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A convenient solid phase synthesis of *S*-palmitoyl transmembrane peptides

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Abstract—*S*-Palmitoylated peptides are important tools as models for integral membrane proteins to study peptide–lipid interactions. Herein, we report a convenient solid phase synthesis of *S*-palmitoyl transmembrane peptides. The highly acid labile *S*-(4-methoxytrityl) group is preferred over the *S*-(*tert*-butylsulfanyl) group for protection of the cysteine side chain since the latter gives rise to quantitative desulfurization during on-resin deprotection. The resulting free thiol function is modified with palmitic acid via a carbodiimide-mediated coupling and the title compounds are obtained in good yields and purity.

The covalent modification of proteins by lipidation (palmitoylation, myristoylation, and prenylation)[1] plays an important role in numerous signal transduction processes across cell membranes since these lipid modifications are used as molecular anchors of the (membrane) proteins. The increased hydrophobicity of lipidated proteins generally results in better interactions with the phospholipids of the cell membrane. These protein–lipid interactions are important for the biological function and activity of membrane proteins. Since integral membrane proteins are characterized by membrane-spanning peptide sequences, the interactions of phospholipids with these transmembrane sequences play an important role in determining the overall conformation and bioactivity of membrane proteins.[2] Model transmembrane peptides are extremely useful for investigation of protein–lipid membrane interactions. Model peptides are chemically well defined and also allow incorporation of biophysical markers (^{2}H-labels, fluorescent probes) at specific positions of the membrane-spanning segment to obtain further molecular insight into protein–lipid interactions. As examples of model transmembrane peptides, Trp-flanked poly-Leu-Ala peptides—inspired by gramicidine A, a small Trp-flanked ion channel—have been introduced by Killian et al.[3] These so-called WALP peptides[4] (e.g., Ac-Gly-[Trp]$_2$-[Leu-Ala]$_n$-Leu-[Trp]$_2$-Ala-NH$_2$, $n = 6$ to 10) form stable α-helices and their interactions with phospholipids have been studied in great detail.[5] The solid phase synthesis, purification by HPLC and analysis by mass spectrometry of these WALP peptides[6] is far from trivial, especially when these peptides carry palmitoyl chains to target them to cholesterol-rich lipid domains.[7] Palmitoylated peptides are useful as models for naturally occurring acylated membrane proteins and, when containing biophysical markers (^{2}H-labels) in the acyl chain, they can be used to probe the interface between a transmembrane peptide and the surrounding lipid bilayer. Based on the papers of Trifilieff et al.,[8] Przybylski and co-workers,[9] and Waldmann and co-workers[10] we describe here a convenient solid phase synthesis to produce *S*-palmitoyl *transmembrane* peptides. Furthermore, an HPLC protocol for purification and a mass spectrometric analysis are also described.

Our first approach for the synthesis of the title compounds consisted of the coupling of *N*-acetyl-*S*-palmitoyl cysteine **1** to the free α-amino functionality of peptide resin **2**. Compound **1** was synthesized (Scheme 1) starting from Ac-Cys-OH, which was acylated with palmitoyl chloride in the presence of TEA (96%)[11] and

