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FACILE SYNTHESIS OF *(S)*-5,5-DIFLUORONORLEUCINE AND ITS INCORPORATION IN BIOLOGICALLY ACTIVE PEPTIDES AS AN METHIONINE MIMETIC

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Abstract – Both Boc and Fmoc protected 5,5-difluoronorleucines (F_2Nle) were easily prepared from commercially available amino acid derivatives. F_2Nle can be viewed as a mimic of methionine in which the sulfur atom is replaced by the CF_2 moiety. Two analogues of methionine-containing biologically active peptides (fMLP and Met-enkephalin) were prepared. The validity of CF_2-substituted methionine analogues was confirmed in their biological assay.

INTRODUCTION

Under physiological conditions, methionine can be oxidized to methionine sulfoxide (MetO). In the presence of a strong oxidant, MetO is further oxidized to form methionine sulfone, $MetO_2$ (Figure 1). The latter process may be only of pathophysiological and/or experimental relevance, whereas the former process is a phenomenon that can happen in living cells.

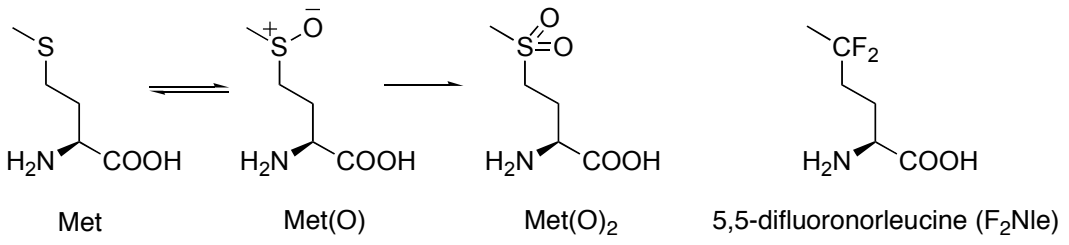


Figure 1. Methionine side-chain oxidation and 5,5-difluoronorleucine as a mimetic of Met.

It is well known, partially oxidized methionine participates in age- or degenerative disease-related protein modifications. In the case of methionine-containing biologically active peptides, oxidation on sulfur atom results in substantial reduction in their activity. Recently, oxidation and the enzymatically-catalyzed reduction of methionine have been in focus as a potential novel molecular mechanism for cellular

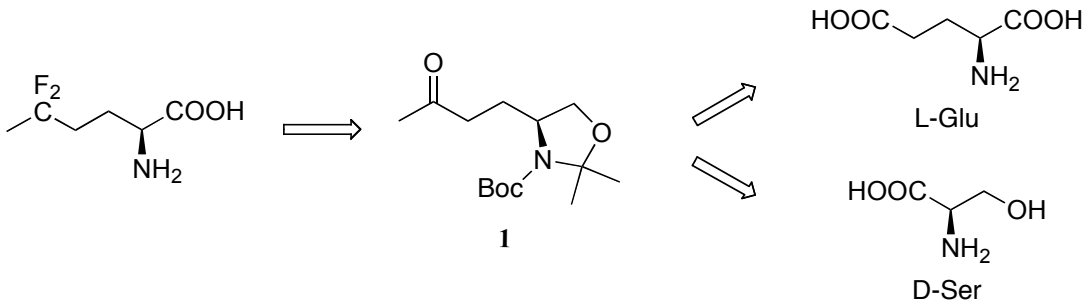
regulation.[1] Thus, partially oxidized methionine residues may not only degenerative modifications, but also play a role in post-translational modification cycles of the proteins involved in cellular signaling, which in turn may give rise to activity-dependent plastic change in cellular excitability.[1b] Synthetic oxidation-resistant methionine analogues may have unique biological activity when they incorporated into peptide, which lead to the potential to evolve into a new strategy or treating a disease, or become a useful tool for gaining insight into the mechanisms of age-related or post-translational protein modifications.

We designed 5,5-difluoronorleucine (F_2Nle) in which the sulfur atom in methionine is replaced by a CF_2 moiety as a methionine mimetic. CF_2 group is often used as an isoster of oxygen,[2] but its van der Waals size is rather close to sulfur. While the sulfur atom is easily oxidized to sulfoxide in physiological conditions, the CF_2 group is inactive under such conditions. Therefore, F_2Nle can be viewed as an oxidation-resistant mimic of methionine.

