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Fluoro-modified Chemotactic Peptides: fMLF Analogues[‡]

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Abstract: A small library of peptide analogues of the chemotactic tripeptide For-Met-Leu-Phe-NH$_2$ modified by substitution of Leu at position 2 by three different fluorinated amino acids varying in content of fluorine, length of the fluorinated side chain, and alkylation degree at the α-carbon atom was synthesized. The influence of the fluorine substitution on the biological activity was investigated by measuring the oxidative activity of neutrophils using a luminol-dependent chemiluminescence assay. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Neutrophils are involved in the first line of defence against bacterial infections [1,2]. They are activated by the binding of chemoattractants to their surface receptors. As a consequence, the neutrophils move toward the bacteria from which the chemoattractants originate. This directed movement of cells along a chemical gradient is called chemotaxis [3].

The hydrophobic *N*-formyl-tripeptide For-Met-Leu-Phe-OH (fMLF) is one of the early recognized chemoattractants [4,5], which is produced by *Escherichia coli* amongst others. It was chosen as the model peptide for measuring chemotaxis and lysosomal enzyme release. Binding of fMLF to G protein-coupled *N*-formylpeptide receptor (FPR) triggers chemotaxis and a subsequent cascade of biochemical events: lysosomal enzyme secretion, i.e. the production of β-glucosaminidase, β-glucuronidase and bacteriocytic proteins, as well as the activation of NADPH oxidase with production of toxic oxygen metabolites, such as superoxide anion radical, hydrogen peroxide and hypochlorous acid, which is considered to be one of the primary physiological responses to bacterial invasion and tissue injury [6–8].

The cellular mechanism by which neutrophils rapidly move to sites of infection signalled by chemoattractants still remains enigmatic, but there is some evidence that the chemoattractant peptide fMLF interacts with a neutrophil receptor [9] with a specificity for hydrophobic *N*-formylated peptides thanks to a hydrophobic pocket in the receptor where the Leu side chain fits [1,10]. Structure–activity studies and conformational analysis [11] revealed that this pocket is large relative to the size of the Leu isobutyl group, since *N*-formylmethionyl-1-aminocyclohexyl-1-carbonyl-phenylalanine methylester (For-Met-Ac$_6$c-Phe-OMe) and *N*-formylmethionyl-C$^{\alpha,\alpha}$-di-*n*-butylglycyl-phenylalanine methylester (For-Met-

Dbg-Phe-OMe) are at least as active as the parent sequence [10,12,13]. Furthermore, chemical modifications provided evidence that the Met side chain fits into a hydrophobic pocket of limited depth [14]. Likewise, the fit of the third pocket to the aromatic ring side chain of the *C*-terminal Phe residue seems to be very tight [15,16]. Modification of the parent sequence fMLF has given clear evidence for the requirement of hydrophobic residues in all of the three positions [1,14,15]. Position 2 is the most versatile for constructing libraries of chemotactic *N*-formyltripeptides of the type For-Met-Xaa-Phe-OMe with high structural variability.

Recently, it was disclosed that hydrophobic interactions are far more essential in ligand–receptor interactions than assumed, while the influence of hydrogen bonding was overestimated [17,18]. The mechanism of these hydrophobic interactions, however, was never elucidated in detail [19]. Side-chain phenyl groups play an essential role in ligand–receptor interactions. When the aromatic system interacts with the side chains of Val, Leu, Ile and Ala, the aromatic π-system functions as a hydrogen bond acceptor. These interactions are denoted as CH/π. This concept has recently been established by Nishio *et al.* [20]. In the series CF_3, CHF_2, CH_2F, CH_3 only CHF_2 and CH_2F can act as a hydrogen bond donor as well as a hydrogen bond acceptor [21,22], with the CHF_2 group being the most potent hydrogen bond donor in this series [23–25]. Furthermore, fluoroalkyl groups may act as a coordinative site in metal complexes.

In this context, incorporation of fluoro-modified amino acids into peptides is of current interest, since it represents an efficient strategy to delay proteolytic degradation, to stabilize secondary structure elements and to improve lipophilicity. Fluoro-modification seems to be complementary to other existing stabilization methods [26]. Therefore, fluoro-modification might serve as a 'final push' towards higher stability after rational design and might also improve ligand–receptor interactions [27]. Herein, a method is described for the incorporation of fluoro-modified amino acids, such as

(S)- and (R)-C$^\alpha$-difluoroethyl glycine (DfeGly) [28], (S)- and (R)-C$^\alpha$-trifluoromethyl alanine, (αTfm)Ala [29,30], and (S)- and (R)-C$^\alpha$-difluoromethyl alanine, (αDfm)Ala [31] into position 2 of For-Met-Leu-Phe-NH$_2$ (Scheme 1). Fluoro-modified For-Met-Leu-Phe-NH$_2$ analogues are expected to be promising candidates for the study of the effect of fluorine on ligand–receptor interactions [32]. The tripeptide analogue containing Aib (α-aminoisobutyric acid) was included in this study in order to distinguish between electronic effects of the fluorinated group and the steric demand of an alkyl substituent.

MATERIALS AND METHODS

General

Starting materials (when not stated otherwise) were purchased from commercial suppliers and were used without purification. Solvents were dried and distilled prior to use. The fluorinated amino acids DfeGly [28], (αTfm)Ala [29,30], and (αDfm)Ala [31] were synthesized by applying literature protocols.

NMR spectra were recorded on a 600 MHz Bruker NMR spectrometer (^{1}H 600 MHz, ^{19}F 565 MHz, ^{13}C 151 MHz), 400 MHz Bruker NMR spectrometer (^{1}H 400 MHz, ^{19}F 376 MHz, ^{13}C 101 MHz) or a 300 MHz Varian Gemini NMR spectrometer (^{1}H 300 MHz, ^{19}F 282 MHz). The Bruker NMR spectrometers were equipped with inverse 5 mm TBI and 5 mm QNP probe head. ^{1}H and ^{13}C chemical shifts were referenced to tetramethylsilane (TMS, $\delta = 0$ ppm), while the ^{19}F chemical shifts were referenced to trichlorofluoromethane ($\delta = 0$ ppm) as the internal standard. For complete assignment, additional COSY, HMQC, HMBC spectra were recorded.

ESI-MS spectra were recorded on a 7 Tesla FT-ICR-MS APEX II Bruker-Daltronics instrument using methanol solution, ESI ionization and positive ion detection.

For HPLC a LaChrom (Merck Hitachi) analytical HPLC instrument was used, equipped with an interface D-7000, a diode array detector L-7450,
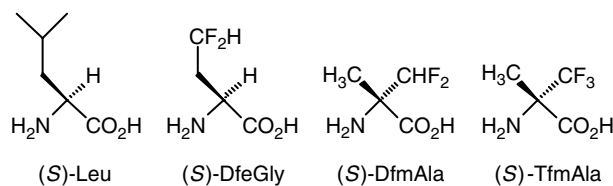
Scheme 1   Chemical structures of fluorinated amino acids used in comparison to Leu.

and a pump A-B L-7100. Eluant system parameters were (gradient method A): flow 1.000 ml/min, eluant A: 95% H_2O, 5% MeCN, 0.1% TFA; eluant B: 95% MeCN, 5% H_2O, 0.1% TFA; gradient: 0.0 min (100% A, 0.0% B), 30.0 min (0% A, 100% B), 40.0 min (100% A, 0% B); column: Vydac C4, 10 μm, 4.6×250 (Separation Products).

For flash chromatography, silica gel (32–63 μm) was used with solvent systems given in the text.

On TLC the compounds were visualized by spraying the plate with a mixture of ceric(IV) sulphate (0.2%), ammonium molybdate (5%) and H_2SO_4 (5%) in water, followed by heating.

Luminol was a product from Boehringer-Mannheim (Heidelberg, Germany). Chemicals for neutrophil isolation and purification, i.e. Hanks' balanced salt solution without phenol red, Ficoll-Hypaque, dextran, heparin and the stimulator For-Met-Leu-Phe-OH were purchased from Sigma (Deisenhofen, Germany).

Tripeptides were dissolved in dimethylsulphoxide at 10^{-2} mol/l. These stock solutions were divided into aliquots and stored at $-20\,°C$. Working solutions of tripeptides were prepared by dilution with Hanks' medium immediately before use.

Peptide Synthesis

Peptide coupling with DIC/HOAt (method A).
To the stirred solution of 1 eq of PG-Xaa-OH (PG: protecting group) in DMF (N,N-dimethylformamide), 1.2 eq HOAt (1-hydroxy-7-aza-1,2,3-benzotriazole) and 1.2 eq of DIC (N,N'-diisopropylcarbodiimide) were added at room temperature. After 5 min 1.2 eq of H-Yaa-PG and 1 eq of DIEA (diisopropyl ethyl amine) were added. The progress of the reaction was monitored by HPLC or ^{19}F-NMR spectrometry. The organic phase was evaporated *in vacuo.* The residue was partitioned between a 10% citric acid solution and ethyl acetate. The phases were separated and the organic phase was washed with 10% citric acid (2x), sat. NaCl solution (3x), 10% $NaHCO_3$ solution (3x) and sat. NaCl solution. (3x). The organic layer was dried with $MgSO_4$, filtered and concentrated under reduced pressure. The peptides were purified by flash chromatography using the solvent mixtures given in the text.

Peptide coupling via mixed anhydrides (method B).
To a stirred solution of 2.2 eq of PG-Xaa-OH in DMF, at $-30\,°C$ 2 eq of NMM (N-methyl morpholine) was added. After stirring for 15 min at $-15\,°C$, 2 eq of CAIBE (isobutyl chloroformate), and after

10 min 1 eq of H-Yaa-PG, were added. The reaction temperature was kept below $0\,°C$ for 1 h, and then the mixture was warmed up to room temperature. The progress of the reaction was monitored by HPLC or ^{19}F-NMR spectrometry. For work-up procedure and purification, see method A.

N^α-Formylation of H-Met-Xaa-Phe-NH$_2$ (method C).
To 1 eq of HCO_2^-. $^+H_2$-Xaa-PG dissolved in DMF, 1.2 eq of CMF (cyanomethyl formate) [33] and 1 eq of triethylamine were added and stirred for 2 h. After completion of the reaction (monitored by HPLC) the solvent was evaporated *in vacuo.* The formyl peptides were purified by flash chromatography using the solvent mixtures given in the text. In the case of **6a** and **6b**, where chromatography was not possible because of the formation of almost insoluble aggregates, the solid was dispersed in water and filtrated on a 45 μm filter. The salt-free, formylated peptides were washed down from the filter with methanol. After evaporating the solvent, the solid materials were lyophilized.

Deprotection of the Z-group.
To a solution of Z-Xaa-Phe-NH$_2$ in methanol, Pd/C (10%) was added. The mixture was stirred under an atmosphere of hydrogen until the reaction was complete (^{19}F-NMR or TLC analysis). After filtration, methanol was evaporated under reduced pressure. The crude peptide was used for the subsequent coupling reaction without further purification.

