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Synthesis and application of *N*-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl] (Fde)-protected amino acids for optimization of solid-phase peptide synthesis using gel-phase ^{19}F NMR spectroscopy

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***N*-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl] (Fde) protected amino acids have been prepared and applied in solid-phase peptide synthesis monitored by gel-phase ^{19}F NMR spectroscopy. The Fde protective group could be cleaved with 2% hydrazine or 5% hydroxylamine solution in DMF as determined with gel-phase ^{19}F NMR spectroscopy. The dipeptide Ac-L-Val-L-Val-NH_2 12 was constructed using Fde-L-Val-OH and no noticeable racemization took place during the amino acid coupling with *N,N'*-diisopropylcarbodiimide and 1-hydroxy-7-azabenzotriazole or Fde deblocking. To extend the scope of Fde protection, the hydrophobic nonapeptide LLLLTVLTV from the signal sequence of mucin MUC1 was successfully prepared using Fde-L-Leu-OH at diagnostic positions. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: solid-phase peptide synthesis; gel-phase ^{19}F NMR spectroscopy; fluorinated α-amino protective group; difficult peptide

Introduction

Gel-phase ^{19}F NMR spectroscopy is a useful and robust method to monitor the progress of reactions on solid support as exemplified in several recent publications [1–9]. Fluorinated linkers and building blocks, and fluorinated protective groups allow quantitative and qualitative analysis of the reaction outcome. Not long ago, we used this analytical tool to optimize the solid-phase synthesis of a 'difficult' nine-residue hydrophobic peptide LLLLTVLTV [10], which is a part of the signal sequence from the mucin MUC1 [11]. The optimization resulted in a modified Fmoc protocol based on HOAt and DIC for amino acid couplings and 2% DBU in DMF for Fmoc removal. Peptide chain aggregation was prevented by introduction of the Fmoc-Leu-Thr($\psi^{Me,Me}$ pro)-OH pseudoproline dipeptide building block. The strategy however required that a small aliquot of the peptide resin be permanently terminated with a fluorinated benzoyl group in order to allow estimation of problems such as aggregation and incomplete couplings and deprotections using gel-phase ^{19}F NMR spectroscopy. Therefore, we decided to improve the practicability and efficiency of the method by the development of a fluorinated alpha-amino protective group compatible with the Fmoc protocol.

Results and Discussion

We based our reasoning on the known Dde [(4,4-dimethyl-2,6-dioxohexylidene)ethyl] protective group [12,13] which has been used for protection of the lysine side-chain [13] and construction of side-chain modified peptides [14]. The Dde group is cleaved with 2% hydrazine in DMF solution and is relatively stable to piperidine and TFA. The strategy was to introduce a fluorine atom by replacement of the two methyl groups at position 4 in the Dde structure with a *p*F-phenyl ring. First, compound **1** [15] was reacted with acetyl chloride giving the enol ester **2**. Subsequent isomerization in the presence of DMAP [16] produced compound **3** in 86% yield (Scheme 1). Compound **3** was then reacted with L-valine in a base catalyzed reaction [17] to furnish *N*-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl]-L-valine **4** (Fde-L-Val-OH) in 95% yield. Protected L-leucine **5** and glycine **6** were prepared in 96 and 65% yield, respectively, using the same conditions. L-Valine and L-leucine were chosen for protection with the Fde group because of their hydrophobic side chains and common presence in 'difficult' peptide sequences and glycine was selected as the simplest and nonchiral amino acid. The lability of the new protective group was tested with 2% hydrazine hydrate and 5% hydroxylamine in DMF in solution. At room temperature the valine derivative **4** was fully deprotected after 40 and 10 min, respectively. The protective group cleavage products **10** and **11** were isolated and the structures were confirmed by LC-MS and NMR spectroscopy (Scheme 2).

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Abbreviations used: *DIC, N,N'-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; HOAt, 1-hydroxy-7-azabenzotriazole; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene.*

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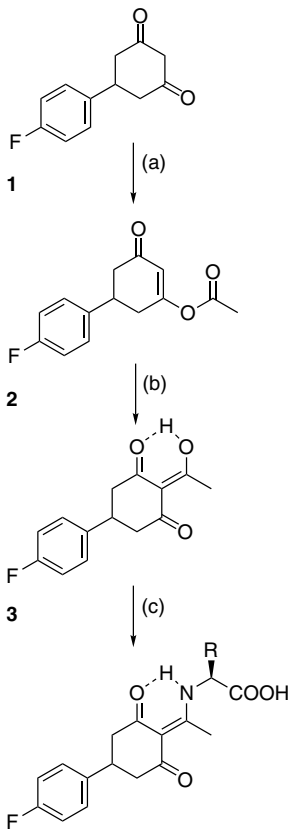


4, R= -CH(CH$_3$)$_2$
5, R= -CH$_2$CH(CH$_3$)$_2$
6, R= -H

Scheme 1. Synthesis of Fde (*N*-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl]) protected amino acids. Reagents and conditions: (a) AcCl, pyridine, CH_2Cl_2, room temperature, (b) DMAP, toluene, reflux (86% yield), (c) triethyl amine, EtOH, reflux (95, 96 and 65% yield for Fde-L-Val-OH **4**, Fde-L-Leu-OH **5**, and Fde-Gly-OH **6,** respectively).