Preparation of racemic[3] and L-form[4] of F_2Nle was reported, however, both synthetic routes utilized highly toxic and hazardous HF and SF_4 to introduce fluorine. Absence of a more practical and facile synthetic method can be one of the reasons that peptide chemists have somehow ignored the use of F_2Nle as a methionine mimic, thus we have developed a facile synthetic method to prepare enantiomerically pure Boc- or Fmoc-protected 5,5-difluoronorleucine, starting from commercially available glutamic acid derivative or D-serine derived Garner's aldehyde.[5] These compounds can be readily utilized for peptide synthesis in both solution and solid-support methods. Due to the feasibility of the oxidation-resistant F_2Nle-containing peptide analogues to replace more vulnerable methionine-containing peptides, incorporation of F_2Nle to biologically active peptide was demonstrated on a chemotactic peptide, fMLP, and an opioid peptide, Met-enkephalin.

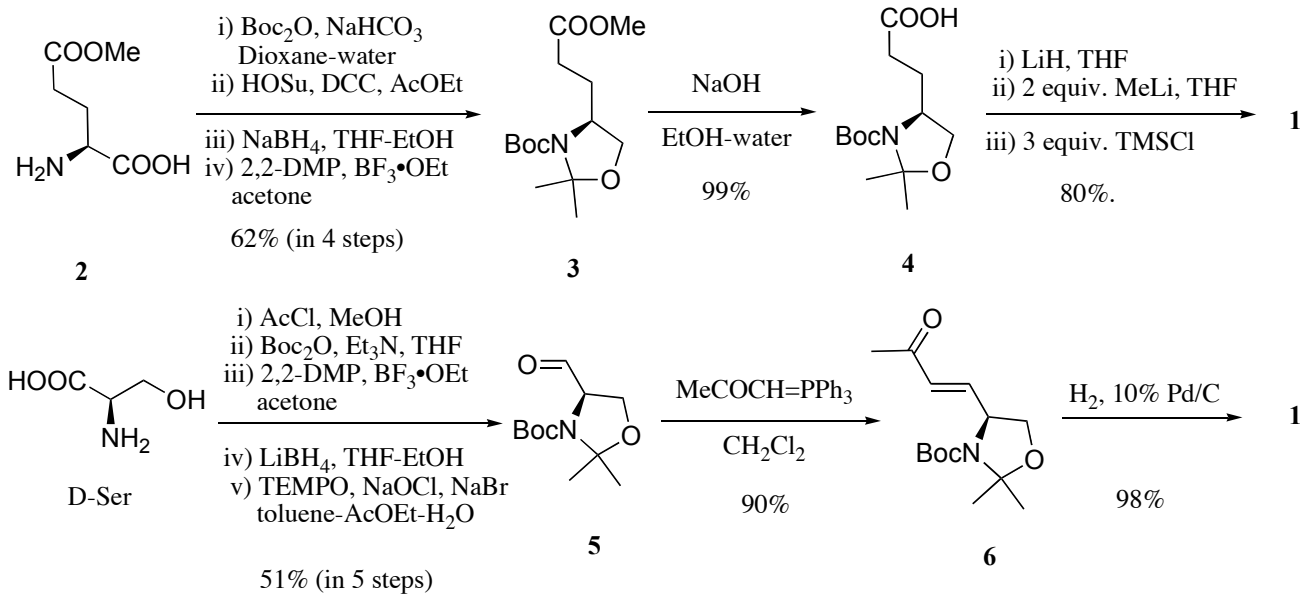
RESULTS AND DISCUSSION

As two fluorine atoms can be easily installed by functional transformation from the corresponding methyl ketone (**1**) (Scheme 1), we started to prepare **1** by utilizing two different chiral building blocks.



Scheme 1.

As shown in Scheme 2, the commercially available γ-methyl L-glutamate (**2**) was transformed to *N,O*-protected ester (**3**) in 62% yield through 4 steps.[6] Saponification of the side-chain ester by NaOH followed by acidification gave the free acid (**4**). The carboxylic acid (**4**) was initially converted to lithium carboxylate using LiH, treated with excess methyllithium, then quenched by addition of chlorotrimethylsilane[7] to give the methyl ketone (**1**) in 80% yield. Direct addition of three equivalents of methyllithium to carboxylic acid (**4**) and aqueous work-up at the last step lowered the yield (maximum 55%, with tendency to form a certain amount of *tert*-alcohol as a byproduct). Direct addition of methyllithium to ester (**3**) at low temperatures also gave ketone (**1**), however, chromatographic separation of the desired ketone (**1**) and unreacted ester (**3**) was hard to accomplish because of their close R_f values.

