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Application of gel-phase ^{19}F NMR spectroscopy for optimization of solid-phase synthesis of a hydrophobic peptide from the signal sequence of the mucin MUC1

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Abstract: This paper describes the manual Fmoc/*t*-Bu solid-phase synthesis of a difficult nine-residue hydrophobic peptide LLLLTVLTV from one of the signal sequences that flank the tandem repeat of the mucin MUC1. Gel-phase ^{19}F NMR spectroscopy was used as a straightforward method for optimization of the solid-phase synthesis. Different approaches were applied for comparative studies. The strategy based on modified solid-phase conditions using DIC/HOAt for coupling, DBU for Fmoc deprotection, and the incorporation of the pseudo proline dipeptide Fmoc-Leu-Thr($\psi^{Me,Me}$ pro)-OH as a backbone-protecting group was found to be superior according to gel-phase ^{19}F NMR spectroscopy. Implementation of the optimized Fmoc protocol enabled an effective synthesis of signal peptide LLLLTVLTV. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid-phase peptide synthesis; difficult sequence; gel-phase ^{19}F NMR; pseudo proline

INTRODUCTION

The tumor-associated human mucin MUC1 is a heavily *O*-glycosylated transmembrane protein expressed on the surfaces of many types of glandular tissues. Its expression is upregulated in many carcinomas, notably ovarian, breast, prostate and pancreatic cancers [1,2]. It is intensively studied as a broadly applicable and potential target for antigen-specific immunotherapy of cancer [3–6]. The peptide LLLLTVLTV derived from the signal sequence of MUC1 [5] has been subjected to computer-assisted analysis, and the peptide presumably has a high probability of binding to the MHC I (human class I major histocompatibility) molecule, HLA-A2 [7]. Recently, it was found that T-cells recognizing this peptide, identified in the human system, are expanded in breast cancer patients [8].

Solid-phase synthesis is today used to prepare a wide variety of organic molecules [9–13]. Although solid-phase peptide synthesis (SPPS) has been developed during last four decades, challenges still remain. Certain peptides resist facile synthesis and a class of peptides known as *difficult sequences* has been identified [14–18]. Their properties are a consequence of interchain association of the intermediate resin-bound peptides to form hydrogen-bonded β-sheets [16,19], sequence-related incomplete aminoacylations [17,19] and difficult coupling steps due to steric hindrance at single amino acids with bulky side chains or protecting groups [15]. In addition, incomplete Fmoc deblocking by standard piperidine solution in DMF is frequently encountered [20,21]. It has been demonstrated that the stronger and nonnucleophilic amidine DBU is a suitable alternative to the more commonly used piperidine for the efficient and rapid cleavage of the terminal N^{α}-Fmoc group in the standard Fmoc protocol [22]. However, it should be noted that basic conditions might result in side reactions such as aspartimide formation in aspartic acid-containing peptides [23].

Several approaches aimed at overcoming intramolecular aggregation have been reported and include certain solvents or solvent mixtures that disrupt internal aggregation [24–26], the use of chaotropic salts as additives [27], the use of a fully solvated polymer matrix, which favors the random coil formation [28], elevated temperatures, solubilizing protecting groups and backbone protection of amino acid building blocks [16,29]. Peptides that contain proline residues spaced along a peptide chain often result in prosperous synthesis by, without much doubt, disrupting secondary structures [30] and hence preventing interchain hydrogen bonding. On the basis of this fact, reagents devised to break apart secondary structures, e.g. Hmb-protected amino acids and the Ser/Thr-derived oxazolidine and Cys-derived thiazolidine building blocks (pseudoproline dipeptides ψ-Pro) have been developed [31,32]. Comparative studies with Hmb backbone protection indicated that pseudoprolines can permit better and faster amino acid couplings [33].