Subsequently the model peptide amide Ac-L-Val-L-Val-NH$_2$ **12** where the *N*-terminal valine was protected with the novel Fde group was synthesized (Scheme 2). The solid-phase synthesis was carried out in a continuous flow NMR-tube reactor [18]. First, resin **7** was prepared from TentaGel coupled with activated Fmoc-L-*m*F-Phe-OH containing the fluorinated marker, followed by the Rink amide linker. The amino acids (4 equiv.) were activated with HOAt and DIC (4 equiv. each) and coupled to resin **7** to produce resin **8** (Scheme 2). Fmoc deprotections were carried out with 20% piperidine in DMF. The gel-phase ^{19}F NMR spectrum recorded for resin **8** shows two resonances at −113.60 and −116.68 ppm originating from the *m*F-phenylalanine and Fde group, respectively (Figure 1(a)). Integration of the ^{19}F signals indicated ~90% yield of the resin-bound Fde-protected dipeptide. Subsequently, the Fde group was removed with 2% hydrazine hydrate solution in DMF. Gel-phase ^{19}F NMR spectra were recorded periodically after each continuous flow deprotection cycle (Figure 1). The spectrum in Figure 1(e) demonstrates that the Fde group was cleaved completely after 45 min flow of hydrazine solution to give resin **9**. Fde deprotection was also carried out with 5% hydroxylamine in DMF. Gel-phase ^{19}F spectra were recorded after 15 and 20 min (Figure 2) as described for the deprotection with hydrazine hydrate. Figure 2(c) shows that the Fde group is cleaved completely after 20 min of hydroxylamine treatment. Interestingly, the fluorine signal from the *m*F-phenylalanine reference signal at −113.60 ppm is split during cleavage with both hydrazine and hydroxylamine (Figures 1(b)–(d) and 2(b)–(c)). The underlying reason for this observation is unknown. The appearance of the split signal during hydrazine treatment appears to be reversible (Figure 1) and therefore racemization of the *m*F-phenylalanine is unlikely in this case. In contrast, cleavage with hydroxylamine results in a nonreversible process (Figure 2) and racemization cannot be excluded. Another explanation is that the cleavage reagents affect the resin and thus produce rotamers or different environments for the fluorine atoms depending on their localization. Resin **9** prepared both with hydrazine hydrate and hydroxylamine was then acetylated with Ac_2O in DMF and subsequently treated with 90% TFA solution to produce the crude peptide amide **12**. The split *m*F-phenylalanine fluorine signal observed during Fde cleavage did not affect the structure and purity of the product **12**. Its identity and purity was assessed with LC-MS. In order to investigate if any racemization took place during the coupling of activated Fde-L-Val-OH **4**, we also prepared Ac-L-Val-L-Val-NH$_2$ **12** and its diastereoisomer Ac-D-Val-L-Val-NH$_2$ **13** using Fmoc-L-Val-OH and Fmoc-D-Val-OH activated with HOAt/DIC and 20% piperidine in DMF for Fmoc deprotection. Crude **12** and **13** were analyzed by reversed-phase HPLC, LC-MS, and ^{1}H NMR spectroscopy. As observed in Figure 3(a) and (b), two diastereomeric peptides had different retention times. Careful inspection of the analytical HPLC chromatogram of peptide **12** prepared with Fde-L-Val-OH and hydrazine hydrate in DMF for Fde deprotection (Figure 3(c)) did not reveal any trace of the epimerized product **13**. Peptide **12** prepared with Fde-L-Val-OH and deprotection with hydroxylamine solution gave the same result (Figure 3(d)). Neither analysis of the ^{1}H NMR spectrum showed any traces of Ac-D-Val-L-Val-NH$_2$ **13** in the crude peptide **12** prepared with Fde-L-Val-OH. No difference in the overall performance between Fde-L-Val-OH and Fmoc-L-Val-OH was observed. Acylations were monitored with bromophenol blue [19] and the Fde group was found to be fully compatible with this monitoring technique and the general Fmoc protocol for solid-phase peptide synthesis. The results demonstrate the applicability of Fde-protected amino acids in solid-phase peptide synthesis. In addition, the Fde group can be applied for side-chain protection of, for example lysine. This would however require full orthogonality to the Fmoc group. The Dde group is essentially stable during Fmoc deblocking with 20% piperidine but small losses of the Dde group can occur and migration of the Dde group to nearby primary amino groups have been described [20]. Both short-comings can be addressed by using more bulky Dde variants that however is more stable and require prolonged cleaving time [13].