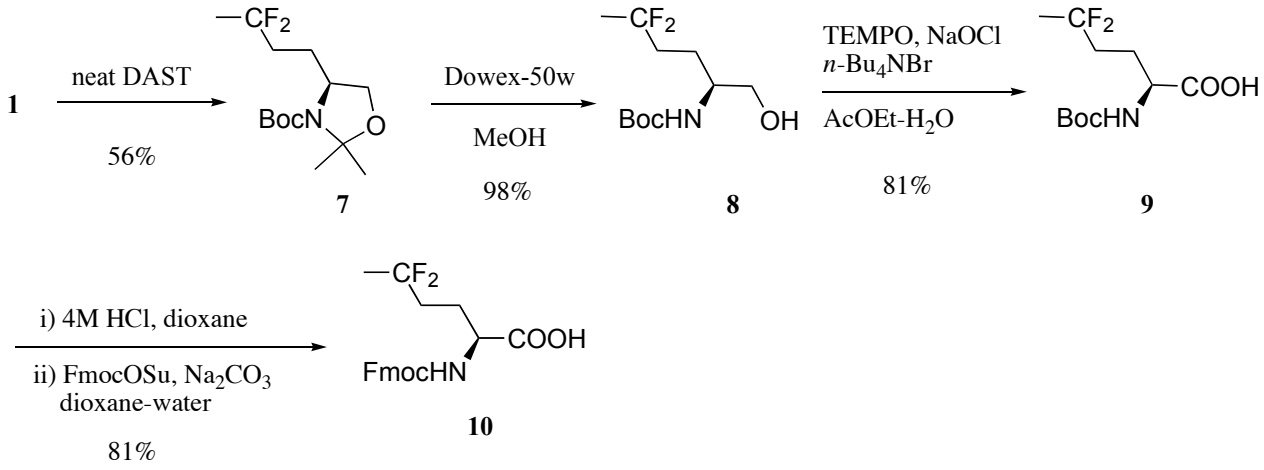


Scheme 2. Synthesis of methyl ketone (**1**) from γ-methyl glutamate or D-serine

Alternatively, treatment of the well-known Garner's aldehyde[5] with $Ph_3P{=}CHCOCH_3$ in dichloromethane gave α,β-unsaturated ketone as a crystalline compound in 90% yield. Subsequent hydrogenation of the double bond was easily achieved over 10% Pd/C to give methyl ketone (**1**) in 98% yield. The latter route is easier to proceed and more efficient, however, Garner's aldehyde for this scheme should be derived from the expensive D-serine in 5 steps.

The carbonyl group of **1** was then converted to CF_2 functionality in neat diethylaminosulfur trifluoride[8] (DAST). The reaction rate is very slow in solution and took a week for consumption of the starting material. Partial acetonide deprotection by trace amounts of HF was observed during the long reaction period, which leads to a complex mixture. Complete acetonide deprotection of the difluoro compound (**7**) was then achieved by treating it with Dowex resin in MeOH to give the amino alcohol (**8**) in 98% yield.

Conversion of Boc-amino alcohol (**8**) to Boc-amino acid (**9**) was cleanly accomplished in 86% yield by environmentally benign TEMPO-NaOCl-*n*-Bu_4NBr oxidation.[9] In contrast to the conventional PDC oxidation in DMF, the biphasic condition of TEMPO oxidation makes it easy to isolate the amino acid by a simple extraction technique, without using chromatographic separation. The *N*-protecting Boc group was readily transformed to Fmoc protection, which is frequently used in solid-support peptide synthesis.



Scheme 3. Synthesis of *N*-Protected F_2Nle

Thus obtained F_2Nle was applied for peptide synthesis. To investigate the validity of CF_2-substituted methionine analogues, F_2Nle analogues of methionine-containing biologically active peptides, fMLP and Met-enkephalin were designed (Figure 2).