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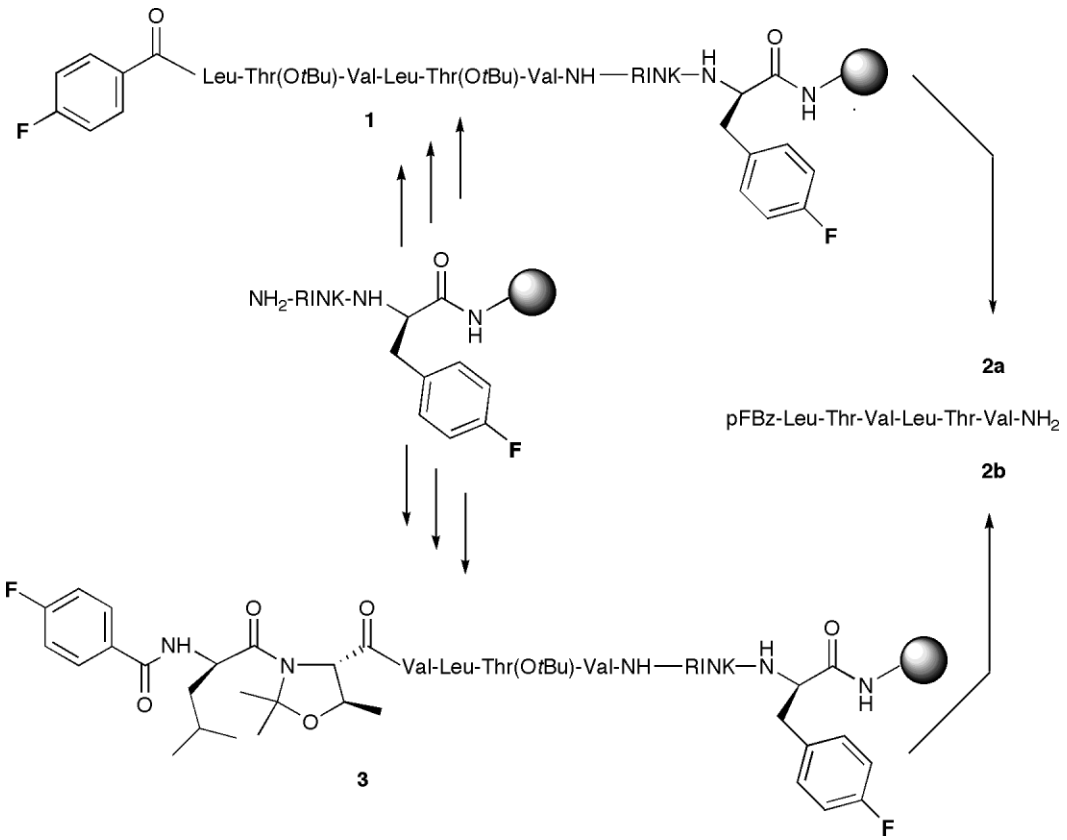

Scheme 1 SPPS of resin **1** and resin **3**. Amino acids activated with HOBt (6 equiv.)/DIC (3.9 equiv.), Fmoc-Leu-Thr($\psi^{Me,Me}$pro)-OH (1.08 equiv.) activated as HOAt (3 equiv.)/DIC (1.2 equiv.); 20% piperidine in DMF as Fmoc deprotecting agent. Cleavage from the support was carried out with TFA/H_2O/thioanisole/ethanedithiol (8.75 : 0.5 : 0.5 : 0.25).

In spite of the developments in the area of solid-phase synthesis, there still exists a need for improved methods for monitoring coupling efficiency. Color test methods, e.g. the Kaiser test [34] and noninvasive continuous acid–base indicators [35], have served as quantitative assessment techniques to determine the amino acid coupling step completion. The utilization of analytical tools such as MALDI-TOF [36,37], infrared spectroscopy [38,39], gel-phase and high-resolution magic-angle-spinning NMR spectroscopy [40–42] is somewhat restricted by the cost and the specialized instruments required. During the last decade ^{19}F NMR spectroscopy was successfully applied as a robust and adaptable monitoring tool in solid-phase chemistry [43–46]. The nucleus ^{19}F has several favorable advantages, such as 100% abundance, a high magnetogyric ratio of approximately 0.9 times that of the proton and wide spectral range, that make the resonances of fluorine nuclei well separated. In addition, the resin and nonfluorinated solvents are transparent during the recording of the spectra. Fluorinated linkers have been turned to practical use as internal standards in combination with fluorinated building blocks [43], including those bearing fluorine-labeled protecting groups [45,47], allowing qualitative and quantitative analysis, including the stereochemical outcome, of reactions performed on the solid support. In this work, we used gel-phase ^{19}F NMR spectroscopy for optimizing and monitoring the progress of manual SPPS of the MUC1-derived peptide LLLLTVLTV.