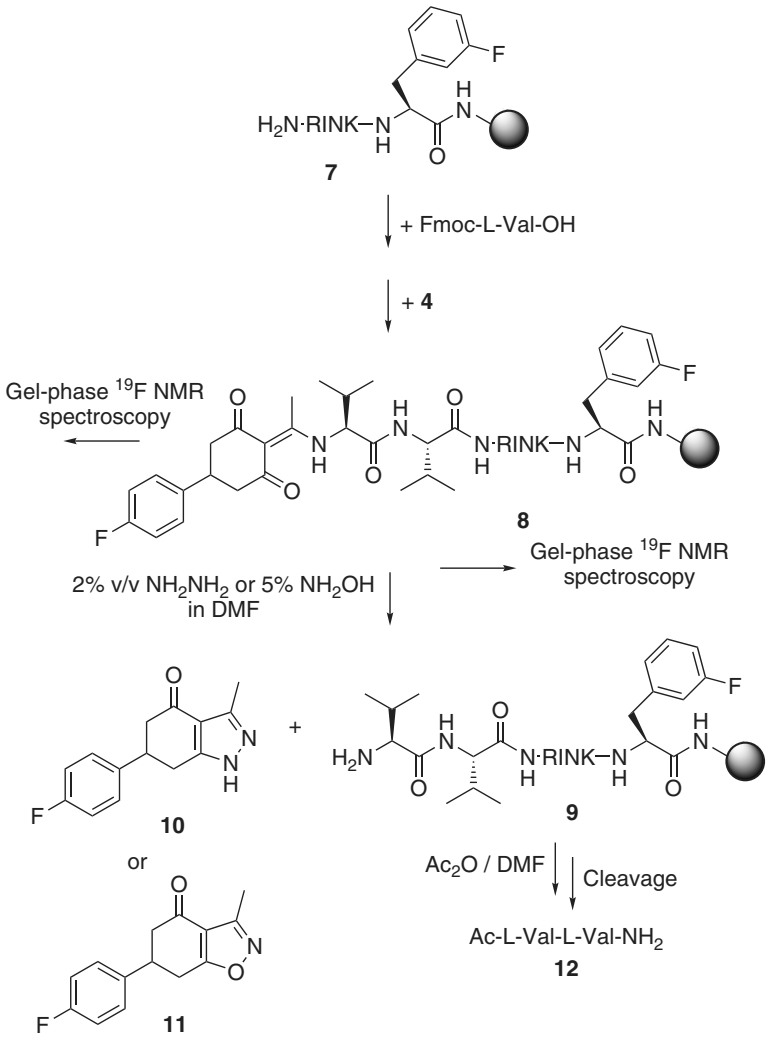
To fully evaluate the scope of Fde protection in solid-phase peptide synthesis, the hydrophobic peptide LLLLTVLTV **16** derived from the signal sequence from the mucin MUC1 was prepared. The synthesis of this difficult peptide has previously been optimized using gel-phase ^{19}F NMR spectroscopy as an analytical tool [10]. The optimization resulted in a modified Fmoc protocol using HOAt and DIC for amino acid couplings and 2% DBU in DMF for Fmoc removal. Peptide chain aggregation was prevented by introduction of the dipeptide pseudoproline building block Fmoc-Leu-Thr($\psi^{Me,Me}$ pro)-OH. The protocol however required that a small aliquot of the peptide resin had to be permanently terminated with a fluorinated benzoyl group to allow gel-phase ^{19}F NMR spectroscopy. Based on this, the synthesis of the peptide was repeated according to the optimized protocol using Fde-protected

amino acids to allow gel-phase ^{19}F NMR spectroscopy without permanent labeling of the peptide resin. Resin **14** was produced from resin **7** using our previously established protocol and Fde-L-Leu-OH activated with HOAt/DIC (Scheme 3). The gel-phase ^{19}F NMR spectrum of resin **14** showed the two fluorine resonances at −113.57 (*m*F-Phe) and −116.58 (Fde-Leu) ppm and indicated a maximum yield of 92% for resin **14** (Figure 4(a)). Lack of aggregation and incomplete couplings and deblockings was evident from the high quality of the ^{19}F NMR spectrum. Thereafter the Fde group was removed with 5% hydroxylamine solution in DMF during 25 min. Complete deprotection was confirmed by the inspection of the gel-phase ^{19}F NMR spectrum (spectrum not shown). Synthesis of resin **15** was accomplished by using standard Fmoc amino acids with the exception of Fde-L-Leu-OH that was coupled in the *N*-terminal position (Scheme 3). Gel-phase ^{19}F NMR of resin **15** afforded the spectra of very good quality indicating a successful synthesis (Figure 4(b)). The integral values for the Fde signal ranged between 90 and 98% of that for the *m*F-Phe reference signal depending on the solvent used. The Fde protective group was readily removed from the leucine in position 9 within 25 min as described above. Subsequently TFA/H_2O cleavage furnished the crude signal peptide **16** (Figure 5(a)). Purification by reversed-phase HPLC furnished peptide **16** in 54% yield based on the original resin loading capacity (Figure 5(b)). The identity and purity were confirmed with ^{1}H NMR, LC-MS, and reversed-phase HPLC, and data were found to be in agreement with those published [10].

To summarize, a new fluorinated amino protective group for monitoring the progress of solid-phase peptide couplings using gel-phase ^{19}F NMR spectroscopy has been developed. The Fde group is readily cleaved by hydrazine or hydroxylamine and Fde-protected amino acids can be used to evaluate and optimize SPPS, e.g. so-called 'difficult' peptides as demonstrated by our synthesis of peptide **16**. Their introduction into a 'difficult' sequence at diagnostic positions and a gel-phase ^{19}F NMR spectroscopy allow the detection of possible problems such as peptide chain aggregation and poor coupling or deblocking efficiency.

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) with linear gradients of



Scheme 2. SPPS of Ac-L-Val-L-Val-NH_2 (**12**). Amino acids were activated with HOAt and DIC (4 equiv. both). Fmoc removal was effected with 20% piperidine in DMF. Fde removal was performed with 2% hydrazine hydrate or 5% hydroxylamine in DMF. Cleavage from the support was carried out with TFA/H_2O (9 : 1).