Deprotection of the Boc-group.
Boc-Met-Xaa-Phe-NH$_2$ was stirred in the presence of an excess of concentrated formic acid. After 30 min the formic acid was evaporated under reduced pressure and the residue was subjected to N^α-formylation without further purification.
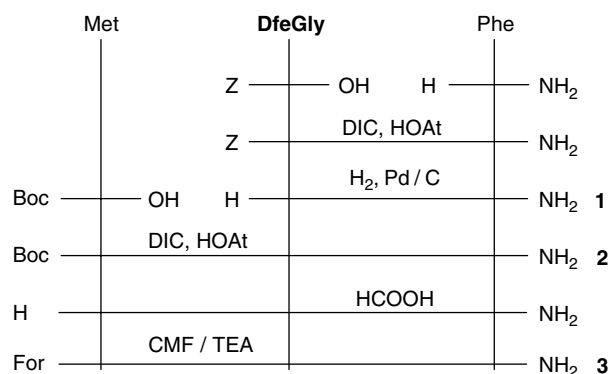
Synthesis of For-Met-DfeGly-Phe-NH$_2$ (3) (Scheme 2).
Chemotactic peptides of the type For-Met-Xaa-Phe-NH$_2$ were synthesized by applying published solution phase procedures. (S)- and (R)-DfeGly were synthesized from (S)- and (R)-Asp using 1,1,1,3,3,3-hexafluoroacetone as the protecting and activating agent [28] and were used as Z-derivatives in peptide synthesis. As carboxylic group protected Phe derivative, the corresponding amide was used, because of the high tendency of peptide amides to crystallize. Extensive studies of a large number of analogues revealed that esterification and amidation of the *C*-terminal carboxylic group does not result in a loss of biological activity [14,15]. The deprotected dipeptide amide (**1**) was coupled with

Boc-Met-OH on treatment with DIC/HOAt to give the Boc-protected tripeptide amide (**2**). The *N*-terminal amino group of **2** was deprotected with concentrated formic acid and subsequently N^α-formylated on treatment with CMF/TEA (triethylamine) [33] to give the fluoro-modified chemotactic tripeptide amide (**3**).

H-(S)-DfeGly-Phe-NH₂ (1a). The dipeptide **1a** was synthesized from Z-(S)-DfeGly-OH [22] (273 mg, 1.0 mmol) and H-Phe-NH₂ (197 mg, 1.2 mmol) applying method A. The Z-protected dipeptide was purified by flash chromatography (eluant: CHCl₃/MeOH, 10 : 1). Z-deprotection was achieved using the above mentioned general route. Yield of **1a**: 122 mg (43%).

^{1}H-NMR (d₆-DMSO, 400 MHz) δ ppm: 1.67–1.85, 1.86–2.03 (m, 2H, DfeGly $C^\beta H_2$), 1.94 (s, 2H, DfeGly NH₂), 2.82 (dd, 1H, $^2J = 13.6$ Hz, $^3J = 9.2$ Hz, Phe $C^\beta H_2$), 3.04 (dd, 1H, $^2J = 13.6$ Hz, $^3J = 4.8$ Hz, Phe $C^\beta H_2$), 3.27 (dd, 1H, $^3J = 9.2$ Hz, $^3J = 4.8$ Hz, DfeGly $C^\alpha H$), 4.47 (m, 1H, Phe $C^\alpha H$), 5.99 (tdd, 1H, $^2J = 57.0$ Hz, $^3J = 5.7$ Hz, $^3J = 4.0$ Hz, CF₂H), 7.11 (s, 1H, Phe NH₂), 7.14–7.29 (m, 5H, Ar-Phe), 7.48 (s, 1H, Phe NH₂), 8.11 (d, 1H, $^3J = 8.2$ Hz, Phe NH). ^{13}C-NMR (d₆-DMSO, 101 MHz) δ ppm: 37.72 (Phe C^β), 38.6 (overl. m, DfeGly C^β), 50.13 (t, $^3J = 5.7$ Hz, DfeGly C^α), 53.10 (Phe C^α), 116.88 (t, $^1J = 236.8$ Hz, CF₂H) 126.12–137.65 (Ar-Phe), 172.66 (C=O, DfeGly), 173.27 (C=O, Phe). ^{19}F-NMR (d₆-DMSO, 376 MHz) δ ppm: −114.4 (ddd, 1F, $^2J = 280.8$ Hz, $^2J = 57.0$ Hz, $^3J = 22.2$ Hz, $^3J = 14.4$ Hz, CF₂H), −115.3 (ddd, 1F, $^2J = 280.8$ Hz, $^2J = 56.8$ Hz, $^3J = 17.2$ Hz, $^3J = 14.2$ Hz, CF₂H).

Analytical HPLC: $R_t = 4.71$ min. Calculated MW = 285.29 for $C_{13}H_{17}F_2N_3O_2$. ESI-MS: *m/z* [M + H]⁺ 286.13606 (286.13616), [M + Na]⁺ 308.11757 (308.11810), [2M + H]⁺ 571.26562 (571.26504), [2M + Na]⁺ 593.24846 (593.24699).

Scheme 2   Synthesis of peptides **1–3**.

H-(R)-DfeGly-Phe-NH₂ (1b). For synthesis of **1b** see **1a**. Yield of **1b**: 134 mg (47%). ^{1}H-NMR (d₆-DMSO, 600 MHz) δ ppm: 1.71–1.85, 1.88–1.99 (m, 2H, DfeGly $C^\beta H_2$), 2.78 (dd, 1H, $^2J = 13.7$ Hz, $^3J = 9.5$ Hz, Phe $C^\beta H_2$), 3.06 (dd, 1H, $^2J = 13.7$ Hz, $^3J = 4.6$ Hz, Phe $C^\beta H_2$), 3.41 (m, 1H, DfeGly $C^\alpha H$), 3.79–4.30 (br s, 2H, DfeGly NH₂), 4.50 (m, 1H, Phe $C^\alpha H$), 5.87 (tdd, 1H, $^2J = 56.6$ Hz, $^3J = 5.7$ Hz, $^3J = 4.0$ Hz, CF₂H), 7.14 (s, 1H, Phe NH₂), 7.16–7.28 (m, 5H, Ar-Phe), 7.48 (s, 1H, Phe NH₂), 8.32 (d, 1H, $^3J = 7.9$ Hz, Phe NH). ^{13}C-NMR (d₆-DMSO, 151 MHz) δ ppm: 37.72 (overl., Phe C^β, DfeGly C^β), 49.32 (DfeGly C^α), 53.41 (Phe C^α), 116.32 (t, $^1J = 233.7$ Hz, CF₂H), 126.26–137.72 (Ar-Phe), 171.72 (C=O, DfeGly), 172.59 (C=O, Phe). ^{19}F-NMR (d₆-DMSO, 376 MHz) δ ppm: −114.3 (ddd, 1F, $^1J = 282.0$ Hz, $^2J = 56.6$ Hz, $^3J = 20.3$ Hz, $^3J = 14.9$ Hz, CF₂H), −115.1 (ddd, br.1F, br. $^1J = 282.0$ Hz, $^2J = 57$ Hz, CF₂H).

Analytical HPLC: $R_t = 7.31$ min. Calculated MW = 285.29 for $C_{13}H_{17}F_2N_3O_2$. ESI-MS: *m/z* [M + H]⁺ 286.13607 (286.13616), [2M + H]⁺ 571.26581 (571.26504).

Boc-Met-(S)-DfeGly-Phe-NH₂ (2a). Tripeptide **2a** was synthesized from Boc-Met-OH (249 mg, 1.0 mmol) and H-(S)-DfeGly-Phe-NH₂ (**1a**) (342 mg, 1.2 mmol) by applying method A. Purification by column chromatography (eluant: CHCl₃/MeOH, 15 : 1). Yield of **2a**: 305 mg (59%).

^{1}H-NMR (d₆-DMSO, 400 MHz) δ ppm: 1.37 (s, 9H, C(CH₃)₃), 1.67–1.88 (m, 2H, Met $C^\beta H$), 2.02 (s, 3H, Met CH₃), 2.03–2.24 (m, 2H, DfeGly $C^\beta H_2$), 2.35–2.47 (m, 2H, Met $C^\gamma H_2$), 2.81 (dd, 1H, $^2J = 13.8$ Hz, $^3J = 9.1$ Hz, Phe $C^\beta H_2$), 3.00 (dd, 1H, $^2J = 13.8$ Hz, $^3J = 4.8$ Hz, Phe $C^\beta H_2$), 3.90–4.00 (m, 1H, DfeGly $C^\alpha H$), 4.35–4.47 (m, 2H, Met $C^\alpha H$, Phe $C^\alpha H$), 5.94 (tdd, 1H, $^2J = 56.5$ Hz, $^3J = 5.9$ Hz, $^3J = 3.8$ Hz, CF₂H), 7.08 (overl., 2H, Met NH, Phe NH₂), 7.14–7.28 (m, 5H, Ar-Phe), 7.43 (s, 1H, Phe NH₂), 8.10 (d, 1H, $^3J = 7.7$ Hz, Phe NH), 8.17 (d, 1H, $^3J = 7.7$ Hz, DfeGly NH). ^{13}C-NMR (d₆-DMSO, 101 MHz) δ ppm: 14.52 (Met CH₃), 28.04 (C(CH₃)₃), 29.59 (Met C^γ), 31.03 (Met C^β), 36.32 (DfeGly C^β), 37.33 (Phe C^β), 47.55 (DfeGly C^α), 53.24 (Met C^α), 53.81 (Phe C^α), 115.90 (t, $^1J = 236.9$ Hz, CF₂H), 126.16–137.63 (Ar-Phe). ^{19}F-NMR (d₆-DMSO, 376 MHz) δ ppm: −114.4 (ddd, 1F, $^1J = 282.3$ Hz, $^2J = 56.5$ Hz, $^3J = 21.9$ Hz, $^3J = 14.2$ Hz, CF₂H), −115.3 (br ddd, 1F, $^1J = 282.3$ Hz, $^2J = 56$ Hz, CF₂H).

Analytical HPLC: $R_t = 16.93$ min. TLC (CHCl₃/MeOH, 15 : 1): $R_f = 0.11$. Calculated MW = 516.61 for $C_{23}H_{34}F_2N_4O_5S$. ESI-MS: *m/z* [M + H]⁺ 517.22901 (517.22907), [M + Na]⁺ 539.21076

(539.21102), [M + K]$^+$ 555.18588 (555.18496), [2M + Na]$^+$ 1055.43128 (1055.43282), [2M + K]$^+$ 1071.41125 (1071.40676), [3M + Na]$^+$ 1571.64627 (1571.65462).

Boc-Met-(R)-DfeGly-Phe-NH$_2$ (2b). Tripeptide **2b** was synthesized from Boc-Met-OH (249 mg, 1.0 mmol) and H-(R)-DfeGly-Phe-NH$_2$ (342 mg, 1.2 mmol) using method A. Purification by column chromatography (eluant: CHCl$_3$/MeOH, 15:1). Yield of **2b**: 237 mg (46%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.37 (s, 9H, C(CH$_3$)$_3$), 1.70–1.87 (m, 2H, Met C$^\beta$H$_2$), 1.87–2.04 (m, 2H, DfeGly C$^\beta$H$_2$), 2.02 (s, 3H, Met CH$_3$), 2.39–2.48 (m, 2H, Met C$^\gamma$H$_2$), 2.74 (dd, 1H, ^2J = 13.4 Hz, ^3J = 10.5 Hz, Phe C$^\beta$H$_2$), 3.08 (1H, dd, ^2J = 13.4 Hz, ^3J = 14.2 Hz, Phe C$^\beta$H$_2$), 3.96 (1H, m, Met C$^\alpha$H), 4.39 (1H, m, DfeGly C$^\alpha$H), 4.45 (m, 1H, Phe C$^\alpha$H), 5.69 (br. tdd, 1H, ^2J = 56 Hz, CF$_2$H), 7.07 (d, 1H, ^3J = 7.2 Hz, Met NH), 7.15 (s, 1H, Phe NH$_2$), 7.16–7.26 (m, 5H, Ar-Phe), 7.40 (s, 1H, Phe NH$_2$), 8.11 (d, 1H, ^3J = 8.1 Hz, DfeGly NH), 8.16 (d, 1H, ^3J = 8.8 Hz, Phe NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 14.10 (Met CH$_3$), 27.70 (C(CH$_3$)$_3$), 29.18 (Met C$^\gamma$), 30.70 (Met C$^\beta$), 35.42 (t, ^2J = 20.4 Hz, DfeGly C$^\beta$), 37.13 (Phe C$^\beta$), 47.17 (DfeGly C$^\alpha$), 53.41 (overl. br. s, Met C$^\alpha$, Phe C$^\alpha$), 77.93 (C(CH$_3$)$_3$), 115.53 (t, ^1J = 233.4 Hz, CF$_2$H), 125.80–137.46 (Ar-Phe), 155.08 (C=O, Boc), 169.04 (C=O, DfeGly), 171.33 (C=O, Met), 172.16 (C=O, Phe). ^{19}F-NMR(d$_6$-DMSO, 376 MHz) δ ppm: −113.9 (ddd, 1F, ^2J = 282.4 Hz, ^2J = 56.5 Hz, ^3J = 21.0 Hz, ^3J = 14.8 Hz, CF$_2$H), −115.0 (br ddd, 1F, ^2J = 282.4 Hz, ^2J = 56 Hz, CF$_2$H).