RESULTS AND DISCUSSION

Our goal was to find an optimal procedure for the synthesis of the peptide LLLLTVLTV in a mechanically agitated standard reactor. Since the sequence contains a significant number of hydrophobic amino acids, which may contribute to the internal association of the resin-bound intermediates during peptide assembly, we decided to investigate potential complications by means of gel-phase ^{19}F NMR spectroscopy. First, the reference amino acid Fmoc-*p*-fluoro-phenylalanine followed by the RINK amide linker was coupled to a Tentagel S NH_2 solid support with HOBt/DIC in DMF (Scheme 1). Tentagel contains long PEG tethers that play a key role in obtaining good-quality spectra and exhibits good swelling properties and high reaction rates as demonstrated in previous studies [45,48]. First, we studied if any aggregation did occur before the full sequence was completed, by the synthesis of resin **1**

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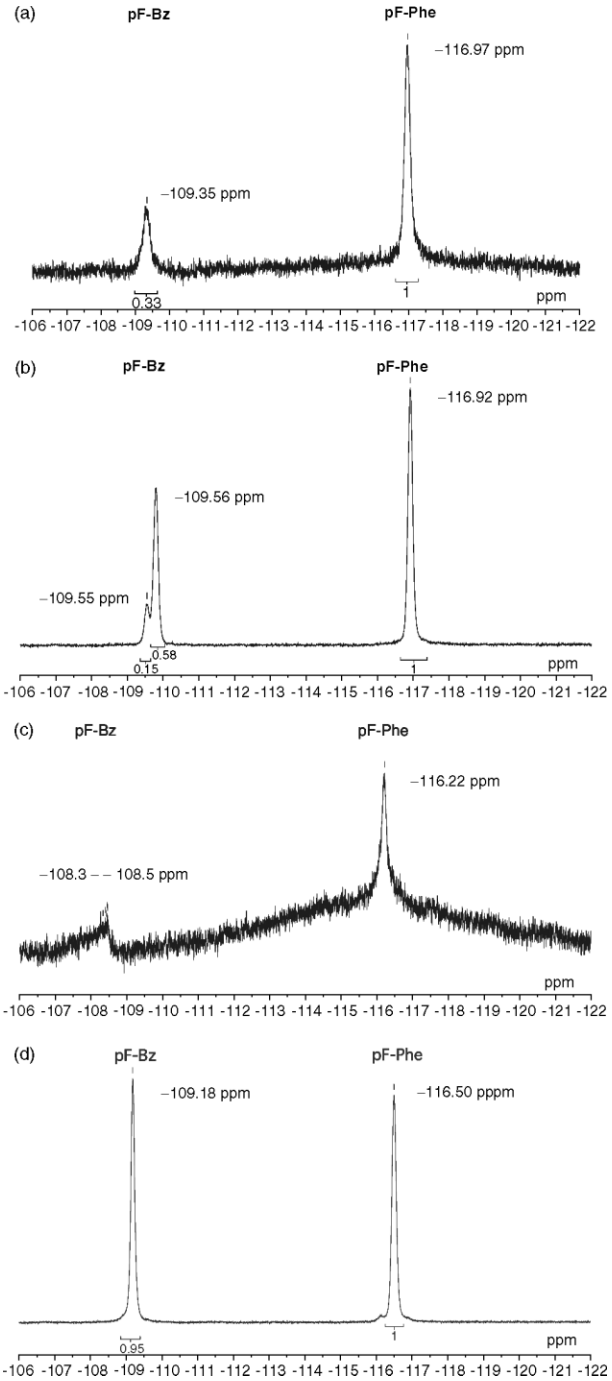

Figure 1 ^{19}F NMR spectra of resins (a) **1**, (b) **3**, (c) **4** and (d) **6** in DMF-d_7.