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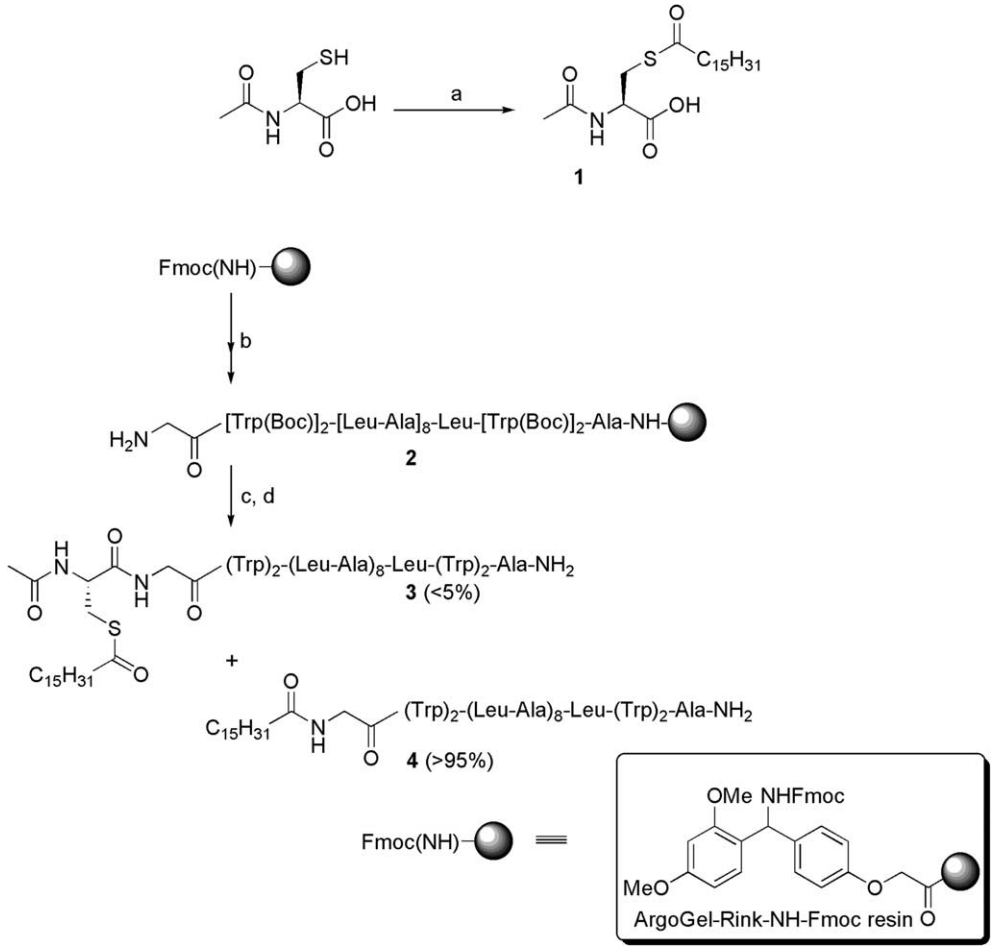
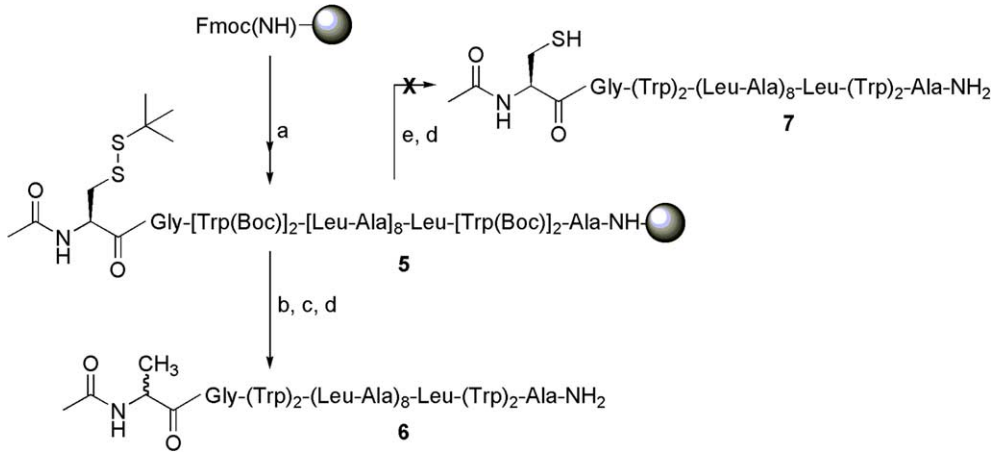


Scheme 1. Reaction conditions: (a) Pal-Cl, TEA in CH_2Cl_2, 0 °C → rt, 16 h, 96%. (b) Fmoc/^{t}Bu SPPS. (c) **1**, DIC, HOBt in NMP, rt, 16 h. (d) TFA/TIS/H_2O (95:2.5:2.5 v/v/v), rt, 3 h.