Analytical HPLC: R_t = 17.05 min. TLC (CHCl$_3$/MeOH, 15:1): R_f = 0.17. Calculated MW = 516.61 for C$_{23}$H$_{34}$F$_2$N$_4$O$_5$S. ESI-MS: m/z [M + Na]$^+$ 539.21109 (539.21102), [2M + Na]$^+$ 1055.43142 (1055.43282), [2M + K]$^+$ 1071.40762 (1071.40676), [3M + Na]$^+$ 1571.65698 (1571.65462), [4M + Na]$^+$ 2087.87048 (2087.87641).

For-Met-(S)-DfeGly-Phe-NH$_2$ (3a). Tripeptide **2a** (516 mg, 1.0 mmol) was deprotected with concentrated formic acid as described above and then N$^\alpha$-formylated by method C to give **3a**. Yield of **3a**: 204 mg (46%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.72–1.80, 1.83–1.91 (m, 2H, Met C$^\beta$H$_2$), 2.02 (s, 3H, Met CH$_3$), 2.03–2.24 (s, 2H, DfeGly C$^\beta$H$_2$), 2.36–2.47 (m, 2H, Met C$^\gamma$H$_2$), 2.82 (dd, 1H, ^2J = 13.9 Hz, ^3J = 9.2 Hz, Phe C$^\beta$H$_2$), 3.01 (dd, 1H, ^2J = 13.9 Hz, ^3J = 4.7 Hz, Phe C$^\beta$H$_2$), 4.33–4.45 (overl. m, 3H,

Phe C$^\alpha$H, DfeGly C$^\alpha$H, Met C$^\alpha$H), 5.96 (tdd, 1H, ^2J = 56.2 Hz, ^3J = 5.7 Hz, ^3J = 3.8 Hz, CF$_2$H), 7.10 (s, 1H, Phe NH$_2$), 7.16–7.28 (m. 5H, Ar-Phe), 7.40 (s, 1H, Phe NH$_2$), 8.01 (d, 1H, ^3J = 8.2 Hz, Phe NH), 8.03 (s, 1H, H-For), 8.33 (d, 1H, ^3J = 8.2 Hz, DfeGly NH), 8.35 (d, 1H, ^3J = 8.2 Hz, Met NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 14.16 (Met C$^\varepsilon$), 28.92 (Met C$^\gamma$), 31.15 (Met C$^\beta$), 35.41 (t, ^2J = 22.2 Hz, DfeGly C$^\beta$), 36.96 (Phe C$^\beta$), 47.40 (DfeGly C$^\alpha$), 50.17 (Met C$^\alpha$), 53.45 (Phe C$^\alpha$), 115.72 (t, ^1J = 238.6 Hz, CF$_2$H), 125.81–137.28 (Ar-Phe), 160.80 (C=O, For), 169.16 (C=O, DfeGly), 170.34 (C=O, Met), 172.06 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: −114.4 (ddd, 1F, ^2J = 282.4 Hz, ^2J = 56.6 Hz, ^3J = 22.2 Hz, ^3J = 14.4 Hz, CF$_2$H), −115.4 (ddd, 1F, ^2J = 282.4 Hz, ^2J = 55.8 Hz, ^3J = 16.4 Hz, ^3J = 13.3 Hz, CF$_2$H).

Analytical HPLC: R_t = 11.75 min. TLC (CHCl$_3$/MeOH/acetone, 5:1:1): R_f = 0.37. Calculated MW = 444.50 for C$_{19}$H$_{26}$F$_2$N$_4$O$_4$S. ESI-MS: m/z [M + Na]$^+$ 467.15414 (467.15350), [2M + Na]$^+$ 911.32037 (911.31724).

For-Met-(R)-DfeGly-Phe-NH$_2$ (3b). Tripeptide **2b** (516 mg, 1.0 mmol) was deprotected with concentrated formic acid as described above and then N$^\alpha$-formylated by applying method C to give **3b**. Yield of **3b**: 222 mg (50%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.72–2.06 (overl. m, 4H, DfeGly C$^\beta$H$_2$, Met C$^\beta$H$_2$), 2.02 (s, 3H, Met CH$_3$), 2.56–2.40 (m, 2H, Met C$^\gamma$H$_2$), 2.76 (dd, 1H, ^2J = 13.8 Hz, ^3J = 10.6 Hz, Phe C$^\beta$CH$_2$), 3.07 (dd, 1H, ^2J = 13.8 Hz, ^3J = 4.2 Hz, Phe C$^\beta$H$_2$), 4.33–4.48 (m, 3H, overl.-Phe C$^\alpha$H, DfeGly C$^\alpha$H, Met C$^\alpha$H), 5.73 (br tdd, 1H, ^2J = 56 Hz, ^3J = 5 Hz, CF$_2$H), 7.14 (s, 1H, Phe NH$_2$), 7.15–7.27 (m, 5H, Ar-Phe), 7.40 (s, 1H, Phe NH$_2$), 8.03 (s, 1H, H-For), 8.21 (d, 1H, ^3J = 8.7 Hz, Phe NH), 8.40 (overl. d, 2H, DfeGly NH, Met NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 14.10 (Met CH$_3$), 28.88 (Met C$^\gamma$) 31.03 (Met C$^\beta$), 35.25 (t, ^2J = 20.4 Hz, DfeGly C$^\beta$), 37.06 (Phe C$^\beta$), 47.20 (br. s, DfeGly C$^\alpha$), 50.42 (Met C$^\alpha$), 53.50 (Phe C$^\alpha$), 115.61 (t, ^1J = 236.7 Hz, CF$_2$H), 125.79–137.54 (Ar-Phe), 160.94 (C=O, For), 169.06 (C=O, DfeGly), 170.31 (C=O, Met), 172.25 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: −114.0 (ddd, 1F, ^2J = 282.4 Hz, ^2J = 56.3 Hz, ^3J = 20.8 Hz, ^3J = 14.5 Hz, CF$_2$H), −115.2 (ddd, 1F, ^2J = 282.4 Hz, ^2J = 56.2 Hz, ^3J = 18.2 Hz, ^3J = 13.4 Hz, CF$_2$H).

Analytical HPLC: R_t = 13.00 min. TLC (CHCl$_3$/MeOH/acetone, 5:1:1): R_f = 0.37. Calculated MW = 444.50 for C$_{19}$H$_{26}$F$_2$N$_4$O$_4$S. ESI-MS: m/z [M +

Na]$^+$ 467.15368 (467.15350), [2M + Na]$^+$ 911.31950 (911.31779), [3M + Na]$^+$ 1355.48134 (1355.48207).

Synthesis of For-Met-(αTfm)Ala-Phe-NH$_2$ (6)

The synthesis of **6** was carried out according to Scheme 3.

Z-(R)-(αTfm)Ala-Phe-NH$_2$ (4a). Dipeptide **4a** was synthesized from Z-(S,R)-(αTfm)Ala-OH (1.46 g, 5.0 mmol) and H-Phe-NH$_2$ (984 mg, 6.0 mmol) using method A. Purification of the diastereomeric mixture was achieved by column chromatography (eluant: CHCl$_3$/MeOH, 10 : 1). Diastereomers were separated by reverse-phase flash chromatography (silica gel 60, silanized, 0.063–0.200 mm; eluant: H$_2$O/MeOH, 1 : 1). Yield of **4a**: 655 mg (30%), first fraction. Determination of the absolute configuration of (αTfm)Ala in this dipeptide was performed by x-ray diffraction analysis.

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.44 (s, 3H, TfmAla CH$_3$), 2.99 (dd, 1H, ^2J = 13.9 Hz, ^3J = 9.6 Hz, C$^\beta$H$_2$), 3.10 (dd, 1H, ^2J = 13.9 Hz, ^3J = 4.0 Hz, Phe C$^\beta$H$_2$), 4.31 (m, 1H, Phe C$^\alpha$H), 5.00 (b. s, 2H, CH$_2$-Z), 7.12–7.25 (m, 5H, Ar-Phe), 7.15 (s, 1H, Phe NH$_2$), 7.26 (s, 1H, Phe NH$_2$), 7.30–7.40 (m, 5H, Ar-Z), 8.06 (d, 1H, ^3J = 7.4 Hz, Phe NH), 8.30 (s, 1H, TfmAla NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 18.33 (TfmAla CH$_3$), 35.60 (Phe C$^\beta$), 54.19 (Phe C$^\alpha$), 65.61 (CH$_2$-Z), 125.67–137.64 (Ar-Z, Ar-Phe), 154.30 (C=O, Z), 165.32 (C=O, TfmAla), 171.91 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: −73.0 (s, 3F, CF$_3$).

Analytical HPLC: R_t = 16.97 min. TLC (CHCl$_3$/MeOH, 10 : 1): R_f = 0.49. Calculated MW = 437.42 for C$_{21}$H$_{22}$F$_3$N$_3$O$_4$.. ESI-MS: m/z [M + H]$^+$ 438.16382 (438.16352), [M + Na]$^+$ 460.14559 (460.14546), [M + K]$^+$ 476.11942 (476.11940), [2M + H]$^+$ 875.32116 (875.31976), [2M + Na]$^+$ 897.30292

(897.30170), [2M + K]$^+$ 913.27810 (913.27564), [3M + Na]$^+$ 1334.46015 (1334.45794).

X-ray crystallographic data: Single crystals were grown from chloroform–hexane. Orthorhombic, space group P2$_1$2$_1$2$_1$, T = 223(2) K; a = 8.5815(5) Å, b = 11.0121(7) Å, c = 22.1372(14) Å; V = 2092.0(2) Å^3; Z = 4; D_c = 1.389 M.gm^{-3}; CCD-diffractometer (Bruker AXS), 10928 data collected, 4085 independent reflections (R_{int} = 0.0197), structure solution by direct methods, anisotropic refinement [34] for all non-hydrogen atoms, hydrogen atoms refined isotropically, $R1$ = 0.0330, $wR2$ = 0.0777 [I > 2σ(I)]; and $R1$ = 0.0441, $wR2$ = 0.0818 for all data [35].

Z-(S)-(αTfm)Ala-Phe-NH$_2$ (4b). Diastereomers were separated by reverse-phase flash chromatography (**4b** was eluted as the second fraction). Yield of **4b**: 590 mg (27%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.14 (s, 3H, TfmAla CH$_3$), 2.82 (dd, 1H, ^2J = 14.0 Hz, ^3J = 11.4 Hz, Phe C$^\beta$H$_2$), 3.27 (br. dd, 1H, Phe C$^\beta$H$_2$), 4.48 (m, 1H, Phe C$^\alpha$H), 5.01, 5.08 (dd, 2H, ^2J = 12.3 Hz, CH$_2$-Z), 7.14–7.27 (m, 5H, Ar-Phe), 7.32–7.42 (m, 5H, Ar-Z), 8.30 (d, 1H, ^3J = 7.8 Hz, Phe NH), 8.36 (s, 1H, TfmAla NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 19.17 (TfmAla CH$_3$), 35.66 (Phe C$^\beta$), 53.11 (Phe C$^\alpha$), 65.80 (CH$_2$-Z), 125.68–138.01 (Ar-Z, Ar-Phe), 154.66 (C=O, Z), 165.97 (C=O, TfmAla), 172.29 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm. −72.9 (s, 3F, CF$_3$).