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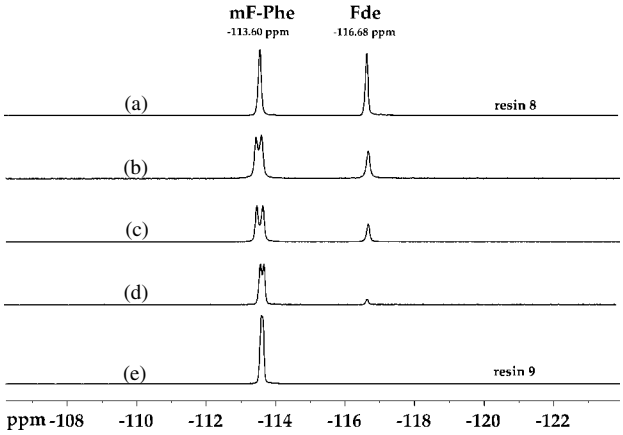

Figure 1. ^{19}F NMR spectra of resin **8** ($CDCl_3$): (a) after coupling of Fde-L-Val-OH (Fde : *m*F-Phe integrals 0.86 : 1.00), and after the deprotection of the Fde group with 2% hydrazine in DMF for (b) 15 min, (c) 20 min, (d) 30 min, and (e) 45 min to give resin **9**.

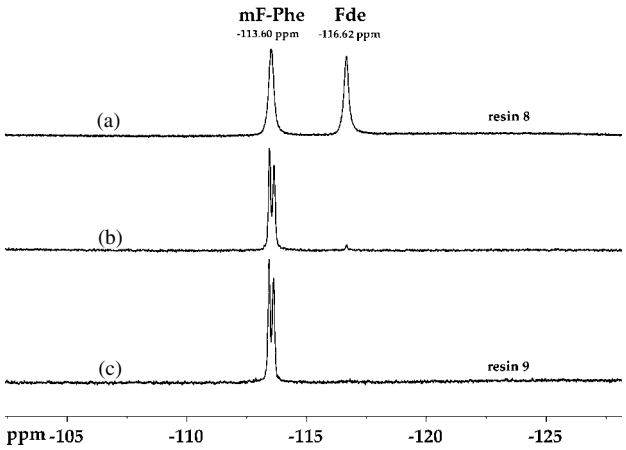

Figure 2. ^{19}F NMR spectra of resin **8** ($CDCl_3$): (a) after coupling of Fde-L-Val-OH (Fde : *m*F-Phe integrals 0.97 : 1.00), and after the deprotection of Fde group with a flow of 5% hydroxylamine solution in DMF for (b) 15 min, (c) 20 min to give resin **9**.

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). Analytical reversed-phase LC-MS was performed on a Waters System using an XTerra C-18 column (50 × 4.9 mm, 5 µm, 125 Å) 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). Mass spectra were recorded on a Waters Micromass ZQ using positive electrospray ionization (ES^+). Silica gel (Matrex, 60 Å, 35–70 mm, Grace Amicon) and solvents of analytical grade were used for flash column chromatography.

^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-400. 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)]. ^{19}F NMR spectra were recorded with Bruker DRX-400 spectrometer equipped with a probe for fluorine detection operating at 376 MHz for resin suspensions in $CDCl_3$, DMSO-d_6, and DMF-d_7 [$CFCl_3$ (δ_F 0.00 ppm) as an internal standard] at 298 K. Two peaks appear in the spectra at approximately 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.

2-Acetyl-5-(4-fluorophenyl)-1,3-cyclohexanedione (3)

To a solution of 5-(4-fluorophenyl)cyclohexane-1,3-dione **1** [15] (3.78 g, 18.33 mmol) in CH_2Cl_2 (50 ml) and pyridine (1.48 ml, 20.16 mmol), acetyl chloride (1.42 ml, 20.13 mmol) was added dropwise at room temperature and the mixture was stirred for 3 h at this temperature. The solution was washed with HCl (0.5 M, 20 ml), once with water (20 ml) and then with brine (20 ml), dried over Na_2SO_4 and evaporated. The obtained crude enol ester **2** was dissolved in dry toluene (50 ml) and DMAP (0.095 g, 0.78 mmol) and the mixture was stirred for 6 h at 95 °C. The solution was then cooled to room temperature and the solvent was evaporated. The residue was dissolved in ether (50 ml) and treated with NaOH (1 M, 76 ml). The aqueous layer was acidified with concentrated HCl. The precipitate was filtered off with suction and washed with water and subsequently was purified with flash column chromatography (petroleum ether/ethyl acetate, 2 : 1) to give 3.92 g of pure **3** in 86% yield.

^{1}H NMR ($CDCl_3$) δ_H: 2.64–2.94 (m, 4H), 2.64 (s, 3H), 3.36 (tt, J = 4.4 Hz, J = 12.0 Hz, 1H), 7.10 (m, 2H), 7.21 (m, 2H); ^{13}C NMR ($CDCl_3$) δ_C: 28.79, 36.22, 40.74, 46.02, 113.04, 115.93 (d, J = 21.4 Hz), 128.12 (d, J = 8.1 Hz), 137.53 (d, J = 3.4 Hz), 161.98 (d, J = 244.9 Hz), 194.31, 197.63, 203.11; ^{19}F NMR ($CDCl_3$) δ_F: −115.66; MS (ES^+) calculated for $C_{14}H_{13}FO_3$ $(M + H)^+$ 249.09, found 249.03; m.p. 110–112 °C.

