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Synthesis of cleavable peptides with authentic C-termini: an application for fully automated SPOT synthesis

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Abstract—A simple method for the synthesis of carboxyl-free peptides on cellulose membranes was improved and adapted for fully automated SPOT synthesis. Using 1,1′-carbonyl-di-imidazole (CDI) or 1,1′-carbonyl-di-(1,2,4-triazole) (CDT) as an activator within a defined period of time, we were able to reduce the formation of di- or oligomerization of the C-terminal amino acid. The soluble peptides are obtained in a purity range of 60–95% and could be used directly for different biological assays (e.g., CD8 T-cell epitope) that require authentic C-termini.

In the last few years it has become more and more important to screen huge peptide libraries for CD8 T-cell epitope mapping, which provides important information for vaccine development.[1,2] To determine a positive T-cell response (e.g., for CD8 T-cells), it is necessary to synthesize peptide libraries with free (or authentic) C-termini.[3,4]

The standard method to generate cleavable peptides uses glycine or β-alanine for the amino-functionalization of the cellulose membrane by esterification.[5,6] However, the peptide sequences synthesized by standard SPOT synthesis[7] can only be cleaved without authentic C-termini (β-alanine or glycine as additional C-terminal amino acid). Another method uses S-modified cellulose membranes and a Fmoc-amino acid-3-bromopropyl ester as the C-terminal amino acid.[8] But this chemistry is time-consuming, gives low yields and Fmoc-amino acid-3-bromopropyl esters are not commercially available.

Other esterification methods on cellulose membranes such as 2,6-dichlorobenzoyl chloride[9] or, 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazolide (MSNT),[10] or the Mitsunobu reaction[11] are not suitable due to the noxious reagents and/or low coupling yields.[12] Fmoc-amino acid fluorides have been shown to be highly reactive, but their synthesis is time-consuming and arginine cannot be produced.[13] Furthermore, they are not stable enough during SPOT synthesis.

To overcome these problems, we present here an improved method to generate cleavable peptides with free C-termini, which is furthermore adapted to fully automated SPOT synthesis. Peptide sequences containing all 20 different C-terminal L-amino acids could be synthesized directly on one cellulose membrane, with good coupling yields, using CDI or CDT as an activator. This modification of the SPOT synthesis procedure now allows protein-wise epitope screening in biological assays requiring soluble peptides with authentic C-termini (e.g., CD8 T-cell epitope screening[14]).

We therefore envisaged directly coupling the 0.2 M Fmoc-amino acid-OH activated with 1 equiv CDI and 1 equiv *N*-methylimidazole (NMI) using a fully automated pipette robot (MultiPep, INTAVIS Bioanalytical Instruments AG, Cologne, Germany). The first analytical results of the 20 L-amino acids show coupling efficiencies between 112 nmol/cm^2 for glycine and 10 nmol/cm^2 for proline (Fig. 1, white bars).

Unfortunately, we observe C-termini elongations as side reactions (dimerization or oligomerization of the C-terminal amino acid). These elongations appear mainly on small hydrophobic amino acids such as alanine, glycine, proline and valine. Furthermore, we curiously also observe oligomerization products with threonine as the C-terminal amino acid (data not shown).

Keywords: CDI; CDT; SPOT synthesis; T-cell epitope mapping.
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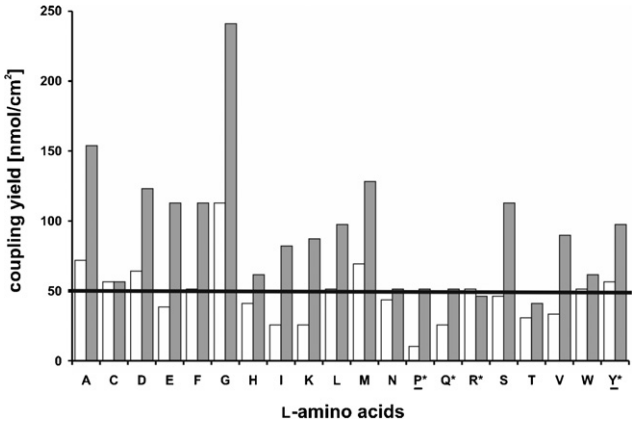

Figure 1. Determination of the coupling efficiency for the 20 C-terminal L-amino acids. White columns: 0.2 M Fmoc-AA-OH activated in DMF with 1 equiv CDI and 1 equiv NMI as activators, coupled four times. Grey columns: 0.4 M Fmoc-amino acid-OH in DMF activated with 3 equiv CDI, coupled four times. Amino acids denoted with * were coupled eight times. Underlined amino acids were activated with 3 equiv CDT. Quantification is achieved by measuring the Fmoc-piperidine complex[15] cleaved from one spot (0.25 cm^2).