Analytical HPLC: R_t = 17.11 min. TLC (CHCl$_3$/MeOH, 10 : 1): R_f = 0.42. Calculated MW = 437.42 for C$_{21}$H$_{22}$F$_3$N$_3$O$_4$. ESI-MS: m/z [M + H]$^+$ 438.16410 (438.16352), [M + Na]$^+$ 460.14546 (460.14546), [M + K]$^+$ 476.11952 (476.11940), [2M + H]$^+$ 875.32045 (875.31976), [2M + Na]$^+$ 897.30302 (897.30170), [2M + K]$^+$ 913.27689 (913.27564), [3M + Na]$^+$ 1334.46127 (1334.45794).

Boc-Met-(S)-(αTfm)Ala-Phe-NH$_2$ (5a). Tripeptide **5a** was obtained from Boc-Met-OH (548 mg, 2.2 mmol) and H-(S)-(αTfm)Ala-(S)-Phe-NH$_2$ (303 mg, 1.0 mmol) (method B). Purification by column chromatography (eluant: CHCl$_3$/MeOH, 15 : 1). Yield of **5a**: 342 mg (64%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.32 (s, 3H, TfmAla CH$_3$), 1.37 (s, 9H, C(CH$_3$)$_3$), 1.73–1.82 (m, 1H, Met C$^\beta$H$_2$), 1.85–1.94 (m, 1H, Met C$^\beta$H$_2$), 2.04 (s, 3H, Met CH$_3$), 2.39–2.53 (m, 2H, Met C$^\gamma$H$_2$), 2.82 (dd, 1H, ^2J = 14.1 Hz, ^3J = 10.9 Hz, Phe C$^\beta$H$_2$), 3.26 (dd, 1H, ^2J = 14.1 Hz, ^3J = 3.7 Hz, Phe C$^\beta$H$_2$), 4.04 (m, 1H, Met C$^\alpha$H), 4.44 (m, 1H, Phe C$^\alpha$H),

Scheme 3   Synthesis of peptides **4**–**6** and **8**–**10**.

7.09 (s, 1H, Phe NH$_2$), 7.14–7.27 (m, 5H, Ar-Phe), 7.20 (s, 1H, Phe NH$_2$), 7.78 (d, 1H, ^3J = 8.3 Hz, Phe NH), 8.66 (s, 1H, TfmAla NH). ^{13}C-NMR(d$_6$-DMSO, 151 MHz) δ ppm: 14.30 (Met CH$_3$), 18.43 (TfmAla CH$_3$), 27.66 (C(CH$_3$)$_3$), 29.19 (Met C$^\gamma$) 29.98 (Met C$^\beta$), 35.77 (Phe C$^\beta$), 53.19 (Phe C$^\alpha$), 53.48 (Met C$^\alpha$), 60.59 (q, ^2J = 30 Hz, TfmAla C$^\alpha$), 78.08 (C(CH$_3$)$_3$), 124.09 (q, ^1J = 287 Hz, TfmAla CF$_3$), 125.72–137.73 (Ar-Phe), 155.31 (C=O, Boc), 165.38 (C=O, TfmAla), 171.96 (C=O, Phe), 172.25 (C=O, Met). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: −72.8 (s, 3F, CF$_3$).

Analytical HPLC: R_t = 17.98 min. TLC (CHCl$_3$/MeOH, 15 : 1): R_f = 0.30. Calculated MW = 534.60 for C$_{23}$H$_{33}$F$_3$N$_4$O$_5$S. ESI-MS: m/z [M + Na]$^+$ 557.20154 (557.20160), [2M + H]$^+$ 1069.43430 (1069.43203), [2M + Na]$^+$ 1091.41467 (1091.41397), [2M + K]$^+$ 1107.39077 (1107.38791), [3M + Na]$^+$ 1625.62529 (1625.62635), [3M + K]$^+$ 1641.60696 (1641.60029), [4M + Na]$^+$ 2159.84978 (2159.83873).

Boc-Met-(R)-(αTfm)Ala-Phe-NH$_2$ (5b).

Tripeptide **5b** was obtained from Boc-Met-OH (548 mg, 2.2 mmol) and H-(R)-(αTfm)Ala-Phe-NH$_2$ (303 mg, 1.0 mmol) (method B). Purification by column chromatography (eluant: CHCl$_3$/MeOH, 15 : 1). Yield of **5b**: 208 mg (39%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.36 (s, 9H, C(CH$_3$)$_3$), 1.45 (s, 3H, TfmAla, CH$_3$), 1.71–1.88 (m, 2H, Met C$^\beta$H$_2$), 2.03 (s, 3H, Met CH$_3$), 2.37–2.48 (m, 2H, Met C$^\gamma$H$_2$), 2.95 (dd, 1H, ^2J = 14.1 Hz, ^3J = 9.2 Hz, Phe C$^\beta$H$_2$), 3.13 (dd, 1H, ^2J = 14.1 Hz, ^3J = 4.7 Hz, Phe C$^\beta$H$_2$), 4.06 (m, 1H, Met C$^\alpha$H), 4.30 (m, 1H, Phe C$^\alpha$H), 7.12 (d, 1H, ^3J = 7.0 Hz, Met NH), 7.13–7.26 (m, 5H, Ar-Phe), 7.70 (d, 1H, ^3J = 7.7 Hz, Phe NH), 8.67 (s, 1H, TfmAla NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 14.19 (Met CH$_3$), 17.54 (TfmAla, CH$_3$), 27.67 (C(CH$_3$)$_3$), 29.09 (Met C$^\gamma$) 30.24 (Met C$^\beta$), 35.71 (Phe C$^\beta$), 53.21 (Met C$^\alpha$), 54.13 (Phe C$^\alpha$), 61.08 (q, ^2J = 29 Hz, TfmAla C$^\alpha$), 78.05 (C(CH$_3$)$_3$), 124.06 (q, ^1J = 286 Hz, TfmAla CF$_3$), 125.72–137.52 (Ar-Phe), 155.27 (C=O, Boc), 164.69 (C=O,-TfmAla), 171.63 (C=O, Met), 172.11 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: −73.1 (s, 3F, CF$_3$).

Analytical HPLC: R_t = 17.94 min. TLC (CHCl$_3$/MeOH, 15 : 1): R_f = 0.30. Calculated MW = 534.60 for C$_{23}$H$_{33}$F$_3$N$_4$O$_5$S. ESI-MS: m/z [M + H]$^+$ 535.21968 (535.21965), [M + Na]$^+$ 557.20191 (557.20160), [M + K]$^+$ 573.17578 (573.17553), [2M + Na]$^+$ 1091.41677 (1091.41397), [2M + K]$^+$ 1107.39187 (1107.38791), [3M + Na]$^+$ 1625.63540 (1625.62635), [4M + Na]$^+$ 2159.83542 (2159.83873).

For-Met-(S)-(αTfm)Ala-Phe-NH$_2$ (6a).

Tripeptide **5a** (267 mg, 0.5 mmol) was deprotected by the above mentioned general route and then N^α-formylated by applying method C to give **6a**. Purification by column chromatography (eluant: CHCl$_3$/MeOH, 15 : 1). Yield of **6a**: 155 mg (67%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.29 (s, 3H, TfmAla CH$_3$), 1.73–1.82, 1.95–2.03, (m, 2H, Met C$^\beta$H$_2$), 2.05 (s, 3H, Met CH$_3$), 2.38–2.45 (m, 2H, Met C$^\gamma$H$_2$), 2.82 (dd, 1H, ^2J = 14.2 Hz, ^3J = 10.7 Hz, Phe C$^\beta$H$_2$), 3.25 (dd, 1H, ^2J = 14.2 Hz, ^3J = 4.1 Hz, Phe C$^\beta$H$_2$), 4.41–4.49 (overl. m, 2H, Met C$^\alpha$H, Phe C$^\alpha$H), 7.07 (s, 1H, Phe NH$_2$), 7.15–7.29 (m, 5H, Ar-Phe), 7.81 (d, 1H, ^3J = 8.4 Hz, Phe NH), 8.01 (s, 1H, For H), 8.45 (d, 1H, ^3J = 7.3 Hz, Met NH), 8.86 (s, 1H, TfmAla NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 14.33 (Met CH$_3$), 18.50 (TfmAla CH$_3$), 28.99 (Met C$^\gamma$) 30.58 (Met C$^\beta$), 35.82 (Phe C$^\beta$), 50.57 (Met C$^\alpha$), 53.19 (Phe C$^\alpha$), 60.62 (q, ^2J = 29 Hz, TfmAla C$^\alpha$), 124.12 (q, ^1J = 286 Hz, TfmAla CF$_3$), 125.73–137.77 (Ar-Phe), 161.01 (C=O, For), 165.31 (C=O, TfmAla), 171.22 (C=O, Met), 172.00 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ-ppm: 72.7 (s, 3F, CF$_3$).

Analytical HPLC: R_t = 13.51 min. TLC (CHCl$_3$/MeOH, 15 : 1): R_f = 0.15. Calculated MW = 462.49 for C$_{19}$H$_{25}$F$_3$N$_4$O$_4$S. ESI-MS: m/z [M + H]$^+$ 463.16247 (463.16214), [M + Na]$^+$ 485.14425 (485.14408), [M + K]$^+$ 501.11852 (501.11802), [2M + Na]$^+$ 947.30160 (947.29894), [2M + K]$^+$ 963.27412 (963.27288), [3M + Na]$^+$ 1409.45676 (1409.45381), [4M + Na]$^+$ 1871.62426 (1871.60867).

For-Met-(R)-(αTfm)Ala-Phe-NH$_2$ (6b).

Tripeptide **5b** (267 mg, 0.5 mmol) was deprotected as described above and then N^α-formylated by method C to give **6b**. Purification by column chromatography (eluant: CHCl$_3$/MeOH, 15 : 1). Yield of **6b**: 169 mg (73%).

^{1}H-NMR (d$_6$-DMSO, 600 MHz) δ ppm: 1.46 (s, 3H, TfmAla CH$_3$), 1.72–1.81, 1.87–1.96 (m, 2H, Met C$^\beta$H$_2$), 2.03 (s, 3H, Met CH$_3$), 2.37–2.48 (m, 2H, Met C$^\gamma$H$_2$), 2.96 (dd, 1H, ^2J = 14.1 Hz, ^3J = 9.0 Hz, Phe C$^\beta$H$_2$), 3.11 (dd, 1H, ^2J = 14.1 Hz, ^3J = 4.9 Hz, Phe C$^\beta$H$_2$), 4.30 (m, 1H, Phe C$^\alpha$H), 4.49 (m, 1H, Met C$^\alpha$H), 7.13–7.27 (m, 5H, Ar-Phe), 7.72 (d, 1H, ^3J = 7.6 Hz, Phe NH), 8.03 (s, 1H, For H), 8.38 (d, 1H, ^3J = 7.8 Hz, Met NH), 8.89 (s, 1H, TfmAlaNH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ ppm: 14.21 (Met CH$_3$), 17.77 (TfmAla CH$_3$), 28.80 (Met C$^\gamma$) 30.99 (Met C$^\beta$), 35.73 (Phe C$^\beta$), 50.30 (Met C$^\alpha$), 54.06 (Phe C$^\alpha$), 61.13 (q, ^2J = 30 Hz, TfmAla C$^\alpha$), 124.06 (q, ^1J = 286 Hz, TfmAla CF$_3$), 125.72–137.47 (Ar-Phe), 160.89 (C=O, For), 164.63 (C=O, TfmAla),

171.05 (C=O, Met), 171.67 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: -72.9 (s, 3F, CF$_3$).