peptide resin **2** was synthesized using Fmoc/^{t}Bu solid phase peptide chemistry on an ArgoGel-Rink®-NH-Fmoc resin to yield C-terminal peptide amides[12] (Scheme 1). Since thio-esters are very labile under basic conditions, **1** was coupled in the absence of additional base with DIC/HOBt.[13] After deprotection and cleavage from the resin with TFA, mass analysis showed that instead of *S*-palmitoylated **3**, the *N*-palmitoylated peptide **4** had been formed. Apparently, aminolysis of the thio-ester is much faster than the carbodiimide-mediated peptide coupling.[14]

The next approach was based on the on-resin deprotection and modification of the N-terminal cysteine moiety (Scheme 2). Removal of the cysteine side-chain protection should be compatible with the Rink amide linker



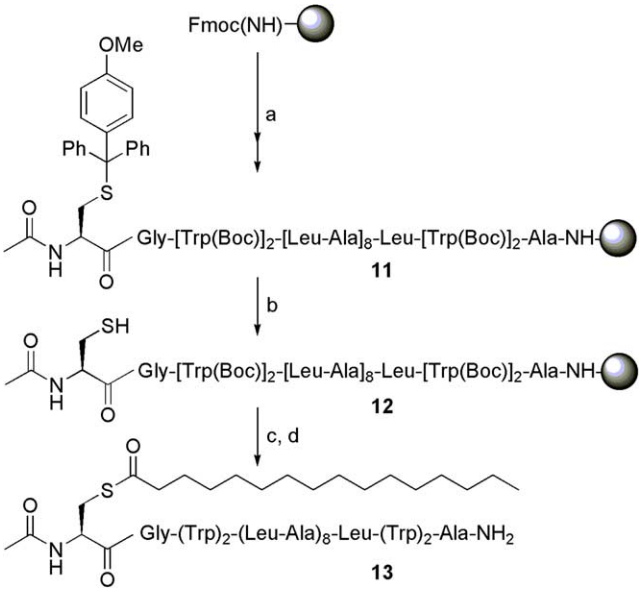
Scheme 2. Reaction conditions: (a) Fmoc/^{t}Bu SPPS. (b) $P(Bu)_3$ in NMP/H_2O (95:5 v/v), rt, 16 h. (c) Palmitic acid, DIC, HOBt in NMP/DCE (1:1 v/v), rt, 16 h. (d) TFA/TIS/H_2O (95:2.5:2.5 v/v/v), rt, 3 h. (e) β-Mercaptoethanol in NMP (1:1 v/v), rt, 16 h.

and the Trp(Boc) functionality. Therefore, Cys(Trt) was not used since the applied deprotection conditions (50% TFA)[10] were too harsh. For this purpose, cysteine was protected as its *S*-(*tert*-butylsulfanyl) derivative[15] since the on-resin deprotection of Cys(S^{t}Bu) was recently reported either with β-mercaptoethanol[8,9,16] or by treatment with a phosphine.[10,16b] Peptide resin **5** was treated with $P(Bu)_3$ in the presence of NMP/H_2O under oxygen-free conditions by flushing with a stream of nitrogen. After washing the resin, palmitic acid was coupled to the free sulfhydryl moiety with DIC/HOBt in NMP/1,2-dichloroethane (DCE). This solvent system was chosen to avoid precipitation of the palmitic acid carbodiimide-/HOBt active ester intermediate. Finally, the resin was treated with TFA to obtain the *S*-palmitoylated transmembrane peptide. However, mass analysis showed that the peptide thus obtained contained an N-terminal alanine residue (compound **6**) and not the expected Cys(Pal) moiety. Desulfurization of Cys(S^{t}Bu) to alanine as a side reaction was reported earlier[17] but not as a nearly quantitative reaction.