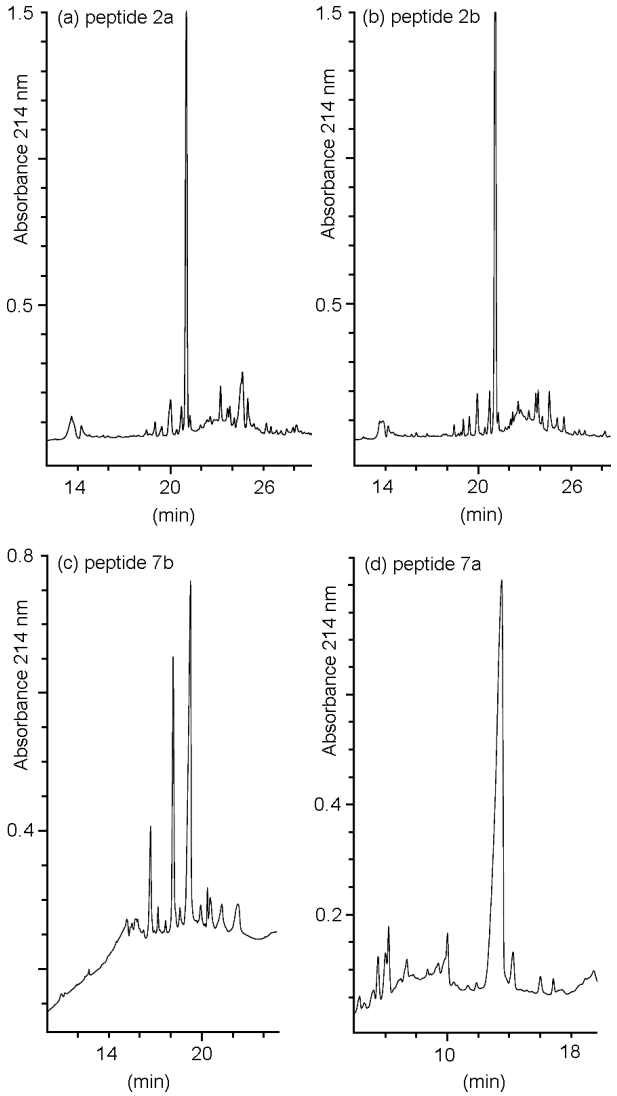

Figure 2 Analytical reversed-phase HPLC chromatograms of crude cleaved peptides: (a) **2a**, gradient 0 → 100% CH_3CN in H_2O, both containing 0.1% TFA, for 40 min; (b) **2b**, gradient 0 → 100% CH_3CN in H_2O, both containing 0.1% TFA, for 40 min; (c) **7b**, gradient 0 → 100% CH_3CN in H_2O, both containing 0.1% TFA, for 20 min; and (d) **7a**, gradient 50 → 100% CH_3CN in H_2O, both containing 0.1% TFA, for 30 min.

comprising the first six amino acids terminated by a *p*-fluoro-benzoyl group (Scheme 1). The amino acids were activated with HOBt/DIC, and 20% piperidine in DMF was used as Fmoc deprotecting agent. *p*-Fluoro-benzoic acid was coupled to a small aliquot of the hexapeptide resin. After completion of the synthesis of resin **1**, a gel-phase ^{19}F NMR spectrum was recorded. Although it was possible to observe the two fluorine resonances at −109.35 and −116.97 ppm (Figure 1(a)) originating from *p*-fluoro-benzoic acid and *p*-fluoro-phenylalanine, respectively, the spectral quality was low, and integration of the ^{19}F signals indicated a maximum yield of 30%. Resin **1** was treated with a TFA:H_2O cleavage cocktail and the identity of the resulting peptide **2a** (Scheme 1) was confirmed by analytical LC-MS. Besides the peptide **2a**, LC-MS analysis (HPLC UV profile spectrum in Figure 2(a)) showed small amounts of the *N*-terminal deletion peptide (TVLTV) and the Fmoc-hexapeptide resulting from incomplete Fmoc deprotection. As judged by the results above, we decided to synthesize resin **3** (Scheme 1) in which the fifth and the sixth