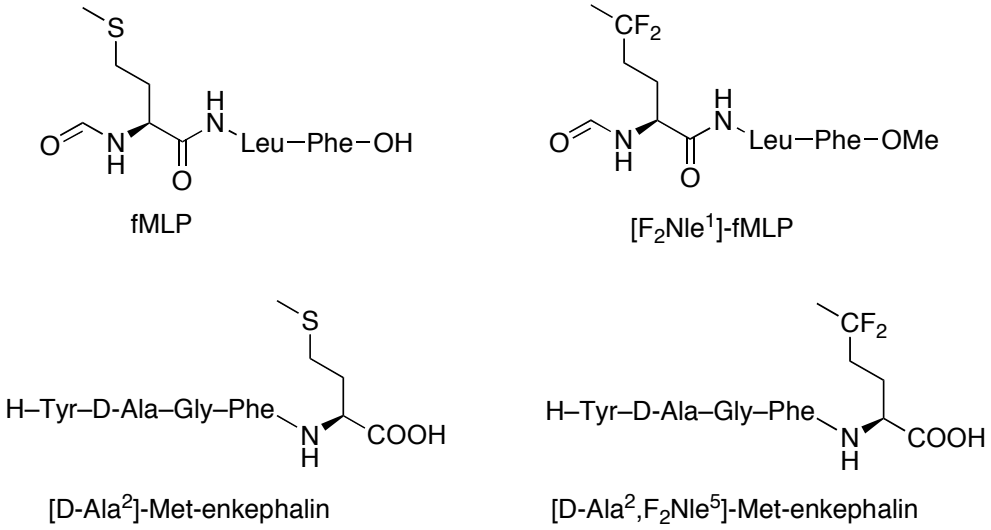


Figure 2. F_2Nle analogues of two biologically active peptides

As a chemotactic peptide fMLP analog, HCO-F_2Nle-Leu-Phe-OMe ([F_2Nle^1]-fMLP) was prepared by conventional coupling procedure between Boc-F_2Nle-OH and H-Leu-Phe-OMe using EDCI and HOBt,

and successive conversion on *N*-terminal Boc to formyl by HCOOH and EEDQ. For a Met-enkephalin analogue, considering ease of biological assay, [D-Ala^2]-Met-enkephalin was chosen for the reference compound, and its analog with F_2Nle substitution at methionine residue was prepared by solid-support peptide synthesis procedure using Wang resin.

Table 1. Superoxide production assay of fMLP analogues

	superoxide production (EC_{50})
fMLP	20 nM
[Nle^1]-fMLP	50 nM
[F_2Nle^1]-fMLP	5 nM

Table 2. Competition binding assay of Met-Enkephalin analogues

	Binding Potency (IC_{50})		
	δ-OR	μ–OR	κ–OR
[D-Ala^2]-Met-enkephalin	4 nM	4 nM	6200 nM
[D-Ala^2,Nle^5]-Met-enkephalin	4 nM	5 nM	5800 nM
[D-Ala^2,F_2Nle^5]-Met-enkephalin	5 nM	6 nM	> 10,000 nM

Biological evaluation on F_2Nle analogues of methionine-containing biologically active peptides, fMLP and Met-enkephalin, are shown in Tables 1 and 2, respectively. In both cases, Nle analogues were independently prepared and used for evaluating feasibility of CF_2 group.

Superoxide production assay were applied for fMLP analogues.[10] Interestingly, superoxide production of neutrophils stimulated by [F_2Nle^1]-fMLP (EC_{50}=5 nM) was more potent than fMLP (EC_{50}=20 nM). Some evidences are reported that stimulated neutrophils inactivate fMLP by oxidizing the sulfur of the methionine residue, and it has been proposed that such oxidation may be a mechanism by which neutrophils modulate the inflammatory response.[11] Slightly enhanced activity of F_2Nle analog may imply that the oxidation-resistant difluoromethylene group mimicking sulfur atom without loss of its activity.

For enkephalin analogues, binding potency of peptides for the δ, μ, and κ-opioid receptors (ORs) were evaluated by the competition binding assay using selective antagonists and COS-7 cells expressing opioid receptors.[12] In this case, three enkephalin analogues had similar potency and selectivity trends, thus no outstanding effect due to the CF_2 substitution was observed in compared with CH_2 substitution. It can be concluded that the sulfur atom in the Met-enkephalin is not a key group for the receptor subtype

discrimination. More detailed biological analyses for the downstream events are being undertaken, and will be reported elsewhere.

In conclusion, we have developed a facile synthesis of 5,5-difluoronorleucine as a mimetic of methionine, and demonstrated its sulfur atom mimicking in two biologically active peptides. Generally, Nle substitution at Met is the first choice for increasing stability in biologically active peptides. When it results in a decrease in activity, CF_2 substitution may be one consideration for mimicking the sulfur atom in designing stable isoster.