N-[1-(4-(4-Fluorophenyl)-2,6-dioxocyclohexylidene)ethyl]-L-valine (4)

L-Valine (0.176 g, 1.5 mmol) was suspended in a solution of 2-acetyl-5-(4-fluorophenyl)-1,3-cyclohexanedione **3** (0.484 g, 1.95 mmol) in absolute EtOH (10 ml) and triethyl amine (0.31 ml, 2.25 mmol) was added. The mixture was refluxed for 18 h. The light yellow solution was cooled and concentrated under reduced pressure and the residue was taken up in CH_2Cl_2 (15 ml), washed twice with HCl (0.5 M, 2 × 5 ml). CH_2Cl_2 was removed under reduced pressure and the residue was dissolved in saturated $NaHCO_3$ (5 ml), washed with CH_2Cl_2 three times (3 × 2 ml) to remove organic impurities. The aqueous layer was acidified with 0.5 M HCl until pH 2–3. The precipitate was filtered off with suction and washed with water three times to give 0.492 g of white solid in 95% yield.
$[\alpha]^{25}_D$ = +20 (c = 1.0 mg/100 ml $CDCl_3$); ^{1}H NMR ($CDCl_3$) δ_H: 1.06 (d, J = 6.8 Hz, 3H), 1.10 (d, J = 6.8 Hz, 3H), 2.40 (m, 1H), 2.54 (s, 3H), 2.63–2.85 (m, 4H), 2.42 (m, 1H), 4.30 (dd, J = 4.8 Hz, J = 8.0 Hz, 1H), 7.02 (m, 2H), 7.18 (m, 2H), 8.28 (bs, 1H), 13.82 (d, J = 8.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ_C: 17.67, 18.81, 19.12, 31.36, 36.06, 45.90, 62.16, 108.65, 115.57 (d, J = 21.2 Hz), 128.04 (d, J = 8.0 Hz), 138.34 (d, J = 3.1 Hz), 161.65 (d, J = 244.0 Hz), 171.62, 175.00; ^{19}F NMR ($CDCl_3$) δ_F: −116.42; MS (ES^+) calculated for $C_{19}H_{22}FNO_4$ $(M + H)^+$ 348.16, found 348.08; m.p. 74–76 °C.

N-[1-(4-(4-Fluorophenyl)-2,6-dioxocyclohexylidene)ethyl]-L-leucine (5)

Compound **5** was produced in 96% yield essentially as described for compound **4**.

$[\alpha]^{25}_D$ = +9.5 (c = 1.0 mg/100 ml $CDCl_3$);, ^{1}H NMR ($CDCl_3$) δ_H: 0.94 (d, J = 6.4 Hz, 3H), 1.00 (d, J = 6.4 Hz, 3H), 1.79 (m, 1H), 1.87 (m, 2H), 2.56 (s, 3H), 2.62–2.84 (m, 4H), 3.31 (tt, J = 4.4 HZ, J = 11.6 Hz, 1H), 4.42 (m, 1H), 6.25 (bs, 1H), 7.02 (m, 2H), 7.14–7.21

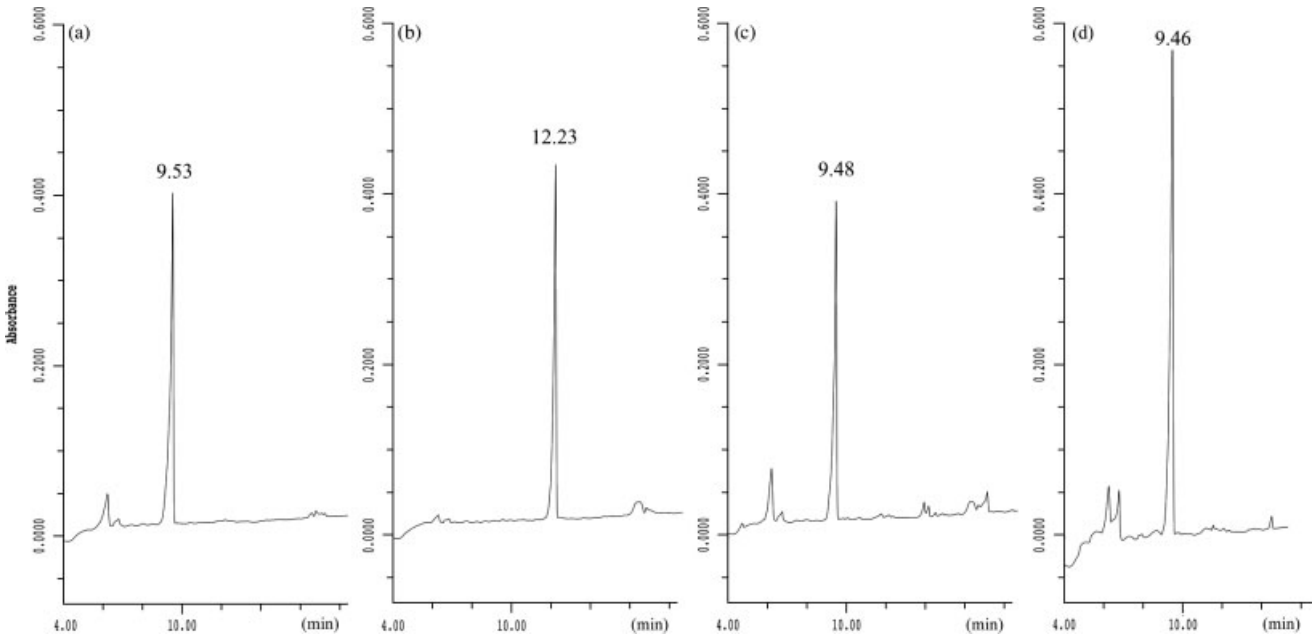

Figure 3. Analytical reversed-phase HPLC chromatograms of crude cleaved peptides (a) Ac-L-Val-L-Val-NH_2 (**12**) prepared with Fmoc-L-Val-OH, (b) Ac-D-Val-L-Val-NH_2 (**13**) prepared with Fmoc-L-Val-OH and Fmoc-D-Val-OH, (c) **12** prepared with Fmoc-L-Val-OH and Fde-L-Val-OH and 2% hydrazine hydrate in DMF for Fde deblocking, (d) **12** prepared with Fmoc-L-Val-OH and Fde-L-Val-OH and 5% hydroxylamine in DMF for Fde deblocking. Gradient 0 → 100% MeCN in H_2O during 50 min, both solvents contained 0.1% TFA.