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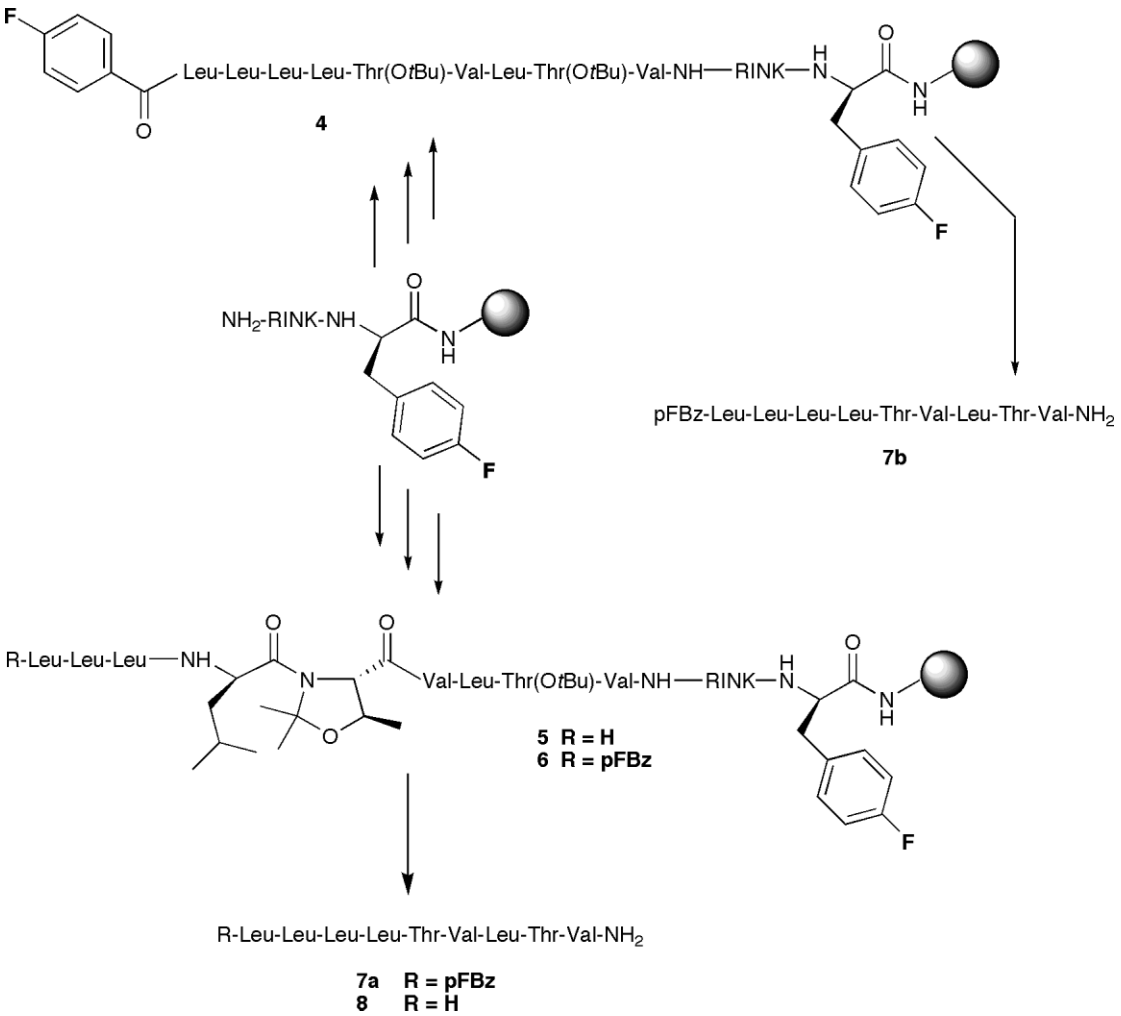

Scheme 2 SPPS of resin **4** and resin **6**. Amino acids activated with HOAt (4 equiv.)/DIC (4 equiv.), Fmoc-Leu-Thr($\psi^{Me,Me}$pro)-OH (1.08 equiv.) activated as HOAt (3 equiv.)/DIC (1.2 equiv.); 2% DBU in DMF as Fmoc deprotecting agent. Cleavage from the support was carried out with TFA/H_2O/thioanisole/ethanedithiol (8.75 : 0.5 : 0.5 : 0.25) for peptide **7b** and TFA/H_2O (9 : 1) for **7a** and **8**.

amino acid (threonine and leucine, respectively) were replaced with the commercially available pseudo proline dipeptide Fmoc-Leu-Thr-($\psi^{Me,Me}$pro)-OH. This type of building block breaks interchain hydrogen bonding by introducing a conformational kink in the peptide chain during SPPS [32,49]. HOAt and DIC were used for the formation of the pseudo proline active ester that was coupled to valine in position four. Finally *p*-fluoro-benzoic acid was coupled to the peptide resin as described for resin **1**. For an aliquot of resin **3** ^{19}F NMR spectrum was recorded. As shown in Figure 1(b) the quality of the spectrum of resin **3** was considerably better than that for resin **1** (Figure 1(a)). Besides the *p*-fluoro-phenylalanine signal, the ^{19}F NMR spectrum for resin **3** had a split peak corresponding to the *p*-fluoro-benzoyl group. The integral value for the *p*-fluoro-benzoyl signals was approx. 75% of that for the *p*-fluoro-phenylalanine reference signal, showing a maximum 75% yield of the fluorobenzoylated peptide. Identity of the cleaved fluoro-tagged peptide **2b** (Scheme 1) was confirmed with analytical LC-MS (HPLC UV profile in Figure 2(b)). A small trace of Fmoc-hexapeptide was identified as the main impurity. Introduction of the pseudo proline building block clearly improved the yield, presumably by disruption of secondary structure, but the overall result was still unsatisfactory. A reasonable explanation for the modest yield could be the incomplete Fmoc deprotection by standard piperidine solution in DMF. DBU in low concentration in DMF has served as a suitable alternative to piperidine [20,22,50,51] and it has been successfully applied in the syntheses of many hydrophobic sequences in automated syntheses [50,51]. Therefore we attempted to synthesize the new resins **4** and **6** with terminal *p*-fluoro-benzoic acid as a fluorous tag placed on leucine in the ninth position using 2% DBU solution in DMF as outlined in Scheme 2. Resin **4** was produced from standard amino acids and *p*-fluoro-benzoic acid that were activated with HOAt/DIC. For an aliquot of resin **4**, a gel-phase ^{19}F NMR spectrum was recorded.