EXPERIMENTAL

Melting points were recorded on Yanako micro melting point apparatus MPS3 and are uncorrected. All commercial reagents were used without further purification unless otherwise noted. The ^{1}H-NMR, ^{13}C-NMR and ^{19}F-NMR spectra were determined on a JEOL AL-300 spectrometer (300 MHz, 75 MHz and 283MHz, respectively). All chemical shifts are reported in ppm as δ value relative to internal tetramethylsilane (^{1}H and ^{13}C) or benzotrifluoride (^{19}F) in $CDCl_3$ unless otherwise noted. Optical rotations were recorded on a JASCO DIP-370 polarimeter. IR spectra were recorded on a JASCO IR-700 spectrophotometer as KBr disks or Nujol mulls. HRMS spectra were obtained on a JEOL GCmateII mass spectrometer. Molecular weight of synthetic peptides was determined by MALDI-TOF MS analysis on Voyager DERF (PerSeptive Biochem). Tetrahydrofuran was distilled from sodium benzophenone ketyl in prior to use. All the manipulations with air-sensitive reagents were carried out under a dry argon atmosphere. Analytical TLC was performed using Merck Silica Gel 60 F_{254} plates (0.25 mm on glass). Flash column chromatography was performed using either Wakogel C-300 (45-75 μm) or Silica Gel 60 (40–63 μm). Following abbreviations are used; DIEA, *N,N*-diisopropylethylamine; EEDQ, *N*-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinone; EDCI•HCl; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, *N*-(9-fluorenylmethoxycarbonyl) succinimide; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; NMP, *N*-methylpiperidone; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy free radical; TIS, triisopropylsilane.

(S, E)-tert-Butyl 2,2-dimethyl-4-(3-oxobut-1-enyl)oxazolidine-3-carboxylate (6)

To a solution of *(R)-tert*-butyl 4-formyl-2,2-dimethyloxazolidine-3-carboxylate (Garner's aldehyde, 2.30 g, 10.0 mmol) in dichloromethane (60 mL) was added (acetylmethylene)triphenylphosphorane (4.00 g, 12.5 mmol) at rt with stirring. After stirring for 3 days, the solvent was evaporated *in vacuo* and 50 mL of ether was added to the residue to precipitate triphenylphosphine oxide. Precipitates were filtered off, and the filtrate was concentrated and purified by flash chromatography (hexane/ethyl acetate = 3/1) to give

title compound as colorless crystals (2.42 g, 60%). mp 76-77°C (P. ether); $[\alpha]_D$ +62.0° (c 1.0, MeOH); ^{1}H-NMR (mixture of Boc rotamers) δ 6.68–6.65 (m, 1H), 6.15 (t, *J* =15.9 Hz, 1H), 4.55–4.48 (m, 1H), 4.11 (dd, *J* = 6.7, 6.5 Hz, 1H), 3.80 (dd, *J* = 9.2, 2.0 Hz, 1H), 2.28 (s, 3H), 1.67-1.42 (m, 15H); ^{13}C-NMR (mixture of Boc rotamers) δ 198.1, 151.5, 144.8, 144.5, 131.2, 130.9, 94.5, 94.0, 80.7, 80.2, 67.4, 67.2, 58.2, 28.3, 27.1, 26.5, 24.5, 23.5; IR (neat film from CH_2Cl_2) ν 2970, 2926, 2870, 1690, 1627, 1473, 1450, 1374, 1249, 1202, 1171, 1093, 1053, 974, 852, 768 cm^{-1}; HRMS (FAB) $(M)^+$ calculated for $C_{14}H_{23}NO_4$ 269.1627, found 269.1610.

***(S)-tert*-Butyl 2,2-dimethyl-4-(3-oxobutyl)oxazolidine-3-carboxylate (1)**

1) *via* ester (**3**).

To a solution of methyl ester (**3**) (3.10 g, 10.7 mmol) dissolved in 5 mL of ethanol was added NaOH aqueous solution (0.46 g / 12 mL) at rt, then mixture was stirred for 14 h. The aqueous solution was washed with ether, acidified by using 6M HCl aqueous solution. Free carboxylic acid was extracted with ether, washed with brine, and dried over $MgSO_4$. Solvent was removed *in vacuo* and the residue was azeotroped with benzene to give colorless oil (2.91 g). Homogeneity of the acid was confirmed by TLC analysis (hexane/ethyl acetate = 3/1 and $CHCl_3$/MeOH/AcOH = 95/5/1). The carboxylic acid (**4**) (1.93 g) dissolved in 20 mL of THF was added to a suspension of 90% LiH (66 mg, 7.4 mmol) in THF (10 mL) at rt. After stirring 10 min at the ambient temperature, the mixture was cooled to 0°C, a solution of methyllithium (1.2 M ether solution, 12 mL, 14 mmol) was added with vigorous stirring. After 1 h at 0°C, 3.0 mL (22 mmol) of chlorotrimethylsilane was added rapidly and the mixture was gradually warm up to rt. The mixture was poured onto saturated NH_4Cl aqueous solution. Phases were separated, the aqueous layer was extracted with ether. Combined organic phases were washed with saturated $NaHCO_3$, brine, then dried. Solvent was removed *in vacuo* and flash chromatography (hexane/ethyl acetate = 3/1) gave 1.52 g of methyl ketone (**1**) (80%) as colorless oil.