Analytical HPLC: $R_t = 13.49$ min. TLC (CHCl$_3$/ MeOH, 15 : 1): $R_f = 0.17$. Calculated MW = 462.49 for C$_{19}$H$_{25}$F$_3$N$_4$O$_4$S. ESI-MS: m/z [M + H]$^+$ 463.16268 (463.16214), [M + Na]$^+$ 485.14433 (485.14408), [M + K]$^+$ 501.11869 (501.11802), [2M + Na]$^+$ 947.30112 (947.29894), [2M + K]$^+$ 963.27793 (963.27288), [3M + Na]$^+$ 1409.45730 (1409.45381).

For-Met-Aib-Phe-NH$_2$ (7)

The synthesis of **7** was carried out according to Scheme 3.

^{1}H-NMR (d$_6$-DMSO, 400 MHz) δ ppm: 1.17 (s, 3H, Aib CH$_3$), 1.21 (s, 3H, Aib CH$_3$), 1.69–1.81, 1.85–1.97 (m, 2H, Met C$^\beta$H$_2$), 2.04 (s, 3H, Met CH$_3$), 2.36–2.49 (m, 2H, Met C$^\gamma$H$_2$), 2.89 (dd, 1H, $^2J = 14.0$ Hz, $^3J = 10.3$ Hz, Phe C$^\beta$H$_2$), 3.16 (dd, 1H, $^2J = 14.0$ Hz, $^3J = 4.3$ Hz, Phe C$^\beta$H$_2$), 4.25–4.35 (m,2H, Phe C$^\alpha$H, Met C$^\alpha$H), 7.06–7.28 (m, 7H, Ar-Phe, Phe NH$_2$), 7.41 (d, 1H, $^3J = 8.4$ Hz, Phe NH), 8.04 (s, 1H, For H), 8.23 (s, 1H, Aib NH), 8.35 (d, 1H, $^3J = 7.6$ Hz, Met NH). ^{13}C-NMR (d$_6$-DMSO, 101 MHz) δ 14.59 (Met CH$_3$), 24.27 (Aib CH$_3$), 25.05 (Aib CH$_3$), 29.33 (Met C$^\gamma$), 31.23 (Met C$^\beta$), 36.17 (Phe C$^\beta$), 51.15 (Met C$^\alpha$), 53.87 (Phe C$^\alpha$), 55.90 (Aib C$^\alpha$), 126.01–138.33 (Ar-Phe), 161.39 (C=O, For), 171.03 (C=O, Met), 172.87 (C=O, Phe), 173.15 (C=O, Aib).

Analytical HPLC: $R_t = 11.25$ min. Calculated MW = 408.52 for C$_{19}$H$_{28}$N$_4$O$_4$S. ESI-MS: m/z [M + Na]$^+$ 431.17296 (431.17235), [M + K]$^+$ 447.14712 (447.14629), [2M + Na]$^+$ 839.35793 (839.35492),

Synthesis of For-Met-(αDfm)Ala-Phe-NH$_2$ (10)

The synthesis of **10** was carried out according to Scheme 3.

Z-(αDfm)Ala-Phe-NH$_2$ (Diastereomer I) (8a).
Dipeptide **8** was synthesized from Z-(R,S)-(αDfm)Ala-OH (273 mg, 1.0 mmol) and H-Phe-NH$_2$ (197 mg, 1.2 mmol). Yield of **8a**: 134 mg (32%), first fraction. Separation of the diastereomers by MPLC (medium-pressure liquid chromatography).

^{1}H-NMR (d$_6$-DMSO, 400 MHz) δ ppm: 1.27 (br s, 3H, DfmAla CH$_3$), 2.95 (dd, 1H, $^2J = 13.9$ Hz, $^3J = 8.7$ Hz, Phe C$^\beta$H$_2$), 3.04 (dd, 1H, $^2J = 13.9$ Hz, $^3J = 4.9$ Hz, Phe C$^\beta$H$_2$), 4.34 (ddd, 1H, $^3J = 8.7$ Hz, $^3J = 7.9$ Hz, $^3J = 4.9$ Hz, Phe C$^\alpha$H), 4.93–5.07 (br. m, 2H, CH$_2$-Z), 6.23 (br. t, 1H, $^2J = 57$ Hz, CF$_2$H), 7.10–7.40 (overl. m, 12H, Ar-Z, Ar-Phe, NH$_2$-Phe), 7.81 (d, 1H, $^3J = 7.9$ Hz, Phe NH), 7.96 (br. s,

DfmAla NH). ^{13}C-NMR (d$_6$-DMSO, 101 MHz) δ ppm: 16.33 (DfmAla CH$_3$), 36.56 (Phe C$^\beta$), 54.11 (Phe C$^\alpha$), 60.21 (t, $^2J = 20$ Hz, DfmAla C$^\alpha$), 113.84 (t, $^1J = 247$ Hz, CF$_2$H), 126.18–137.92 (Ar-Phe), 155.25 (C=O, Z), 168.94 (C=O, DfmAla), 172.36 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: -126.7 (dd, 1F, $^2J = 276.4$ Hz, $^2J = 56.8$ Hz, CF$_2$H), -130.4 (dd, 1F, $^2J = 276.4$ Hz, $^2J = 56.8$ Hz, CF$_2$H).

Analytical HPLC: $R_t = 15.53$ min. Calculated MW = 419.43 for C$_{21}$H$_{23}$F$_2$N$_3$O$_4$. ESI-MS: m/z [M + H]$^+$ 420.17377 (420.17294), [M + Na]$^+$ 442.15539 (442.15488), [M + K]$^+$ 458.13036 (458.12882), [2M + Na]$^+$ 861.32199 (861.32000).

Z-(αDfm)Ala-Phe-NH$_2$ (Diastereomer II) (8b).
Dipeptide **8b** was synthesized from Z-(R,S)-DfmAla-OH (273 mg, 1.0 mmol) and H-Phe-NH$_2$ (197 mg, 1.2 mmol). Yield of **8b**: 125 mg (30%), second fraction. Separation of the diastereomers by MPLC.

^{1}H-NMR(d$_6$-DMSO, 600 MHz) δ ppm: 1.04 (br s, 3H, DfmAla CH$_3$), 2.85 (dd, 1H, $^2J = 13.9$ Hz, $^3J = 10.7$ Hz, Phe C$^\beta$H$_2$), 3.21 (dd, 1H, $^2J = 13.9$ Hz, $^3J = 4.0$ Hz, Phe C$^\beta$H$_2$), 4.47 (ddd, 1H, $^3J = 10.7$ Hz, $^3J = 8.6$ Hz, $^3J = 4.0$ Hz, Phe C$^\alpha$H), 5.02 (br. m, 2H, CH$_2$-Z), 6.19 (br t, 1H, $^2J = 56$ Hz, CF$_2$H), 7.12–7.42 (12H, overl. m, Ar-Z, Ar-Phe, NH$_2$-Phe), 8.07 (br. s, 1H, DfmAla NH), 8.13 (d, 1H, d, $^3J = 8.6$ Hz, Phe NH). ^{13}C-NMR (d$_6$-DMSO, 151 MHz) δ 16.91 (DfmAla CH$_3$), 36.28 (Phe C$^\beta$), 53.24 (Phe C$^\alpha$), 59.75 (t, $^2J = 21$ Hz, DfmAla C$^\alpha$), 113.92 (t, $^1J = 246$ Hz, CF$_2$H), 126.14–138.33 (Ar-Phe), 155.64 (C=O, Z), 169.38 (C=O, DfmAla), 172.66 (C=O, Phe). ^{19}F-NMR (d$_6$-DMSO, 376 MHz) δ ppm: -128.1 (dd, 1F, $^2J = 275.1$ Hz, $^2J = 55.6$ Hz, CF$_2$H), -130.2 (dd, 1F, $^2J = 276.4$ Hz, $^2J = 56.8$ Hz, CF$_2$H).

Analytical HPLC: $R_t = 15.79$ min. Calculated MW = 419.43 for C$_{21}$H$_{23}$F$_2$N$_3$O$_4$. ESI-MS: m/z [M + H]$^+$ 420.17335 (420.17294), [M + Na]$^+$ 442.15535 (442.15488), [M + K]$^+$ 458.12966 (458.12882), [2M + H]$^+$ 839.34149 (839.33805), [2M + Na]$^+$ 861.32169 (861.32000).

Boc-Met-(αDfm)Ala-Phe-NH$_2$ (diastereomer I) (9a).
9a was synthesized from Boc-Met-OH (548 mg, 2.2 mmol) and H-(αDfm)Ala-Phe-NH$_2$ (diastereomer I) (285 mg, 1.0 mmol) (method B). Purification by column chromatography (eluant: CHCl$_3$/MeOH, 20 : 1). Yield of **9a**: 268 mg (52%).

^{1}H-NMR (d$_6$-DMSO, 400 MHz) δ ppm: 1.29 (br s, 3H, DfmAla CH$_3$), 1.36 (s, 9H, C(CH$_3$)$_3$), 1.70–1.88 (m, 2H, Met C$^\beta$H$_2$), 2.02 (s, 3H, Met CH$_3$), 2.38–2.47 (m, 2H, Met C$^\gamma$H$_2$), 2.91 (dd, 1H, $^2J = 14.1$ Hz, $^3J = 9.1$ Hz, Phe C$^\beta$H$_2$), 3.08 (dd, 1H, $^2J = 14.1$ Hz,

$^3J = 5.0$ Hz, Phe $C^\beta H_2$), 4.03 (m, 1H, Met $C^\alpha H$) 4.26 (m, 1H, Phe $C^\alpha H$), 6.25 (br. t, 1H, $^2J = 57$ Hz, CF_2H), 7.07 (d, 1H, $^3J = 6.8$ Hz, Met NH), 7.10–7.28 (overl. m, 7H, Ar-Phe, Phe NH_2), 7.61 (d, 1H, $^3J = 7.9$ Hz, Phe NH), 8.41 (s, 1H, DfmAla NH). ^{13}C-NMR (d_6-DMSO, 101 MHz) δ ppm: 14.60 (Met CH_3), 16.10 (DfmAla, CH_3), 28.11 (C($\underline{C}H_3)_3$), 29.40 (Met C^γ), 31.30 (Met C^β), 36.46 (Phe C^β), 53.40 (Met C^α), 54.48 (Phe C^α), 60.42 (t, $^2J = 30$ Hz, DfmAla C^α), 78.49 ($\underline{C}(CH_3)_3$), 113.63 (t, $^1J = 249$ Hz, CF_2H), 126.15–138.10 (Ar-Phe), 168.35 (C=O, DfmAla), 172.16 (C=O, Phe), 172.71 (C=O, Met). ^{19}F-NMR (d_6-DMSO, 376 MHz) δ ppm: -126.5 (dd, 1F, $^2J = 276.4$ Hz, $^2J = 56.4$ Hz, CF_2H), -129.4 (dd, 1F, $^2J = 276.4$ Hz, $^2J = 57.2$ Hz, CF_2H).

Analytical HPLC: $R_t = 16.54$ min. TLC (CHCl$_3$/MeOH, 15 : 1): $R_f = 0.30$. Calculated MW = 516.61 for $C_{23}H_{34}F_2N_4O_5S$. ESI-MS: m/z $[M + Na]^+$ 539.21171 (539.21102), $[2M + Na]^+$ 1055.43515 (1055.4322).

Boc-Met-(αDfm)Ala-Phe-NH$_2$ (diastereomer II) (9b).

Tripeptide **9b** was synthesized from Boc-Met-OH (548 mg, 2.2 mmol) and H-(αDfm)Ala-Phe-NH$_2$ (diastereomer II) (285 mg, 1.0 mmol) (Method B). Purification by column chromatography (eluant: CHCl$_3$/MeOH, 20 : 1). Yield of **9b**: 330 mg (64%).