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As shown in Figure 1(c) the spectrum recorded in DMF-d_7 was of bad quality presumably because of the extensive aggregation of resin-bound peptides and very poor yield of the fluoro-tagged peptide. Performing additional ^{19}F NMR experiments in other solvents such as DMSO-d_6 and $CDCl_3$ and at elevated temperatures did not result in spectra of satisfactory quality (not shown). Obviously the application of DBU without using the pseudo proline building block was insufficient. Besides the main fluoro-tagged peptide **7b**, analytical LC-MS detected significant amounts of the truncated octapeptide and its Fmoc-protected derivative (HPLC UV profile in Figure 2(c)). A small aliquot of resin **6** was prepared from standard amino acids and the isopropylidene dipeptide Fmoc-Leu-Thr-($\psi^{Me,Me}$pro)-OH activated with HOAt/DIC, which was introduced in position five and six in the peptide sequence, as shown in Scheme 2. Before coupling of the *p*-fluoro-benzoic acid, a portion of resin **5** was set aside for subsequent cleavage to give the target peptide **8**. Gel-phase ^{19}F NMR of resin **6** in DMF-d_7 afforded a spectrum of very good quality as shown in Figure 1(d). Aggregation was clearly thwarted and two resonances at −109.18 and −116.50 ppm could be easily assigned to the terminal *p*-fluoro-benzoic acid and *p*-fluorophenyl tag, respectively. Moreover, the integral value of the *p*-fluoro-benzoyl group signal indicated an almost quantitative yield of the peptide resin **6**. Clearly DBU solution in DMF enhanced the efficiency of removal of Fmoc-protecting groups. It can be rationalized by the greater basicity of DBU and the increased DMF concentration, which provide better solvation of the support and affords facile reactions. The purity and identity of the fluoro-tagged peptide **7a** were confirmed with LC-MS and HPLC (for UV profile see Figure 2(d)) after cleavage from the solid support. These observations prompted us to proceed with the cleavage (TFA/H_2O) of the remaining resin **5** not flanked by the fluorobenzoic tag. The crude product was purified on a reversed-phase HPLC column, which gave peptide **8** (Scheme 2 and Figure 3) in 37% yield based on resin loading. Peptide **8** was fully characterized by means of LC-MS (ES), fast atom bombardment (FAB) spectrometry, amino acid analysis, tandem LC-MSMS and ^{1}H NMR spectroscopy. On the basis of two-dimensional NMR experiments, we were able to assign all important chemical shifts for all amino acids (Table 1).

Gel-phase ^{19}F NMR spectroscopy has been used as a tool to assess the favorable outcome of SPPS of a hydrophobic peptide from the mucin MUC1. Further improvements of this methodology would benefit from a base-sensitive fluorinated N$^{\alpha}$-protective group and use of a recently described NMR tube reactor [52]. Standard Fmoc strategy led to the formation of β-sheets and low yields of intermediate peptides as determined by ^{19}F NMR. Application of a modified Fmoc protocol with 2% DBU in DMF as Fmoc deblocking agent in combination with the isopropylidene dipeptide Fmoc-Leu-Thr-($\psi^{Me,Me}$pro)-OH was the key to the successful synthesis of peptide **8**.