2) *via* unsaturated ketone (**5**)

To a solution of unsaturated ketone (**5**) (1.33 g 4.93 mmol) in ethanol (50 mL) containing 10% Pd/C (80 mg) was stirred for 6 h at rt under hydrogen atmosphere (1 atm). Catalyst was filtered off, solvent was removed *in vacuo* and flash chromatography (hexane/ethyl acetate = 3/1) gave colorless oil (1.31g, 98%). $[\alpha]_D$ +21.8° (c 1.0, MeOH); ^{1}H-NMR (mixture of Boc rotamers) δ 3.86 (m, 2H), 3.62 (d, *J* =7.5 Hz, 1H), 2.39 (t, *J* = 7.6 Hz, 2H), 2.09 (s, 3H), 1.90-1.76 (m, 2H), 1.49-1.41 (m, 15H); ^{13}C-NMR (mixture of Boc rotamers) δ 208.0, 207.8, 152.5, 93.8, 93.3, 80.0, 79.7, 67.0, 56.5, 56.4, 40.1, 39.9, 30.8, 29.8, 28.3, 26.6, 24.3, 22.9; IR (neat) ν 2968, 2928, 2870, 1686, 1473, 1448, 1387, 1307, 1251, 1202, 1169, 1099, 1077, 1048, 1025, 853, 767 cm^{-1}; HRMS (FAB) $(M)^+$ $C_{14}H_{25}NO_4$ 271.1784, found 271.1760.

(*S*)-*tert*-Butyl 4-(3,3-difluorobutyl)-2,2-dimethyloxazolidine-3-carboxylate (7)

A solution of methyl ketone (**1**) (1.08 g, 3.97 mmol) in diethylaminosulfur trifluoride (2 mL) was stirred for 20 h at rt. The reaction mixture was diluted with 20 mL of ether, and the solution was carefully added to a saturated $NaHCO_3$ aqueous solution in dropwise. The two-phase mixture was stirred for 30 min, the ether layer was washed with $NaHCO_3$ aqueous solution and brine, then dried over Na_2SO_4. Concentration and flash chromatography (hexane/AcOEt = 4/1) gave 647 mg (56%) of pale yellow oil. $[\alpha]_D$ +27.9° (c 1.0, MeOH); ^{1}H-NMR (mixture of Boc rotamers) δ 3.98-3.85 (m, 2H), 3.71 (d, *J*=7.6 Hz, 1H), 2.00-1.72 (m 4H), 1.60 (t, *J*=18.4 Hz, 3H), 1.56 (brs, 3H), 1.54 (s, 12H); ^{13}C-NMR (mixture of Boc rotamers) δ 152.2, 151.7, 123.8 (t, *J*=238 Hz) 93.8, 93.3, 80.0, 79.6, 66.7, 66.5, 56.7, 56.6, 34.4 (t, *J*=26 Hz), 28.3, 27.5, 26.6, 26.3, 24.3, 23.5, 23.2, 22.9, 22.8; ^{19}F-NMR δ -92 (m); IR (neat) ν 2970, 2932, 2870, 1690, 1450, 1386, 1319, 1251, 1206, 1171, 1094, 1055, 905, 852, 805, 767 cm^{-1}; HRMS (FAB) $(M+H)^+$ calculated for $C_{14}H_{26}NO_3F_2^+$ 294.1875, found 294.1879.

(*S*)-2-(*N*-*tert*-Butyloxycarbonyl)amino-5,5-difluorohexan-1-ol (8)