^{1}H-NMR (d_6-DMSO, 400 MHz) δ ppm: 1.18 (br. s, 3H, DfmAla CH_3), 1.37 (s, 9H, C($CH_3)_3$), 1.68–1.83 (m, 1H, Met $C^\beta H_2$), 1.83–1.96 (m, 1H, Met $C^\beta H_2$), 2.04 (s, 3H, Met CH_3), 2.37–2.54 (m, 2H, m, Met $C^\gamma H_2$), 2.82 (dd, 1H, dd, $^2J = 14.0$ Hz, $^3J = 10.3$ Hz, Phe $C^\beta H_2$), 3.21 (dd, 1H, $^2J = 14.0$ Hz, $^3J = 3.4$ Hz, Phe $C^\beta H_2$), 4.03 (m, 1H, Met $C^\alpha H$) 4.43 (m, 1H, Phe $C^\alpha H$), 6.20 (br. t, 1H, $^2J = 57$ Hz, CF_2H), 7.10 (d, 1H, $^3J = 7.1$ Hz, Met NH), 7.00–7.30 (overl. m, 7H, Ar-Phe, Phe NH_2), 7.69 (d, 1H, $^3J = 8.4$ Hz, Phe NH), 8.47 (s, 1H, DfmAla NH). ^{13}C-NMR (d_6-DMSO, 101 MHz) δ ppm: 14.61 (Met CH_3), 16.17 (DfmAla CH_3), 28.03 (C($\underline{C}H_3)_3$), 29.50 (Met C^γ), 30.54 (Met C^β), 36.50 (Phe C^β), 52.91 (Phe C^α), 53.23 (Met C^α), 59.86 (t, $^2J = 32$ Hz, DfmAla C^α), 78.40 ($\underline{C}(CH_3)_3$), 113.37 (t, $^1J = 254$ Hz, CF_2H), 126.12–138.03 (Ar-Phe), 168.92 (C=O, DfmAla), 172.75 (C=O, Phe), 173.05 (C=O, Met). ^{19}F-NMR (d_6-DMSO, 376 MHz) δ ppm: -126.7 (dd, 1F, $^2J = 275.7$ Hz, $^2J = 56.2$ Hz, CF_2H), -130.3 (dd, 1F, $^2J = 275.7$ Hz, $^2J = 57.0$ Hz, CF_2H).

Analytical HPLC: $R_t = 16.79$ min. TLC (CHCl$_3$/MeOH, 15 : 1): $R_f = 0.32$. Calculated MW = 516.61 for $C_{23}H_{34}F_2N_4O_5S$. ESI-MS: m/z $[M + Na]^+$ 539.21169 (539.21102), $[2M + Na]^+$ 1055.43586 (1055.43227).

For-Met-(αDfm)Ala-Phe-NH$_2$ (diastereomer I) (10a).

Tripeptide **9a** (258 mg, 0.5 mmol) was deprotected as described above and then N^α-formylated by method C to give **10a**. Purification by column chromatography (eluant: CHCl$_3$/MeOH, 15 : 1). Yield of **10a**: 173 mg (78%).

^{1}H-NMR (d_6-DMSO, 600 MHz) δ ppm: 1.29 (br. s, 3H, DfmAla CH_3), 1.73–1.81 (m, 1H, Met $C^\beta H_2$), 1.85–1.92 (m, 1H, Met $C^\beta H_2$), 2.03 (s, 3H, Met CH_3), 2.37–2.47 (m, 2H, Met $C^\gamma H_2$), 2.92 (dd, 1H, $^2J = 14.0$ Hz, $^3J = 8.9$ Hz, Phe $C^\beta H_2$), 3.05 (dd, 1H, $^2J = 14.0$ Hz, $^3J = 5.0$ Hz, Phe $C^\beta H_2$), 4.28 (dt, 1H, dt, $^3J = 8.3$ Hz, $^3J = 5.0$ Hz, Met $C^\alpha H$), 4.45 (dt, 1H, $^3J = 7.8$ Hz, $^3J = 5.2$ Hz, Phe $C^\alpha H$), 6.27 (t, 1H, $^2J = 56.2$ Hz, CF_2H), 7.12 (br s, 1H, Phe NH_2), 7.13–7.26 (overl. m, 6H, Ar-Phe, Phe NH_2), 7.63 (d, 1H, $^3J = 7.8$ Hz, Phe NH), 8.03 (s, 1H, For H), 8.36 (br. d, 1H, $^3J = 8$ Hz, Met NH), 8.61 (s, 1H, DfmAla NH). ^{13}C-NMR (d_6-DMSO, 151 MHz) δ ppm: 14.58 (Met CH_3), 16.17 (DfmAla CH_3), 29.11 (Met C^γ), 31.49 (Met C^β), 36.40 (Phe C^β), 50.48 (Met C^α), 54.24 (Phe C^α), 60.35 (t, $^2J = 21$ Hz, DfmAla C^α), 113.51 (t, $^1J = 241$ Hz, CF_2H), 126.13–137.98 (Ar-Phe), 161.28 (C=O, For), 168.32 (C=O, DfmAla), 171.72 (C=O, Met), 172.12 (C=O, Phe). ^{19}F-NMR (d_6-DMSO, 565 MHz) δ ppm: -126.5 (dd, 1F, $^2J = 277.0$ Hz, $^2J = 56.2$ Hz, CF_2H), -129.5 (dd, 1F, $^2J = 277.0$ Hz, $^2J = 56.5$ Hz, CF_2H).

Analytical HPLC: $R_t = 11.61$ min. TLC (CHCl$_3$/MeOH, 15 : 1): $R_f = 0.14$. Calculated MW = 444.50 for $C_{19}H_{26}F_2N_4O_4S$. ESI-MS: m/z $[M + Na]^+$ 467.15402 (467.15350), $[2M + Na]^+$ 911.32037 (911.31724).

For-Met-(αDfm)Ala-Phe-NH$_2$ (diastereomer II) (10b).

Tripeptide **9b** (258 mg, 0.5 mmol) was deprotected as described above and then N^α-formylated by method C to give **10b**. Purification by column chromatography (eluant: CHCl$_3$/MeOH, 15 : 1). Yield of **10b**: 144 mg (65%).

^{1}H-NMR (d_6-DMSO, 600 MHz) δ 1.15 (br. s, 3H, DfmAla CH_3), 1.71–1.80 (m, 1H, Met $C^\beta H_2$), 1.95–2.02 (m, 1H, Met $C^\beta H_2$), 2.05 (s, 3H, Met CH_3), 2.37–2.49 (m, 2H, Met $C^\gamma H_2$), 2.83 (dd, 1H, $^2J = 14.0$ Hz, $^3J = 10.2$ Hz, Phe $C^\beta H_2$), 3.20 (dd, 1H, $^2J = 14.0$ Hz, $^3J = 4.1$ Hz, Phe $C^\beta H_2$), 4.43 (overl. m, 2H, Met $C^\alpha H$, Phe C^α), 6.22 (t, 1H, $^2J = 56.6$ Hz, CF_2H), 7.05 (br. s, 1H, Phe NH_2), 7.16–7.27 (overl. m, 6H, Ar-Phe, Phe NH_2), 7.71 (d, 1H, $^3J = 8.3$ Hz, Phe NH), 8.04 (s, 1H, For H), 8.40 (br. d, 1H, $^3J = 7$ Hz, Met NH), 8.67 (s, 1H, DfmAla NH). ^{13}C-NMR (d_6-DMSO, 151 MHz) δ ppm: 14.66 (Met CH_3), 16.19 (DfmAla CH_3), 29.44 (Met C^γ), 31.10 (Met C^β),

36.49 (Phe C$^\beta$), 50.67 (Met C$^\alpha$), 53.29 (Phe C$^\alpha$), 59.86 (t, $^2J_{CF} = 23$ Hz, DfmAla C$^\alpha$), 113.30 (t, $^1J = 241$ Hz, CF$_2$H), 126.19–138.06 (Ar-Phe), 161.42 (C=O, For), 168.97 (C=O, DfmAla), 172.22 (C=O, Met), 172.43 (C=O, Phe). ^{19}F-NMR(d$_6$-DMSO, 565 MHz) δ ppm: -126.5 (dd, 1F, $^2J = 276.1$ Hz, $^2J = 56.5$ Hz, CF$_2$H), -130.5 (dd, 1F, $^2J = 276.1$ Hz, $^2J = 56.5$ Hz, CF$_2$H).

Analytical HPLC: $R_t = 12.21$ min. TLC (CHCl$_3$/ MeOH, 15:1): $R_f = 0.12$. Calculated MW = 444.50 for C$_{19}$H$_{26}$F$_2$N$_4$O$_4$S. ESI-MS: m/z [M + Na]$^+$ 467.15403 (467.15350), [2M + Na]$^+$ 911.32061 (911.31724).

Cell preparation. Polymorphonuclear leukocytes (PMNs, neutrophils) were isolated from freshly heparinized (10 U/ml) blood of healthy volunteers. The preparation included a dextran-enhanced sedimentation of red blood cells, Ficoll-Hypaque density centrifugation, lysis of remaining red blood cells with distilled water and washing of cells with Hanks' balanced salt solution. PMNs were stored in Hanks' medium at a concentration of 4×10^6 cells/ml at 4°C. The cells were used within 2 h after preparation. For each experiment, PMNs were purified from at least three different donors.

Luminol chemiluminescence. All luminescence measurements were performed on a microplate luminometer MicroLumat LB 96 P (EG & G Berthold, Wildbad, Germany) using white microtitre plates. Cells (10^5 cells/well) were preincubated with luminol (5×10^{-5} mol/l, final concentration) at 37°C for 5 min. 50 µl of a working solution of tripeptide was added to 200 µl cell suspension in order to activate the cells via the formyl peptide receptor. The luminol-dependent chemiluminescence, which indicates the oxidative activity of stimulated PMNs, was followed during the next 11 min. All experiments were run in triplicate.

RESULTS AND DISCUSSION

Analogues of the chemotactic peptide fMLF containing a fluorinated amino acid at position 2 instead of Leu were synthesized to impose stereochemical restrictions on the peptide backbone as well as to investigate the effect of fluorine on the biological activity of small peptides. The corresponding Aib peptide was synthesized and studied in the biological assay as a reference in view of the fact that its structural properties and biological activity have been very well investigated [36,37]. Incorporation of

an αTfm and αDfm group, respectively, into amino acids is known to induce considerable polarization effects on neighbouring substituents. Due to the high electron density, fluoroalkyl substitution implies the capability of peptide interaction with receptor subsites in a manner which is completely different from that of non-fluorinated analogues. Moreover, substitution of the C$^\alpha$-proton of an amino acid by both the Tfm and Dfm groups was found to exert conformational restrictions on the peptide chain. Thus, backbone torsion angles determined from several crystal structures indicate the formation of β-turns [38,39]. These structural alterations result in an increased proteolytic stability of peptides [40].

Bacterial peptides related to the fMLF-prototype act via binding to the formyl peptide receptors (FPR). Upon ligand binding to FPR different signalling events are induced, resulting in activation of NADPH oxidase [41–44]. Furthermore, NADPH oxidase reduces molecular oxygen to superoxide anion radicals and gives rise to the formation of all other reactive oxygen species produced by PMNs [45]. These reactive oxygen species modify luminol under the emission of light. Therefore, luminol-dependent chemiluminescence is a very sensitive method for detecting newly generated reactive oxygen species by these cells. fMLF and related peptides act at the start of this cascade. The FPR receptor recognizes not only fMLF but also a variety of other structurally related peptides [46–48].

Typical kinetics of the luminol-dependent chemiluminescence of tripeptide stimulated PMNs are shown in Figure 1.

In particular, the chemotactic tripeptide fMLF is known to produce several time-resolved maxima of luminescence upon PMN stimulation while the intensity of these maxima depends on cell concentration and the cell state [49,50]. In accordance with those published data, luminescence curves of the fluorinated analogues investigated here exhibited two maxima under our experimental conditions. The first maximum was usually found during the second minute after cell stimulation. In most cases, this maximum looked like a shoulder. The second maximum was more evident. With our peptides it was found during the fifth minute after the addition of the cell stimulator.

