Di Blasio, B., Pavone, V., Isermia, C., Pedone, C., Benedetti, E., Toniolo, C., Hardy, P.M., & Lingham, I.N. (1992) *J. Chem. Soc., Perkin. Trans. 2*, 523–526
29. Crisma, M., Valle, G., Toniolo, C., Prasad, S., Balaji Rao, R. & Balaram, P. (1994) *Biopolymers* **35**, 1–10
30. Karle, I.L., Balaji Rao, R., Prasad, S., Kaul, R & Balaram, P. (1994) *J. Am. Chem. Soc.* **116**, 10355–10361
31. Prasad, S., Mitra, S., Subramanian, E., Velmurugan, D., Balaji Rao, R. & Balaram, P. (1994) *Biochem. Biophys. Res. Commun.* **98**, 424–430
32. Bergstrand, H., Lundquist, B. & Michelsen, P. (1988). *Eur. J. Haematol.* **41**, 467–477
33. Bergstrand, H., Lundquist, B. & Michelsen, P. (1989) *Allergy* **44**, 6–17
34. Ye, R.D., Quehenberger, O., Thomas, K.M., Navarro, J., Cavanagh, S.L., Prossnitz, E.R. & Cochrane, C.G. (1993) *Immunology*, **150**, 1383–1394
35. Casy, A.F. (1989) *Adv. Drug Res.* **18**, 177–289

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