To a solution of acetonide (**7**) (641 mg, 2.29 mmol) dissolved in 30 mL of methanol was added Dowex-50w resin (600 mg), which was previously washed with methanol prior to use. After the mixture was stirred for 20 h at ambient temperature, the resin was filtered and a few drop of triethylamine was added to the filtrate. Concentration and flash chromatography (hexane/AcOEt = 2/1) gave Boc-amino alcohol (**8**) (500 mg, 90%) as colorless oil. $[\alpha]_D$ -15.0° (c 1.0, MeOH); ^{1}H-NMR δ 4.70 (br s, 1H), 3.68-3.54 (m, 3H), 3.00 (br s, 1H), 2.00-1.81 (m, 2H), 1.80-1.04 (m, 2H), 1.60 (t, *J*=18.3 Hz, 3H), 1.44 (s, 9H); ^{13}C-NMR δ 156.3, 123.9 (t, *J*=238 Hz) 79.9, 65.2, 52.3, 34.6 (t, *J*=26 Hz), 28.3, 28.3, 24.7 (t, *J*=4 Hz), 23.4 (*J*=28 Hz); ^{19}F-NMR δ -93 (m); IR (neat) ν 3428, 3332, 2968, 2930, 2870, 1683, 1522, 1508, 1450, 1389, 1364, 1274, 1243, 1169, 1047, 899 cm^{-1}; HRMS (FAB) $(M+H)^+$ calculated for $C_{11}H_{22}NO_3F_2^+$ 254.1562, found 254.1581.

***N*-*tert*-Butoxycarbonyl-5,5-difluoro-L-norleucine (9)**

To a solution of Boc-amino alcohol (**8**) (480 mg, 1.89 mmol) dissolved in 4 mL of AcOEt and 2 mL of water was added TEMPO (3 mg, 20 μmol), *n*-Bu_4NBr (6 mg, 20 μmol), and $NaHCO_3$ (600 mg, 7.14 mmol). To this biphasic mixture was added dropwise 5 mL of a solution of NaOCl (10% aqueous) with vigorous stirring at 0°C. After the addition, the mixture was stirred for 30 min at 0°C. Phases were separated and organic layer was extracted with saturated $NaHCO_3$ aqueous solution. Combined aqueous phases were treated with $NaHSO_3$ to decompose excess NaOCl, acidified by adding 6M HCl. The suspension was extracted with ether, and the extracts were dried over $MgSO_4$. Solvent was removed *in vacuo*, and the residue was azeotroped with benzene to remove a trace amount of water to give colorless

oil (410 mg, 81%). Compound homogeneity was confirmed by TLC analysis (hexane/ethyl acetate = 3/1 and $CHCl_3$/MeOH/AcOH = 95/5/1). $[\alpha]_D$ -11.0° (c 1.0, MeOH); ^{1}H-NMR (aggregates mixture) δ 9.96 (brs, 1H), 6.95, 5.11 (2 br d, 1H), 4.36, 4.23 (2 br s, 1H), 2.19-1.81 (m, 4H), 1.60 (t, *J*=18.3 Hz, 3H), 1.45 (s, 9H); ^{1}H-NMR (DMSO-d_6) δ 12.54 (brs, 1H), 7.15 (d, *J*=8.1 Hz, 1H), 3.90 (br t, 1H), 2.00-1.58 (m, 4H), 1.58 (t, *J*=18.0 Hz, 3H), 1.45 (s, 9H); ^{13}C-NMR δ 176.7, 175.7, 156.9, 155.5, 123.5 (t, *J*=238 Hz) 82.1, 80.5, 60.5, 53.7, 52.7 33.8, 28.2, 25.6, 23.5 (t, *J*=5 Hz), 14.1; ^{19}F-NMR δ -92 (m); IR (neat film from CH_2Cl_2) ν 3420, 3318, 2970, 1712, 1509, 1449, 1391, 1365, 1235, 1160, 851 cm^{-1}; HRMS (FAB) $(M)^+$ calculated for $C_{11}H_{19}NO_4F_2$ 267.1282, found 267.1275.

***N-tert*-Fluorenylmethoxycarbonyl-5,5-difluoro-L-norleucine (10)**

Boc-F_2Nle-OH (**9**) (133 mg, 0.5 mmol) was dissolved in 4 M HCl/dioxane and the solution was stirred for 30 min at rt. Solvent was removed *in vacuo*, and azeotroped with dioxane to remove excess HCl. Obtained oil was triturated in petroleum ether-ether to give 5,5-difluoro-L-norleucine hydrochloride as a white powder (101 mg). The powder was dissolved in 5 mL of 10% Na_2CO_3 aqueous solution. This solution was added dropwise to a solution of Fmoc-OSu (235 mg) in 3 mL of dioxane. After stirring 7 h at rt, precipitates were filtered. The filtrate was neutralized with 2 M HCl and extracted with EtOAc, the organic extracts were washed with brine and dried over Na_2SO_4. Solvent was removed in vacuo, and the resultant oil was triturated in petroleum ether to give white powder. Thus obtained compound was immediately used for solid-phase peptide synthesis without further purification.