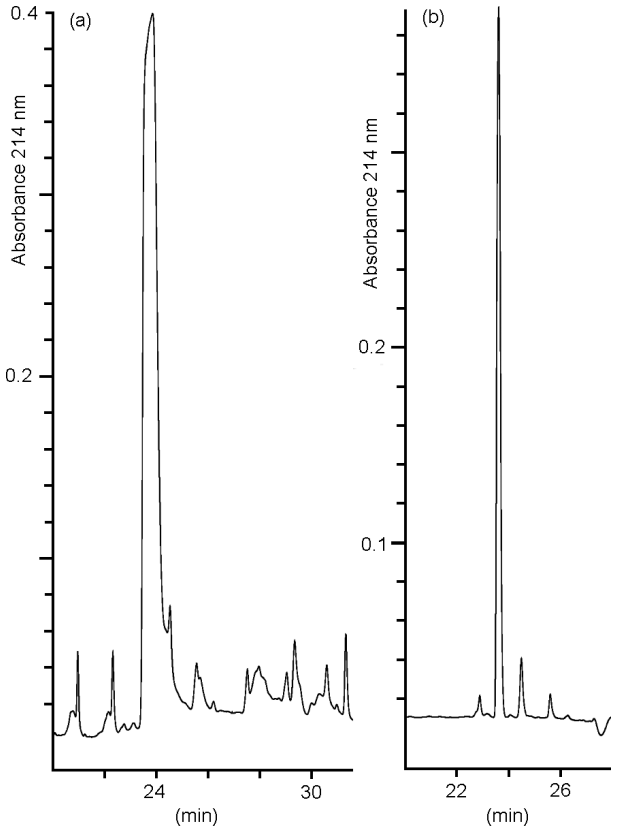

Figure 3 Analytical reversed-phase HPLC chromatograms of (a) crude peptide before purification, gradient 0 → 80% CH_3CN in H_2O, both containing 0.1% TFA, for 40 min and (b) purified nonapeptide LLLLTVLTV, gradient 10 → 90% CH_3CN in H_2O, both containing 0.1% TFA for 45 min.

Table 1 ^{1}H NMR chemical shifts (δ, ppm) for the amino acids in peptide **8**[b]

	NH	Hα	Hβ	Hγ	other
Leu^9	8.11	3.77	1.50[a]	1.50[a]	0.88
Leu^8	8.56	4.43	1.43	1.61	0.88
Leu^7	8.21	4.34	1.44	1.55	0.83
Leu^6	7.98	4.37	1.46	1.58	0.84
Thr^5	7.76	4.25	3.98	1.00	—
Val^4	7.58	4.21	2.00	0.82[a]	—
Leu^3	8.08	4.38	1.48	1.59	0.86
Thr^2	7.77	4.27	4.01	1.00	—
Val^1	7.46	4.12	1.99	0.84[a]	—

[a] Degeneracy has been assumed.
[b] Recorded at 500 MHz, 298 K, with DMSO ($\delta_H = 2.50$) as internal standard.

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MATERIALS AND METHODS

Analytical reversed-phase HPLC of peptides was performed on a Beckman System Gold HPLC, using Supelco Discovery BIO Wide Pore C-18 column (250 × 4.6 mm, 5 μm) and Kromasil C-8 column (250 × 4.6 mm, 5 μm, 100 Å) and using linear gradients of MeCN in H_2O with a flow rate of 1.5 ml min^{-1} and detection at 214 nm (both solvents contained 0.1% trifluoroacetic acid). Preparative purifications of the crude peptides were performed on Supelco Discovery BIO Wide Pore C-18 column (250 × 21.2 mm, 5 μm) using the same eluent system and a flow rate of 11 ml min^{-1} and detection at 214 nm on the same HPLC system. Analytical reversed-phase LC-MS (Waters Micromass ZQ) was performed on a XTerra C-18 column (50 × 4.9 mm, 5 μm, 125 Å), eluted with a linear gradient of MeCN in H_2O with a flow rate of 1.5 ml min^{-1} and detection at 214 nm and 254 nm (both solvents contained 0.2% formic acid).

^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-400 or ARX-500 spectrometer (Massachusetts, USA). All NMR experiments were conducted at 298 K using $CDCl_3$ (residual $CHCl_3$ at 7.26 ppm (δ_H)), DMSO-d_6 (residual DMSO-d_5 at 2.50 ppm (δ_H) and 39.60 ppm (δ_C)). Proton resonances were assigned from appropriate combinations of DQF-COSY, ROESY, TOCSY experiments.

^{19}F NMR spectra were recorded with a Bruker DRX-400 spectrometer operating at 376 MHz for resin suspensions in DMF-d_7, DMSO-d_6 and $CDCl_3$ ($CFCl_3$ (δ_F 0.00 ppm) as internal standard) at 298, 300 and 318 K. The resin was suspended in the NMR solvent and then transferred to a standard NMR tube using a Teflon tube connected to a syringe. Excess solvent was removed by suction with a syringe equipped with a Teflon tube. Two peaks appear in the spectra at approx. 0.00 ppm, one resonance originates from $CFCl_3$ inside the polymer, while the other resonance is derived from $CFCl_3$ outside the polymer. The peak with the highest chemical shift was used as an internal standard.

Mass spectra were recorded on a Waters Micromass ZQ using positive electrospray ionization (ES+). High-resolution mass spectra were recorded on a JEOL SX102 A mass spectrometer. Ions for FABMS were produced by a beam of xenon atoms (6 keV) from a matrix of glycerol and thioglycerol.