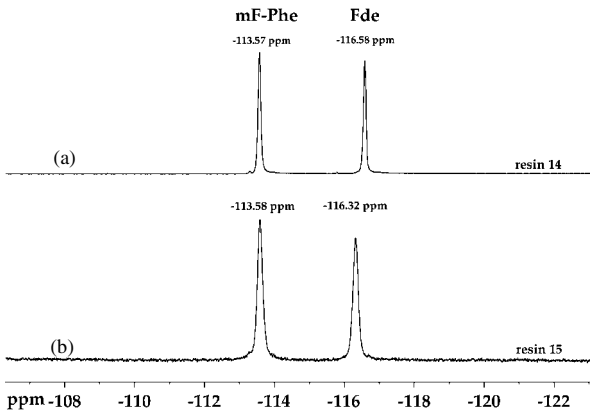

Figure 4. ^{19}F NMR spectra of (a) resin **14** after coupling of Fde-L-Leu-OH in position 3 (Fde : *m*F-Phe integrals 0.92 : 1.00 in $CDCl_3$), (b) resin **15** after coupling of Fde-L-Leu-OH in position 9 (Fde : *m*F-Phe integrals 0.98 : 1.00 in DMSO-d_6).

(m, 2H), 13.70 (d, J = 7.6 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ_C: 18.91, 21.78, 22.72, 24.91, 36.09, 41.27, 45.93, 55.33, 108.60, 115.63 (d, J = 21.3 Hz), 128.07 (d, J = 8.0 Hz), 138.32 (d, J = 3.2 Hz), 161.71 (d, J = 244.0 Hz), 172.54, 174.89; ^{19}F NMR ($CDCl_3$) δ_F: −116.419; MS (ES^+) calculated for $C_{20}H_{24}FNO_4$ $(M + H)^+$ 362.18, found 362.04; m.p. 133–135 °C.

N-[1-(4-(4-Fluorophenyl)-2,6-dioxocyclohexylidene)ethyl]-glycine (6)

Compound **6** was produced in 65% yield essentially as described for compound **4**.

^1H NMR (DMSO-d_6) δ_H: 2.45 (s, 3H), 2.52 (m, 2H), 2.70 (m, 2H), 3.29 (m, 1H), 4.36 (d, J = 5.2 Hz, 2H), 7.12 (m, 2H), 7.34 (m, 2H), 13.24 (t, J = 5.2 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ_C: 18.01, 35.52, 44.83, 107.90, 115.12 (d, J = 21.1 Hz), 128.60 (d, J = 7.7 Hz), 139.81 (d, J = 3.3 Hz), 160.87 (d, J = 240.8 Hz), 169.73, 173.18; ^{19}F NMR (DMSO-d_6) δ_F: −116.210; MS (ES^+) calculated for $C_{16}H_{16}FNO_4$ $(M + H)^+$ 306.11, found 306.06; m.p. 254–255 °C.

6-(4-Fluorophenyl)-3-methyl-6,7-dihydro-1*H*-indazol-4(*5H*)-one (10)

N-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl]-L-valine (0.0356 g, 0.103 mmol) was placed in a 10-ml round flask and 2% hydrazine in DMF (2.5 ml) was added. After stirring for 40 min at room temperature, the reaction mixture was co-evaporated with toluene to remove DMF and then dichloromethane (10 ml) was added. This solution was washed with 5% aqueous $NaHCO_3$ (6.0 ml) and then dried over Na_2SO_4. Flash chromatography (ethyl acetate) of the residue on a column of silica afforded product **10** as a white solid (19 mg, 77% yield).

$[\alpha]^{25}_D$ = −3.9 (c = 1.3 g/100 ml, DMSO); ^1H NMR (DMSO-d_6) δ_H: 2.36 (s, 3H), 2.42 (dd, J = 3.6 Hz, J = 16.4 Hz, 1H), 2.75 (dd, J = 12.0 Hz, J = 16.4 Hz, 1H), 2.94 (d, J = 3.6 Hz, 1H), 2.96 (s, 1H), 3.44 (m, 1H), 7.13 (m, 2H), 7.39 (m, 2H); ^{13}C NMR (DMSO-d_6) δ_C: 12.70, 30.50, 41.53, 46.82, 115.54, 116.07 (d, J = 21.2 Hz), 129.78 (d, J = 8.0 Hz), 140.86 (d, J = 2.8 Hz), 161.89 (d, J = 241.1 Hz), 193.49; ^{19}F NMR (DMSO-d_6) δ_F: −115.920; MS (ES^+) calculated for $C_{14}H_{14}FN_2O$ $(M + H)^+$ 245.11, found 245.13.