Figure 2 summarizes the results of the luminol-dependent chemiluminescence assay (normalized integral values are given for a concentration of 10^{-6} mol/l; strictly similar results, obtained at 10^{-5} mol/l and 10^{-7} mol/l concentrations, are not
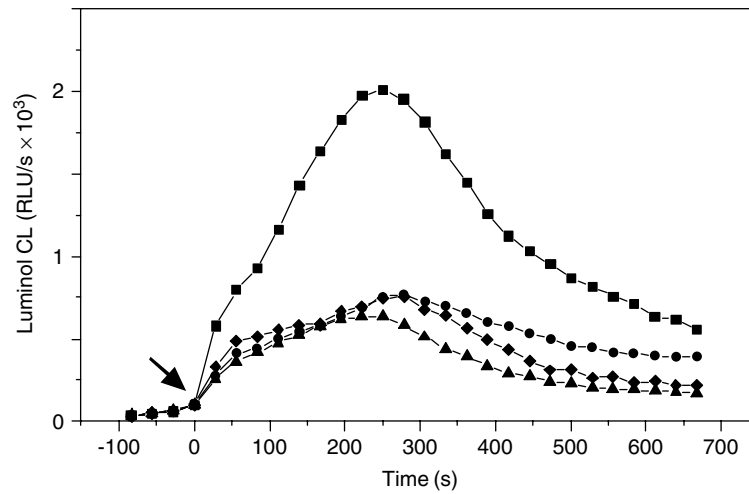
Figure 1   Kinetics of luminol chemiluminescence of oligopeptide stimulated PMNs. The arrow indicates the time of stimulation. The curves of the following tripeptides are reported: For-Met-Leu-Phe-NH$_2$ (■), For-Met-(R)-(αTfm)Ala-Phe-NH$_2$ (♦), For-Met-Aib-Phe-NH$_2$ (●) and For-Met-(αDfm)Ala-Phe-NH$_2$ (diastereomer II) (▲). Representative examples from four different cell preparations are shown.
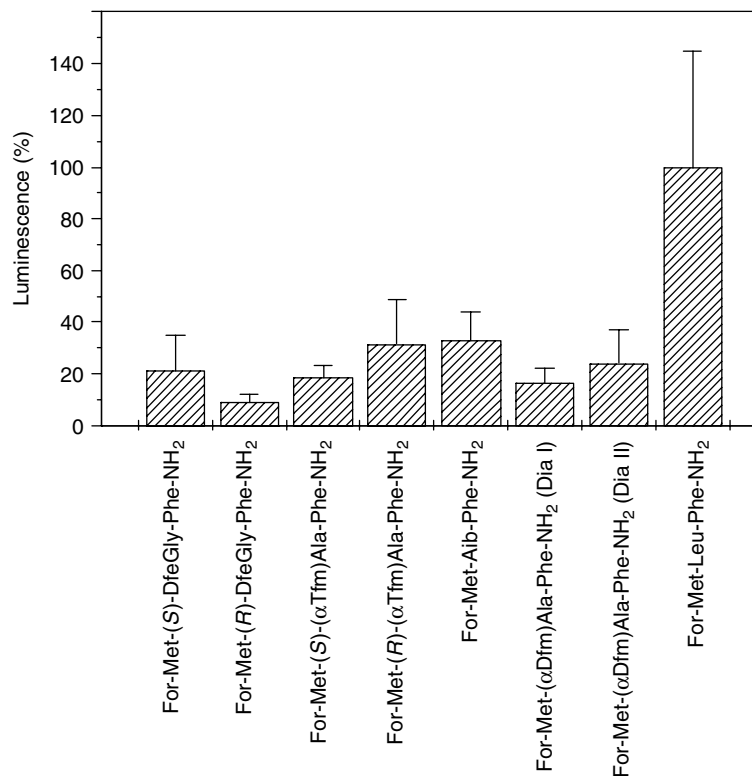

Figure 2   Integral values of luminol chemiluminescence of tripeptide stimulated PMNs. The integrals were determined over a time period of 10 min after stimulation. Mean ± SD of four different cell preparations are indicated. All other experimental conditions were as in Figure 1.

shown) of the fluorinated analogues in comparison to the parent peptide For-Met-Leu-Phe-NH$_2$ and the Aib analogue. It is clearly seen that all of the synthesized analogues were active in generating reactive oxygen species, although For-Met-Leu-Phe-NH$_2$ yielded by far the most intense response in
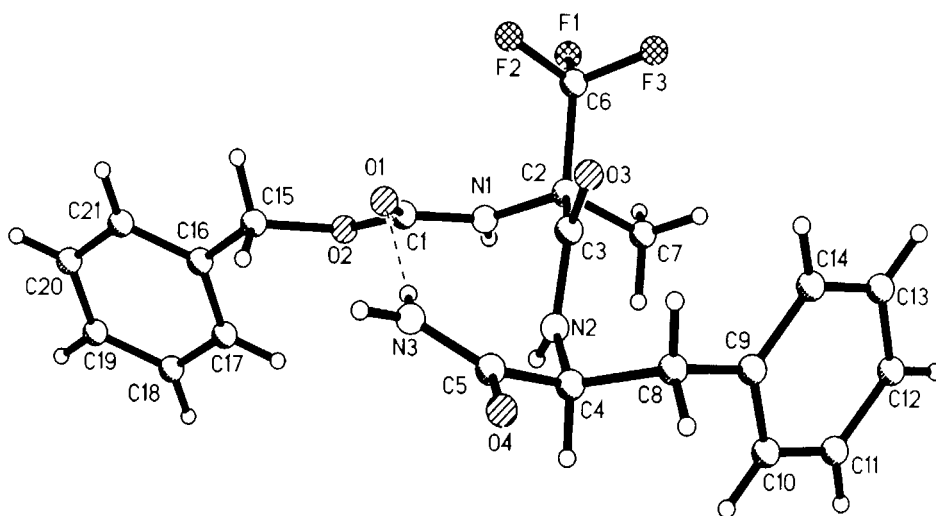
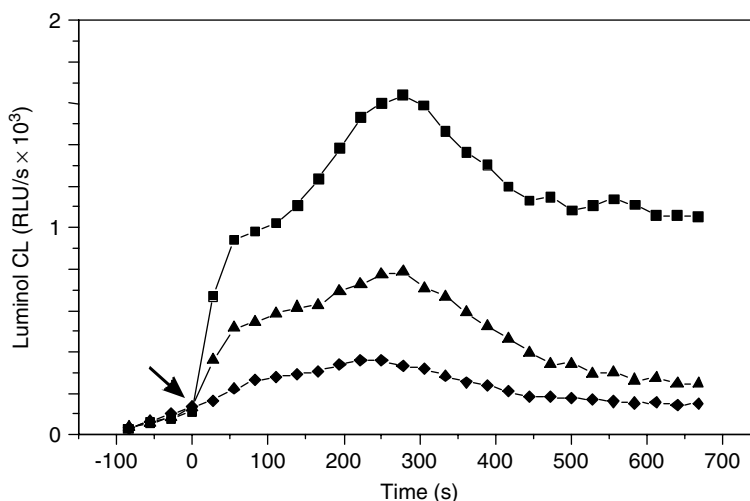Figure 3   Crystal structure of 7-(R)-(αTfm)Ala-Phe-NH$_2$ (**4a**). The intramolecular H-bond is indicated by a dashed line.

Figure 4   Kinetics of luminol chemiluminescence of For-Met-(*R*)-(αTfm)Ala-Phe-NH$_2$ stimulated PMNs as a function of the stimulator concentration. The final peptide concentrations were 10^{-5} mol/l (■), 10^{-6} mol/l (▲) and 10^{-7} mol/l (♦). All other experimental conditions were as in Figure 1.

comparison to all other tripeptides tested. The activity, though, was not only dependent on the nature of the substituent but also on the absolute configuration of the fluorinated amino acid. The most significant difference in inducing an oxidative activity was observed between the two diastereomers of For-Met-DfeGly-Phe-NH$_2$. Interestingly, the activity of the (S)-DfeGly-analogue, having the fluorinated side chain in the same position as it would be for the Leu side chain in For-Met-Leu-Phe-NH$_2$, reached the same order of magnitude as found for the Aib substituted peptide. The activity dropped by more than 50% in the (R)-DfeGly analogue. This finding confirms literature data on the importance

of the hydrophobic interaction between the amino acid at position 2 and the receptor binding pocket. Interestingly, in the case of the (αTfm)Ala peptides the (R)-diastereomer, having the bulkier Tfm side chain in the same position as that of the Leu side chain in For-Met-Leu-Phe-NH$_2$ (Figure 3) was the more active one in producing reactive oxygen species.

The luminescence response increased in a concentration-dependent manner for all the tested peptides. A typical example is given in Figure 4 for the most reactive analogue, For-Met-(R)-(αTfm)Ala-Phe-NH$_2$ (**4a**), among the fluorinated peptides investigated.

CONCLUSION

Site-specific incorporated, highly functionalized amino acids within biologically active peptides can serve as valuable biophysical probes for the investigation of structure–function relationships. Usually, the incorporation of fluorine atoms dramatically influences the physical properties of amino acids and proteins as well as providing the opportunity for studying conformational properties, peptide/protein–membrane interactions, or metabolic processes by ^{19}F-NMR [26,51]. Furthermore, the incorporation of fluoroalkyl amino acids into peptides is known to result in an increased resistance towards proteolysis as well as stabilization of secondary structures [40,52].

A small library of chemotactic peptide analogues of For-Met-Leu-Phe-NH$_2$ substituted at position 2 by different fluorinated amino acids varying in the content of fluorine, the length of the fluorinated side chain, and alkylation degree at the α-carbon atom was synthesized and the influence of the fluorine substitution on the biological activity was investigated. In a luminol-dependent chemiluminescence assay all of the synthesized For-Met-Leu-Phe-NH$_2$ analogues showed activity in the generation of reactive oxygen species. The activity was not only dependent on the nature of the substituent but also on the absolute configuration of the fluorinated amino acid. Remarkably, in the cases of the (αTfm) and DfeGly analogues, the diastereomer having the fluorinated, bulkier side chain in the same position as it would be for the Leu side chain in For-Met-Leu-Phe-NH$_2$ was the more active one. The (αDfm) Ala analogue seems to work in the same way; however, the configuration of the fluorinated amino acid has not been proven by crystal structure analysis yet.

This finding shows again the importance of the hydrophobic interaction between the amino acid at position 2 and the receptor binding pocket. Furthermore, these results establish that chemotactic peptide analogues which favour folded backbone conformations are biologically highly active [1,10,37]. In any case, activity of the fluoroalkyl substituted analogues of For-Met-Leu-Phe-NH$_2$, *e.g.* in inducing chemotaxis, lysosomal enzyme release and histamine release, has to be further investigated as it appears that analogues that are highly active in generating reactive oxygen species are not necessarily active in chemotaxis. The Aib-modified analogue investigated here had already been shown to be highly active for the release of lysozyme from rabbit neutrophils [37]. As the shape of luminescence curves for all of the fluoro-modified peptides tested was comparable to that of the Aib analogue, a common mechanism of cell activation for all of these stimulators can be assumed. Obviously, the preparation of fluoroalkyl substituted For-Met-Leu-Phe-NH$_2$ analogues resulted in structurally constrained peptidomimetics active in the generation of reactive oxygen species and bearing a fluorine label for NMR spectroscopy. Therefore, these conceptually new analogues of the prototype of the chemotactic peptide family provide the opportunity to study the receptor bound conformation of these signal peptides by ^{19}F-NMR.