Solid-Phase Synthesis of 1–7 and a Signal Peptide 8

Peptide chain assembly was carried out in the mechanically agitated reactor on a Tentagel S NH_2 resin (typically 90–120 μmol, Rapp Polymere, Germany) with the RINK amide linker {*p*-[α-fluoren-9-ylmethoxyformamido)-2,4-dimethoxybenzyl]phenoxyacetic acid} [53] (Bachem AG, Switzerland) and pF-phenylalanine as internal standard. Standard N^{α}-Fmoc amino acids (Neosystem, France and Bachem, Switzerland) with the *tert*-butyl (*t*-Bu) protecting group for threonine were used in the synthesis. DMF was distilled before use. Fmoc amino acids or *p*-fluoro-benzoic acid (4 equiv.) were activated as benzotriazolyl esters by using DIC (3.9 equiv.) and HOBt (6 equiv.) in dry DMF for resin **1** and **3** and as 7-azabenzotriazolyl esters by using DIC (4 equiv.) and HOAt (4 equiv.) in dry DMF for resins **4** and **6**. Acylations were performed for 2–24 h and were monitored by using bromophenol blue [35] (20 mM solution in DMF, 1 : 200 v/v) [54] as indicator (the color of the reaction mixture changes from blue to yellow). Reagent solutions were added manually to the reactor. Fmoc removal was achieved with 20% piperidine in DMF flow for 3 min and further by shaking for 5 min during synthesis of resin **1** and **3** and with 2% DBU in DMF flow for 3 min and further by shaking for 5 min during synthesis of resins **4** and **6**. Fmoc removal by a flow of DBU or piperidine in DMF was carried out by applying vacuum to the reactor outlet and by continuous addition of solution during 3 min. Washings between reactions were carried out with DMF, and no intermediate amine capping steps were done. The pseudo proline dipeptide Fmoc-Leu-Thr($\psi^{Me,Me}$pro)-OH (Bachem, Switzerland)(1.08 equiv.) was activated in dry DMF (1 ml) at room temperature for 20 min by addition of DIC (1.2 equiv.) and HOAt (3 equiv.). Activated ester was then double-coupled to the peptide resin for 6–20 h.

After completion of the synthesis, aliquotes (approx. 30 μmol) of peptide resins **1**, **3**, **4**, **5** and **6** were washed with CH_2Cl_2 and dried under vacuum. Cleavage of fluorotagged peptides (**2a**, **2b**, **7b**) from the TentaGel resin and removal of acid labile protective groups were performed with TFA/H_2O/thioanisole/ethanedithiol (8.75 : 0.5 : 0.5 : 0.25, v/v) cocktail, and with TFA/H_2O (9 : 1, v/v) cocktail for peptides **7a** and **8** at room temperature for 3 h. The cleaved resins were washed thoroughly with TFA and AcOH and the combined filtrates were evaporated by a rotatory evaporator. AcOH was added again to filtrates, which were then concentrated after each addition. The residues were dissolved in a mixture of AcOH/H_2O/MeCN and then lyophilized to give white fluffs. The purity and identity of fluorinated intermediate peptides **2a**, **2b**, **7a**, **7b** were confirmed by analytical reversed-phase HPLC and LC-MS.

Resin **1**: ^{19}F NMR (376 MHz, DMF-d_7) δ − 109.35, −116.97 (2 singlets, 1 F each); resin **3**: ^{19}F NMR (376 MHz, DMF-d_7) δ − 109.55, −109.56, −116.92 (3 singlets, 1 F each); resin **4**: ^{19}F NMR (376 MHz, DMF-d_7) δ − 108.3 to −108.5 (broad singlet, 1 F), −116.22 (singlet, 1 F); resin **6**: ^{19}F NMR (376 MHz, DMF-d_7) δ − 109.18, −116.50 (2 singlets, 1 F each).

L-Leucyl-L-Leucyl-L-Leucyl-L-Leucyl-L-Threonyl-L-Valyl-L-Leucyl-L-Threonyl-L-Valine Amide 8

Resin **5** was synthesized according to general procedure, cleaved (90 μmol) and purified by reversed-phase HPLC (gradient 10 → 90% CH_3CN in H_2O, both containing 0.1% TFA for 45 min) giving **8** (33 mg, 70% peptide content, 37% yield). MSFAB : calcd for $C_{48}H_{91}N_{10}O_{11}$ 983.6870 m/z $(M+H)^+$, observed 983.6869. For ^{1}H NMR data, see Table 1. Amino acid analysis: Leu 5.01 (5), Thr 1.99 (2), Val 1.99 (2).

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