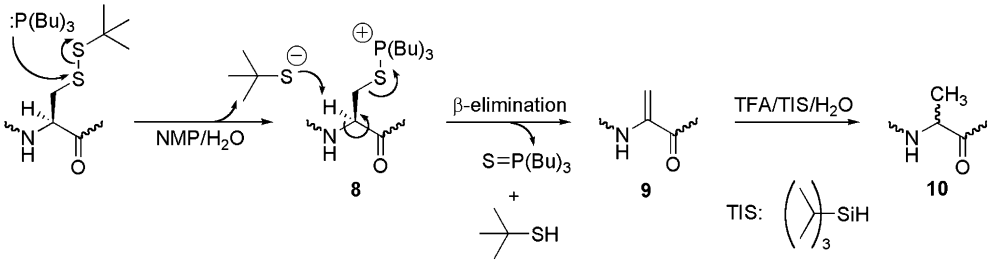
A possible mechanism for the desulfurization of Cys(S^{t}Bu) is shown in Scheme 3. β-Elimination in intermediate **8** may have resulted in formation of dehydroalanine intermediate **9**, which would be protonated by TFA treatment and then reduced by the hydride donor triisopropylsilane to yield an alanine residue in **10**, as is present in peptide **6**. A possible explanation for this surprisingly quantitative desulfurization might be that the phosphonium moiety in **8** was unable to react with H_2O due to the highly hydrophobic nature of the WALP peptide. Evidently, hydration did not take place, since no peptide with an N-terminal serine residue was observed and proton abstraction and subsequent β-elimination mediated by *tert*-butyl thiolate were apparently favored steps.[18]

Alternatively, disulfide bond reduction of the *S*-(*tert*-butylsulfanyl) moiety of resin **5** with β-mercaptoethanol was attempted.[8,9,16b] However, mass analysis did not show any desired product **7** (Scheme 2). This result was in agreement with the report of Trifilieff and Denis[8] since they described the disulfide bond reduction of the *S*-(*tert*-butylsulfanyl) moiety as very sluggish and highly dependent on the amino acid sequence.

We changed our strategy again, now using the *S*-(4-methoxytrityl)cysteine derivative,[19] which can be deprotected under very mild acidic conditions that essentially do not affect other acid-labile protecting groups commonly used in Fmoc/^{t}Bu solid phase peptide chemistry (Scheme 4). The 4-methoxytrityl (Mmt) group has been recommended by Trifilieff and Denis[8] for the on-resin synthesis of *S*-palmitoylated peptides in general. Moreover, the Mmt group was recently successfully used for the solid phase synthesis of *S*-palmitoylated and *S*-farnesylated peptides.[10] Peptide resin **11** was treated with diluted TFA (2%) in CH_2Cl_2 in the presence of triisopropylsilane as a scavenger. The presence of a free sulfhydryl moiety in peptide resin **12** was confirmed with Ellman's reagent.[20] Subsequent carbodiimide-mediated coupling with palmitic acid was complete after 16 h,[21] as incubation with Ellman's reagent did not result in a yellow color. Finally, after cleavage and deprotection, *S*-palmitoylated WALP peptide **13** was obtained in a yield of 39% (purity on HPLC: 90%). Table 1 summarizes the various lipidated WALP peptides **13**, **15**–**18**, which were synthesized in this study. All peptides were obtained in good overall yields and in good purity. Deuterated peptides **15**–**18** were synthesized for biophysical studies.[5c]



Scheme 4. Reaction conditions: (a) Fmoc/^{t}Bu SPPS. (b) TFA/TIS/CH_2Cl_2 (2:5:93 v/v/v), rt, 5 × 2 min. (c) Palmitic acid, DIC, HOBt in NMP/DCE (1:1 v/v), rt, 16 h. (d) TFA/TIS/H_2O (95:2.5:2.5 v/v/v), rt, 3 h.



Scheme 3.

Table 1.

Peptide	R_t (min)[a]	$(M+Na)^{+}$[b]	$(M+Na)^{+}$[c]
Ac-Gly-$[Trp]_2$-$[Leu\text{-}Ala]_8$-Leu-$[Trp]_2$-Ala-NH_2 **14**	43.6	2540.456	2540.658
Ac-Cys-Gly-$[Trp]_2$-$[Leu\text{-}Ala]_8$-Leu-$[Trp]_2$-Ala-NH_2 **7**	42.3	2643.466	2643.478
Ac-Cys(Pal)-Gly-$[Trp]_2$-$[Leu\text{-}Ala]_8$-Leu-$[Trp]_2$-Ala-NH_2 **13**	49.3	2881.695	2881.747
Ac-Cys(Pal-$d_{9,9}$)-Gly-$[Trp]_2$-$[Leu\text{-}Ala]_8$-Leu-$[Trp]_2$-Ala-NH_2 **15**	49.3	2883.708	2883.611
Ac-Cys(Pal-d_{31})-Gly-$[Trp]_2$-$[Leu\text{-}Ala]_8$-Leu-$[Trp]_2$-Ala-NH_2 **16**	49.3	2912.937	2912.904
Ac-Cys(Pal-$d_{13,13}$)-Gly-$[Trp]_2$-$[Leu\text{-}Ala]_8$-Leu-$[Trp]_2$-Ala-NH_2 **17**	49.3	2883.708	2883.376
Ac-Cys(Pal)-Gly-$[Trp]_2$-$[Leu\text{-}Ala]_8$-Leu-$[Trp]_2$-Ala-NH_2 d_3-Ala(13) **18**	49.3	2884.714	2884.309