Acknowledgements

REFERENCES

1. Becker EL, Freer RJ, Toniolo C, Balaram P. The specificity of the chemotactic formyl peptide receptor of rabbit neutrophils. In *Membrane Receptors and Cellular Regulation*, Czech MP, Kahn CR (eds). Liss: New York, 1985; 129–134.
2. Tornoe CW, Sengelov H, Meldal M. Solid-phase synthesis of chemotactic peptides using α-azido acids. *J. Peptide Sci.* 2000; **6**: 314–320.
3. Pirrung MC, Drabik SJ, Ahamed J, Ali H. Caged chemotactic peptides. *Bioconj. Chem.* 2000; **11**: 679–681.
4. Schiffmann E, Corcoran BA, Wahl SM. N-Formylmethionyl peptides as chemoattractants for leukocytes. *Proc. Natl. Acad. Sci. USA* 1975; **72**: 1059–1062.
5. Schiffmann E, Showell HJ, Corcoran BA, Ward PA, Smith E, Becker EL. The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli. J. Immunol.* 1975; **114**: 1831–1837.
6. Toniolo C, Formaggio F, Crisma M, Valle G, Boesten WHJ, Schoemaker HE, Kamphuis J, Temussi PA, Becker EL, Précigoux G. Bioactive and model peptides characterized by the helicogenic (αMe)Phe residue. *Tetrahedron* 1993; **49**: 3641–3653.
7. Becker EL. The formylpeptide receptor of the neutrophil. *Am. J. Path.* 1987; **129**: 15–24.
8. McPhail LC, Harvath L. Signal transduction in neutrophil oxidative metabolism and chemotaxis.

In *The Natural Immune System: The Neutrophil*, Abramson JS, Wheeler JG (eds). Oxford University Press: Oxford, 1993; 63–77.

9. Torrini I, Zecchini GP, Paradisi MP, Lucente G, Gavuzzo E, Mazza F, Pochetti G, Spisani F, Guliani AL. Synthesis and properties of chemotactic peptide analogs. II. HCO-Met-Leu-Phe-OMe analogs containing cyclic α, α-disubstituted amino acids as Met and Phe mimicking residues. *Int. J. Peptide Protein Res.* 1991; **38**: 495–504.

10. Toniolo C, Crisma M, Valle G, Bonora GM, Polinelli S, Becker EL, Freer RJ, Prasad S, Roa RB, Balaram P, Sukumar M. Conformationally restricted formyl methionyl tripeptide chemoattractants: a three-dimensional structure-activity study of analogs incorporating a $C^{\alpha,\alpha}$-dialkylated glycine at position 2. *Peptide Res.* 1989; **2**: 275–281.

11. Benedetti E. X-ray crystallography of peptides. *Biopolymers (Peptide Sci.)* 1996; **40**: 3–44.

12. Sukumar MP, Raz AP, Balaram P, Becker EL. A highly active chemotactic peptide analog incorporating the unusual residue 1-aminocyclohexanecarboxylic acid at position 2. *Biochem. Biophys. Res. Commun.* 1985; **128**: 339–344.

13. Prasad S, Rao RB, Bergstrand H, Lundquist B, Becker EL, Balaram P. Conformation-activity correlations of chemotactic tripeptide analogs incorporating dialkyl residues with linear and cyclic alkyl sidechains at position 2. *Int. J. Peptide Protein Res.* 1996; **48**: 312–318.

14. Freer RJ, Day AR, Schiffmann E, Aswanikumar S, Showell HJ, Becker EL. Further studies on the structural requirements for synthetic peptide chemoattractants. *Biochemistry* 1980; **19**: 2404–2409.

15. Freer RJ, Day AR, Muthukumaraswamy N, Pinon D, Wu A, Showell HJ, Becker EL. Formyl peptide chemoattractants. A model of the receptor in rabbit neutrophils. *Biochemistry* 1982; **21**: 257–263.

16. Day AR, Radding JA, Freer RJ, Showell HJ, Becker EL, Schiffmann E, Corcoran B. Synthesis and binding characteristics of an intrinsically radio-labeled chemotactic acyl tripeptide: N^{α}-formyl-norleucyl-leucyl-phenylalanine. *FEBS Lett.* 1977; **77**: 291–294.

17. Shimohigashi Y. In *Opioid Peptides, Medicinal Chemistry*, Rapka RS, Bernett G, Hawks RL (eds). NIDA Research Monograph 1986; **69**: 65.

18. Hruby VJ, Li G, Haskell-Luevano C, Shenderovich M. Design of peptides, proteins, and peptidomimetic in Chi space. *Biopolymers* 1997; **43**: 219–266.

19. Fujita T, Nose T, Matsushima A, Okada K, Asai D, Yamauchi Y, Shirasu N, Honda T, Shigehiro D, Shimohigashi Y. Synthesis of a complete set of L-difluorophenylalanines, L-(F_2)Phe, as molecular explorers for the CH/π interaction between peptide ligand and receptor. *Tetrahedron Lett.* 2000; **41**: 923–927.

20. Nishio M, Umezawa Y, Hirota M, Takeuchi Y. The CH/π interaction: significance in molecular recognition. *Tetrahedron* 1995; **51**: 8665–8701.

21. Erickson JA, Mc Loughlin JI. Hydrogen bond donor properties of the difluoromethyl group. *J. Org. Chem.* 1995; **60**: 1626–1631.

22. Caminati W, Melandri S, Moreschini P, Favero PG. The C-F···H-C 'anti-hydrogen bond' in the gas phase: microwave structure of the difluoromethane dimer. *Angew. Chem. Int. Ed.* 1999; **38**: 2924–2925.

23. Howard JAK, Hoy VJ, O'Hagan D, Smith GT. How good is fluorine as hydrogen bond acceptor. *Tetrahedron* 1996; **52**: 12 613–12 622.

24. O'Hagan D, Rzepta HS. Some influences of fluorine in bioorganic chemistry. *J. Chem. Soc., Chem. Commun.* 1997; 645–652.

25. Dunitz JD, Taylor R. Organic fluorine hardly ever accepts hydrogen bonds. *Chem. Eur. J.* 1997; **3**: 89–98.

26. Tang Y, Ghirlanda G, Vaidehi N, Kua J, Mainz TD, Goddard WA, III, Degrado WF, Tirrell DA. Stabilization of coiled-coil peptide domains by introduction of trifluoroleucine. *Biochemistry* 2000; **40**: 2790–2796.

27. Degrado WF, Summa CM, Pavone V, Nastri F, Lombardi A. *De novo* design and structural characterization of proteins and metalloproteins. *Annu. Rev. Biochem.* 1999; **68**: 779–819.

28. Winkler D, Burger K. Synthesis of enantiomerically pure D- and L-armentomycin and its difluoro analogues from aspartic acid. *Synthesis* 1996; 1419–1421.

29. Sewald N, Hollweck W, Mütze K, Schierlinger K, Seymour LC, Gaa K, Burger K, Koksch B, Jakubke HD. Peptide modification by introduction of α-trifluoromethyl substituted amino acids. *Amino Acids* 1995; **8**: 187–195.

30. Sewald N, Burger K. Synthesis of β-fluoro-containing amino acids. In *Fluorine-containing Amino Acids, Synthesis and Properties*, Kukhar VP, Soloshonok VA (eds). Wiley: Chichester 1995; 139–162.

31. Osipov SN, Golubev AS, Sewald N, Michel T, Kolomiets AF, Fokin AV, Burger K. A new strategy for the synthesis of β-difluoromethyl substituted α-hydroxy and α-amino acids. *J. Org. Chem.* 1996; **61**: 7521–7528.

32. Preliminary results have been published: Moroni M, Koksch B, Burger K. Analogues of chemotactic peptides containing fluorinated amino acids. *Peptides; Proceedings of the 26th European Peptide Symposium*, Martinez J, Fehrentz J-A (eds.). EDK: Paris 2001; 689–690.

33. Duczek W, Deutsch J, Vieth S, Niclas H-S. A simple and convenient synthesis of N-formyl amino acid esters under mild conditions. *Synthesis* 1996; 37–38.

34. Sheldrick GM. *SHELXS 97. Program Used to Solve and to Refine Structure.* University of Göttingen: Göttingen, Germany, 1997.

35. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre,

CCDC210454. Copies of this information may be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44 1233 336033, e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

36. Bardi R, Piazzesi AM, Toniolo C, Antony Raj P, Raghothama S, Balaram P. Solid state and solution conformation of Boc-L-Met-Aib-L-Phe-OMe. *Int. J. Peptide Protein Res.* 1986; **27**: 229–238.

37. Iqbal M, Balaram P, Showell HJ, Freer RJ, Becker EL. Conformationally constrained chemotactic peptide analogs of high biological activity. *FEBS Lett.* 1984; **165**: 171–174.

38. Koksch B, Sewald N, Burger K, Jakubke H-D. Peptide modification by incorporation of α-trifluoromethyl substituted amino acids. *Amino Acids* 1996; **11**: 425–434.

39. Michel T, Koksch B, Osipov SN, Golubev AN, Sieler J, Burger K. Peptide synthesis with α-(difluoromethyl)-substituted amino acids. *Coll. Czech. Chem. Commun.* 2000; **67**: 1533–1553.

40. Koksch B, Sewald N, Hofmann H-J, Burger K, Jakubke H-D. Proteolytically stable peptides by incorporation of α-Tfm amino acids. *J Peptide Sci.* 1997; **3**: 157–167.

41. Watson F, Robinson JJ, Edwards SW. Sequential phospholipase activation in the stimulation of the neutrophil NADPH-oxidase. *FEMS Microbiol. Immunol.* 1992; **105**: 239–248.

42. Curnutte JT, Erikson RW, Ding J, Badwey JA. Reciprocal interactions between protein kinase C and components of the NADPH-oxidase complex may regulate superoxide production by neutrophils stimulated by a phorbol ester. *J. Biol. Chem.* 1994; **269**: 10 813–10 819.

43. Waite KA, Wallin R, Qualliotine-Mann D, McPhail LC. Phosphatidic acid-mediated phosphorylation of the NADPH oxidase component p47phox. *J. Biol. Chem.* 1997; **272**: 15 569–15 578.

44. Arnhold J, Benard S, Kilian U, Reichl S, Schiller J, Arnold K. Modulation of luminol chemiluminescence of fmet-leu-phe-stimulated neutrophils by affecting dephosphorylation and the metabolism of phosphatidic acid. *Luminescence* 1999; **14**: 1–9.

45. Segal AW, Abo A. The biochemical basis of the NADPH oxidase of phagocytes. *Trends Biochem. Sci.* 1993; **18**: 43–47.

46. Le Y, Yang Y, Cui Y, Yazawa H, Gong W, Qiu C, Wang JM. Receptors for chemotactic formyl peptides as pharmacological targets. *Int. Immunopharmacol.* 2002; **2**: 1–13.

47. Le Y, Oppenheim JJ, Wang JM. Pleiotropic roles of formyl peptide receptors. *Cytokine Growth Factor Rev.* 2001; **12**: 91–105.

48. Dalpiaz A, Scatturin A, Vertuani G, Pecoraro R, Borea PA, Varani K, Traniello S, Spisani S. Met-Ile-Phe-Leu derivatives: full and partial agonists of human neutrophil formylpeptide receptors. *Eur. J. Pharmacol.* 2001; **411**: 327–333.

49. Bender JG, van Epps DE. Analysis of the bimodal chemiluminescence pattern stimulated in human neutrophils by chemotactic factors. *Infect. Immunol.* 1983; **41**: 1062–1070.

50. Dahlgren C. Polymorphonuclear leukocyte chemiluminescence induced by formylmethionyl-leucyl-phenylalanine and phorbol myristate acetate: effects of catalase and superoxide dismutase. *Agent Actions* 1987; **21**: 104–112.

51. Ulrich AS. In *Encyclopedia of Spectroscopy and Spectrometry*, Lindon J, Tranter G, Holmes J (eds). Academic Press: New York, 2000; 813.

52. Koksch B, Sewald N, Jakubke H-D, Burger K. In *Biomedical Frontiers of Fluorine Chemistry*, Ojima I, McCarthy JR, Welch JT (eds). ACS Symposium Series, American Chemical Society: Washington, DC, 1996; **639**: 42–58.