HCO-F_2Nle-Leu-Phe-OMe (11)

To a solution of H-Leu-Phe-OMe (40 mg, 0.12 mmol) in DMF (5 mL), were added Et_3N (12 mg, 0.12 mmol), Boc-F_2Nle-OH (**9**) (33 mg, 0.12 mmol), and HOBt (18 mg, 0.13 mmol). The mixture was stirred and cooled to 0°C, then added EDCI•HCl (25 mg, 0.13 mmol). The reaction mixture was stirred for 30 min at 0°C, and for 5 h at rt. After removing the solvent *in vacuo*, the residue was solidified by adding water. Formed white solid was washed successively with 0.5 M $NaHCO_3$, 5 % $KHSO_4$ and water: yield 42 mg. The Boc-F_2Nle-Leu-Phe-OMe (24 mg, 0.05 mmol) was treated with formic acid (5 mL) for 5 h at rt. When TLC showed absence of starting material, formic acid was removed *in vacuo*, and the residue was dissolved in 2 mL of dry THF. To the solution was added EEDQ (15 mg, 0.06 mmol) in dry THF (2 mL), and the reaction mixture was stirred for 30 min at 0°C and for 5 h at rt. After removing the solvent in vacuo, the residue was solidified by water to give a white solid, which was successively washed with 0.5 M $NaHCO_3$, 5 % $KHSO_4$ and water. Yield 21 mg (91%) Peptide homogeneity was confirmed by RP-HPLC. mp. 150.5-152.0°C; ^{1}H-NMR (DMSO-d_6) δ 8.36 (d, J=7.5 Hz, 1H), 8.29 (d, J=7.5 Hz, 1H), 8.11 (d, J=7.5 Hz, 1H), 8.01 (s, 1H), 7.29-7.18 (m, 5H), 4.50-4.30 (m, 3H), 3.57 (s, 3H), 3.06-2.11 (m,

2H), 1.90-1.50 (m, 7H), 1.19 (m, 1H), 1.43-1.38 (m, 2H), 0.86 (dd, 6H). Amino acid ratios in acid hydrolysate: Leu 1.1, Phe, 0.9. MS (ESI) $(M+Na)^+$ *m/z* calculated for $C_{23}H_{33}N_3O_5F_2Na^+$ 492.23, found 492.85.

H-Tyr-D-Ala-Gly-Phe-F_2Nle-OH (12)

Wang resin (L = 0.73 mmol/g, 71 mg, 0.05 mmol), which previously swelled with DMF, was placed in a reaction vessel, and Fmoc-F_2Nle (**10)** (54 mg, 0.15 mmol) in 1.5 mL of DMF was added and mixed 15 min. Then, pyridine (40 μL, 0.5 mmol) and 2,6-dichlorobenzoyl chloride (40 μL, 0.3 mmol) were added and the mixture was stirred for 24 h. The resin was washed with DMF (5 times), then treated with 0.1M DMAP/DMF (0.1 mL) and benzoic anhydride/NMP (56.5 mg/1 mL) to cap unreacted functional group. The following steps were carried out for elongation of amino acid residues: (1) deprotection of Fmoc group with 20% piperidine in DMF (1 min and then 10 min treatment), (2) washing with DMF (2 mL x 10), (3) coupling with activated acid esters which were prepared from the reaction of the corresponding Fmoc-amino acids and DIEA (2.0 equiv.), 0.45M HBTU-HOBt mixture in DMF (1.0 equiv.), in 2 mL of DMF, and (4) washing, DMF (2 mL x 10). The coupling time was for about 50 min, and usually double coupling was applied. The completion of reaction was monitored by the Kaiser test. After coupling each residue to the resin step by step, the desired peptide was cleaved off by using TFA/water/TIS mixture (95/2.5/2.5, v/v/v) for 90 min. The TFA solution was concentrated and the residue was washed with ethyl ether three times to give a white precipitate. The precipitate was dried and purified by gel permission chromatography (Sephadex G-15, 30% acetic acid) to give desired product (34% yield). HPLC showed the 99% of purity. Amino acid ratios in acid hydrolysate: Tyr 0.9, Ala, 1.3, Gly 1.2, Phe, 1.1. MS (ESI) $(M+H)^+$ *m/z* calculated for $C_{29}H_{38}N_5O_7F_2^+$ 606.27, found 606.11.

Biological assays of synthetic peptides: All of the assay samples of synthetic peptides were HPLC pure. The biological activities of fMLP analogues for human neutrophils were evaluated according to a previously reported procedure.[10] Receptor binding potencies of Met-enkephalin analogues were assessed based on the literature procedure.[12]

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