6-(4-Fluorophenyl)-3-methyl-6,7-dihydrobenzo[*d*]isoxazol-4(*5H*)-one (11)

N-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl]-L-valine(0.0362 g, 0.104 mmol) was placed in a 10-ml round flask and 5% hydroxylamine in DMF (1.43 ml) was added. After stirring for 40 min at room temperature, the reaction mixture was co-evaporated with toluene to remove DMF and then dichloromethane (10 ml) was added. This solution was washed

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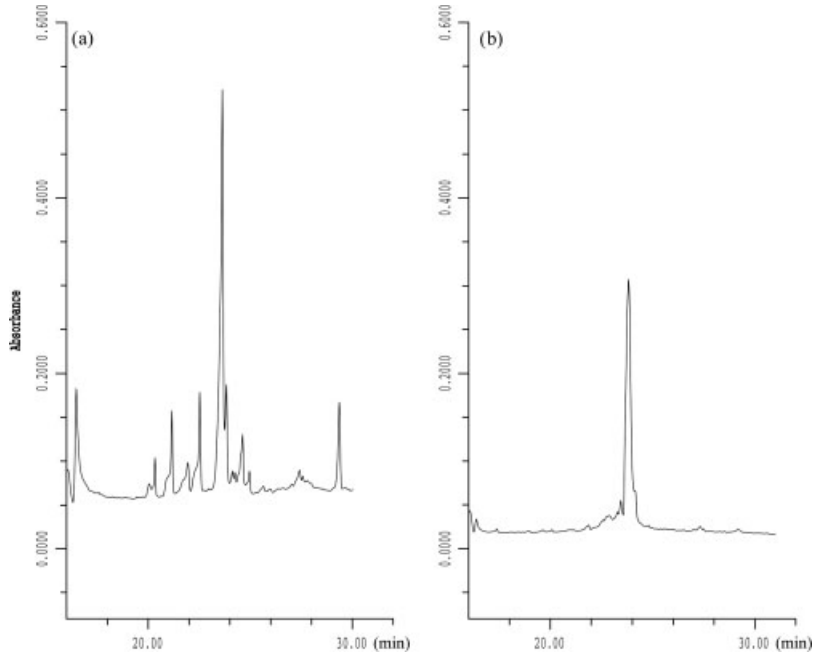


Figure 5. Analytical reversed-phase HPLC chromatograms of (a) crude peptide **16** and (b) purified peptide **16**. Gradient 0 → 80% MeCN in H_2O during 40 min, both solvents contained 0.1% TFA.

with 5% aqueous $NaHCO_3$ (6.0 ml) and then dried over Na_2SO_4. Chromatography (petrolum ether : ethyl acetate = 5 : 1) of the residue on a column of silica afforded white solid (21 mg, 83% yield).

$[\alpha]^{25}_D = -6.3$ (c = 1.4 g/100 ml, $CDCl_3$); ^{1}H NMR ($CDCl_3$) δ_H: 2.49 (s, 3H), 2.74 (d, J = 2.4 Hz, 1H), 2.76 (s, 1H), 3.07 (dd, J = 11.2 Hz, J = 17.6 Hz, 1H), 3.29 (dd, J = 4.8 Hz, J = 17.6 Hz, 1H), 3.57 (m, 1H), 7.06 (m, 2H), 7.24 (m, 2H); ^{13}C NMR ($CDCl_3$) δ_C: 10.88, 30.91, 40.47, 45.53, 115.25, 116.02 (d, J = 21.5 Hz), 128.33 (d, J = 7.9 Hz), 137.25 (d, J = 3.3 Hz), 157.51, 162.09 (d, J = 245.4 Hz), 180.13, 191.58; ^{19}F NMR ($CDCl_3$) δ_F: −115.157; MS (ES^+) calculated for $C_{14}H_{13}FNO_2$ $(M + H)^+$ 246.09, found 246.10.

Solid-phase Synthesis

Peptide chain assembly was carried out in a NMR-tube filter reactor [18] on a TentaGel HL NH_2 resin (0.35 mmol/g, Rapp Polymere, Germany) with circulating reagent solutions. Circulation and washing were performed with the FMI Lab Pump, model RP-G150 (Fluid Metering, Inc.), with custom made transformator to allow for variable speed. The pump speed was set on 0.4 ml/min for circulation and 1.4 ml/min for washings. *m*F-Phe, which acted as a fluorinated internal standard, was followed by the RINK amide linker (*p*-([α-fluoren-9-ylmethoxyformamido]-2,4-dimethoxybenzyl) phenoxyacetic acid) [21] (Bachem AG, Switzerland). DMF was distilled before being used. Fmoc amino acids (4 equiv.) were activated as 7-azabenzotriazolyl esters by using *N*,*N*′-diisopropylcarbodiimide (DIC, 4 equiv.) and 1-hydroxy-7-azabenzotriazole (HOAt, 4 equiv.) in distilled DMF (1–1.5 ml). Acylations were performed for 2–24 h and were monitored by using bromophenol blue (20-mM solution in DMF, 1 : 200 v/v) [19] as an indicator (the color of the reaction mixture changes from blue to yellow) at circulating speed. Extensive washings between reactions were carried out with DMF, CH_2Cl_2, THF, MeOH, DMF, CH_2Cl_2, and $CHCl_3$ (10 ml each) at washing speed and no intermediate amine capping steps were done. Fmoc removal was achieved with 20% piperidine in DMF flow for 5 min at circulating speed followed by washing procedure. Fde group removal was performed by 2% hydrazine hydrate in DMF for 45 min or 5% hydroxylamine in DMF for 20–25 min at circulating speed and was followed by extensive washing with 20% piperidine in DMF (10 ml) and DMF, CH_2Cl_2, THF, MeOH, DMF, CH_2Cl_2, and $CHCl_3$ (10 ml each) at washing flow. Fde group removal was monitored with gel-phase ^{19}F NMR spectroscopy. Final *N*-acylations were performed with Ac_2O/DMF (1 : 2) for 1 h. Peptide cleavage was performed with TFA/H_2O (9 : 1, v/v) solution at room temperature for 2.5 h. The cleaved resins were washed thoroughly with TFA, CH_2Cl_2, and the combined filtrates were evaporated. The purity and identity of the cleaved peptides were confirmed by analytical reversed-phase HPLC, ^{1}H NMR, and LC-MS.