[a] HPLC conditions: analytical HPLC runs were performed on an Adsorbosphere XL C4 300 Å column at a flow rate of 0.75 mL/min using a linear gradient of buffer B (100% in 60 min) from 90% buffer A (buffer A: 50 mM TEAP pH 2.25 in H_2O/CH_3CN 8:2 v/v; buffer B: 50 mM TEAP pH 2.25 in H_2O/CH_3CN/2-propanol 5:50:45 v/v/v).

[b] Calculated monoisotopic mass value.

[c] Found monoisotopic mass value as measured by MALDI-TOF analysis.

However, purification of the lipidated transmembrane peptides was rather difficult due to their high hydrophobicity and their tendency to form stable hydrophobic aggregates in which impurities were enclosed. Moreover, each of the desired peptides was found in several fractions due to excessive peak broadening, which hampered purification. Probably, this was the result of oligomerization, either in solution or on the column. Aqueous acetronitrile buffers, as described previously,[8,10a] could not be used since elution of the highly hydrophobic transmembrane peptides was only possible in the presence of 2-propanol as an organic modifier. An HPLC protocol for the analysis and purification of the lipidated WALP peptides was optimized, based on literature reports.[9,22] Thus, it was found that the title compounds (**13**, **15**–**18**) could be purified using a reverse-phase C4 column and elution with a mixture of an aqueous phosphorous acid/triethylamine buffer pH 2.25/acetonitrile/2-propanol 5:50:45 v/v/v.[23] The retention times of the WALP peptides are given in Table 1. They were characterized by MALDI-TOF analysis.[24] Due to their hydrophobic character, they were obtained as sodium adducts (Table 1).

In conclusion, we have developed a convenient solid phase synthesis of *S*-palmitoylated *transmembrane* peptides. Quantitative desulfurization occurred during deprotection of *S*-(*tert*-butylsulfanyl)cysteine by treatment with tributylphosphine. To circumvent this undesired side reaction, the cysteine residue was protected with the highly acid labile 4-methoxytrityl functionality. Selective removal of this protecting group allowed the on-resin coupling of the free sulfhydryl moiety with palmitic acid and other fatty acids to give the lipidated peptides in good yields and purities.

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11. Ac-Cys(Pal)-OH **1** was recrystallized from acetone and obtained as a white solid (96%). R_f (DCM/MeOH 95:5 v/v): 0.18; mp: 61–63 °C; $[\alpha]_D^{25}$: −5.0 (*c* 1, $CHCl_3$); ES-MS (50 eV) calcd for $C_{21}H_{39}NO_4S$ (401.25): *m/z* (%) 402.45 (100%) $(M+H)^+$, 425.45 (32%) $(M+Na)^+$, 440.55 (61%) $(M+K)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ = 7.98 (br s, 1H), 6.95 (d, 1H, *J* 7.1 Hz), 4.70 (m, 1H), 3.41–3.29 (m, 2H), 2.58 (t, 2H, *J* 7.4 Hz), 2.04 (s, 3H), 1.65 (m, 2H), 1.23 (br s, 24H), 0.88 (t, 3H, *J* 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 199.9, 172.4, 171.7, 52.7, 43.9, 31.8, 29.6, 29.4, 29.3, 29.2, 22.6, 14.0.
12. Peptide resins **2**, **5**, and **11** were synthesized automatically on an Applied Biosystems 433A Peptide Synthesizer using the FastMoc protocol (Fields, C. G.; Lloyd, D. H.; Macdonald, R. L.; Otteson, K. M.; Noble, R. L. *Peptide Res.* **1991**, *4*, 95–101) on a 0.25 mmol scale (single coupling, conditional capping). *N*-α-9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids with the side chain of tryptophan protected by *tert*-butyloxycarbonyl (Boc) and the cysteine side chain protected by either an *S*-

(*tert*-butylsulfanyl: S′Bu) or an *S*-(4-methoxytrityl: Mmt) group were used. Couplings of *N*-α-Fmoc amino acids (1 mmol, 4 equiv) were performed with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole (HBTU/HOBt: Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillesen, D. *Tetrahedron Lett.* **1989**, *30*, 1927–1930) in the presence of 8 equiv *N*,*N*-diisopropylethylamine (DIPEA) in *N*-methylpyrrolidone (NMP) for 45 min. Fmoc removal was carried out with 20% piperidine in NMP for 70 s. Any remaining amino groups after incomplete coupling were acetylated by acetic anhydride/DIPEA/HOBt in NMP. After removal of the final Fmoc group the resin was extensively washed with NMP and CH_2Cl_2 (resin **2**) or acetylated with acetic anhydride (resins **5** and **11**). Finally, the peptide resin was dried in a vacuum desiccator.

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21. Peptide resin **11** (1 equiv) was washed with CH_2Cl_2 (3 × 2 min, 10 mL) and subsequently treated with a mixture of TFA/TIS/CH_2Cl_2 2:5:93 v/v/v (5 × 2 min, 10 mL) to remove the 4-methoxytrityl functionality. Then, the resin was washed with CH_2Cl_2 (3 × 2 min, 10 mL) and NMP (3 × 2 min, 10 mL) followed by addition of palmitic acid (4 equiv), DIC (4 equiv) and HOBt (4.4 equiv) dissolved in NMP/DCE (10 mL, 1:1 v/v). After a reaction time of 16 h, the resin was washed with NMP (5 × 2 min, 10 mL) and CH_2Cl_2 (5 × 2 min, 10 mL). The peptide was deprotected and cleaved from the resin by treatment with TFA/TIS/H_2O 95:2.5:2.5 v/v/v for 3 h. The resulting TFA solution was poured into ice-cold methyl *tert*-butyl ether (MTBE)/hexane 1:1 v/v in order to precipitate the peptide. After centrifugation, the peptide pellet was dissolved in ^{t}BuOH/H_2O 1:1 v/v and lyophilized.
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23. HPLC conditions: *analytical runs*: buffer A: 50 mM TEAP pH 2.25 in H_2O/CH_3CN 8:2 v/v, buffer B: 50 mM TEAP, pH 2.25 in H_2O/CH_3CN/2-propanol 5:50:45 v/v/v, column: Adsorbosphere XL C4 300 Å (250 mm × 4.6 mm), gradient: 10% B → 100% B in 60 min at 0.75 mL/min; *preparative runs*: buffer A: 50 mM TEAP, pH 2.25 in H_2O/CH_3CN 8:2 v/v, buffer B: 50 mM TEAP, pH 2.25 in H_2O/CH_3CN/2-propanol 5:50:45 v/v/v, column: Phenomenex Jupiter C4 300 Å (250 mm × 22 mm), gradient: 10% B → 100% B in 90 min at 4.5 mL/min. Pure fractions were pooled and lyophilized. The residue was eluted over a Sephadex LH 20 column (145 cm × 3 cm) with CH_2Cl_2/MeOH 1:1 v/v at a flow of 2.5 mL/min. Pure peptide fractions were pooled and the solvent was removed in vacuo, the resulting lipopeptide was obtained as a waxy powder.
24. MALDI-TOF analyses were performed on a Kratos Axima CFR apparatus, with ACTH (18-39) as an external reference (monoisotopic $(M+H)^+$: 2465.1989) and α-cyano-4-hydroxycinnamic acid as matrix. The peptide (1 mg) was dissolved in TFA (50 μL) and diluted with TFE (450 μL). 10 μL of this solution was mixed with a saturated solution of matrix (0.1% TFA in H_2O/CH_3CN; 10 μL) and 0.5 μL of this peptide/matrix mixture was spotted on the MALDI plate.