Peptides

Acetyl-L-valyl-L-valine amide 12 prepared with Fmoc-L-valine:
Crude product yield: 10.4 mg, 75% based on the resin capacity; MS (ES^+) calculated for $C_{12}H_{23}N_3O_3$ $(M + H)^+$ 258.18, found 258.16.

Acetyl-D-valyl-L-valine amide 13 prepared with Fmoc-D-valine and Fmoc-L-valine:

Crude product yield: 14 mg, 97% based on the resin capacity; MS (ES^+) calculated for $C_{12}H_{23}N_3O_3$ $(M+H)^+$ 258.18, found 258.16.

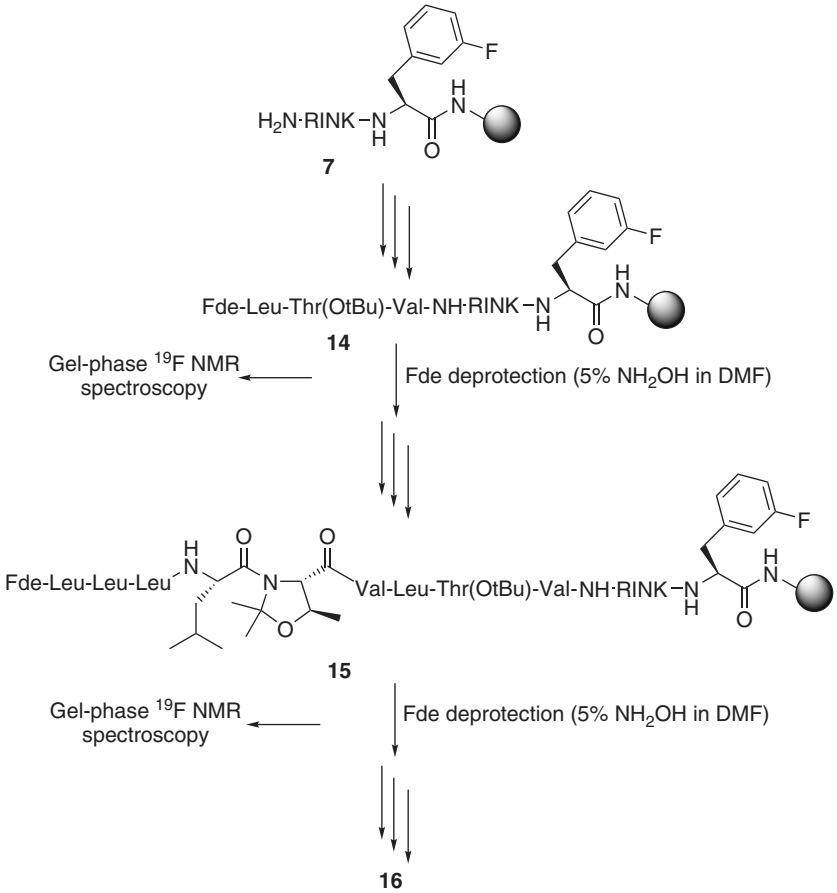
Acetyl-L-valyl-L-valine amide 12 prepared with Fde-L-valine and Fmoc-**L**-valine using 2% hydrazine for Fde removal:

Crude product yield: 10 mg, 97% based on the resin capacity; MS (ES^+) calculated for $C_{12}H_{23}N_3O_3$ $(M+H)^+$ 258.18, found 258.16.

Acetyl-L-valyl-L-valine amide 12 prepared with Fde-L-valine and Fmoc-L-valine using 5% hydroxylamine for Fde removal:

Crude product yield: 12 mg, 118% based on the resin capacity; MS (ES^+) calculated for $C_{12}H_{23}N_3O_3$ $(M+H)^+$ 258.18, found 258.23.

L-Leucyl-L-leucyl-L-leucyl-L-leucyl-L-threonyl-L-valyl-L-leucyl-L-threonyl-L-valine amide 16 was prepared in 54% yield esentially as described previously [10] with the exception that Fde-L-leucine was introduced in position 3 and 9 in the sequence using a



Scheme 3. SPPS of the signal peptide **16** using Fde-L-Leu-OH as building block in position 3 and 9. Amino acids were activated with HOAt and DIC (4 equiv. both). Fmoc removal was effected with 2% DBU in DMF and Fde removal was performed with 5% hydroxylamine in DMF. Cleavage from the support was carried out with TFA/H_2O (9 : 1).

continuous flow NMR-tube reactor [18] and 2% DBU in DMF for Fmoc deblocking and 5% hydroxylamine in DMF for Fde deblocking. The identity and purity of the purified peptide were confirmed with ^{1}H NMR, LC-MS, and reversed-phase HPLC, and data were found to be in agreement with those published [10].

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