The C-terminal elongation is probably due to N-terminal cleavage of the Fmoc-protecting group of the Fmoc-amino acid-OH during the pre-activation period. As reported previously, the Fmoc-group is not stable in dipolar aprotic solvents and in amine containing solvents.[16,17] In our case, we found up to 50% oligomerization when activating the Fmoc-amino acid-OH with CDI in the presence of NMI. This is exemplified with a solution of Fmoc-glycine-OH (0.2 M) in *N*,*N*′-dimethylformamide (DMF) containing NMI (1 equiv) and CDI (1 equiv) after 20 h (Fig. 2A). The ESI mass spectrum (Q-TOFmicro™, Micromass, Manchester, UK) clearly shows glycine oligomerizations: dimers (compound **VI**, **VIII**), trimers (compound **IX**), tetramers (compound **X**) and pentamers (compound **XI**), which represent in total 53% of the applied Fmoc-glycine-OH adduct (compounds **III**–**V**) (all compounds here refer to Fig. 2A).

We assume that during the activation and/or reaction, the imidazole formed from CDI is probably sufficient to partly deprotect the Fmoc-amino acid-OH. Indeed, we observed a stable adduct of dibenzofulven with released imidazole (compound **II**, Fig. 2A). This reaction mechanism is analogous to the Fmoc-cleavage reaction using piperidine.[18]

To inhibit these amino acid oligomerizations, we tested different reaction and time conditions. Due to the fact that oligomerization lengthens proportionally with the reaction time, we determined a best reaction time of 1 h for the complete coupling (30 min pre-activation and 30 min coupling time). Unfortunately, these conditions gave low coupling yields (data not shown). To overcome this problem we increased the amount of amino acids (0.4 M) and CDI (3 equiv). To validate our improved conditions, we analyzed a solution of Fmoc-glycine-OH activated with 3 equiv CDI after an incubation time of 1 h by ESI mass spectrometry (Fig. 2B). Within this

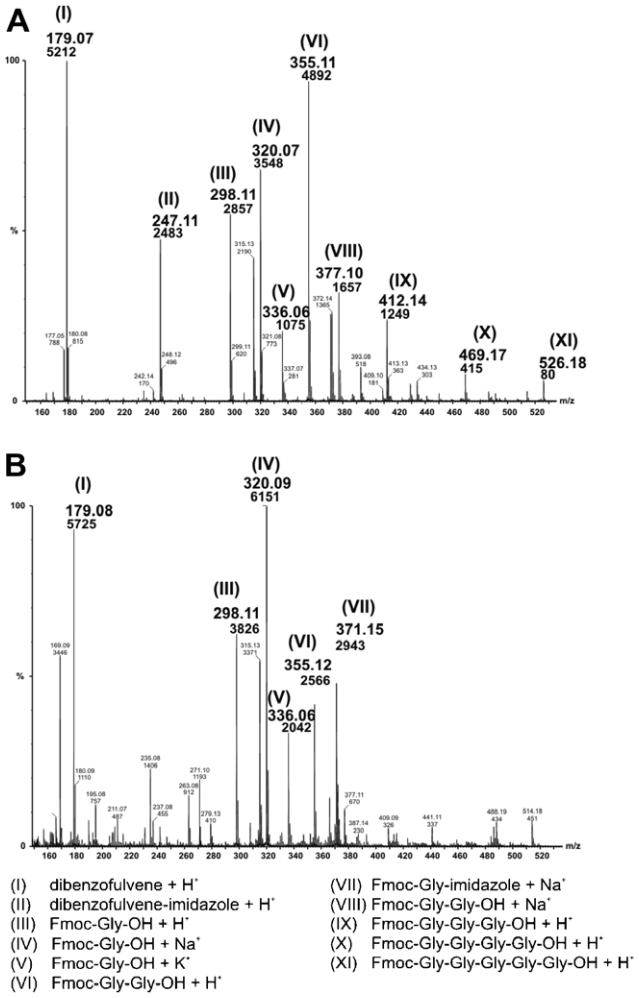

Figure 2. Example of the C-terminal oligomerization of Fmoc-glycine-OH. (A) Solution of 0.2 M Fmoc-glycine-OH in DMF containing 1 equiv NMI and 1 equiv CDI, 20 h at room temperature. Oligomerization represents 53% of the applied Fmoc-glycine-OH. (B) Solution of 0.4 M Fmoc-glycine-OH in DMF containing 3 equiv CDI, 1 h at room temperature.

time, we mainly observed the monomer (compound **III**–**V**), the activated Fmoc-glycine-imidazole adduct (compound **VII**) and the cleaved dibenzofulvene (compound **I**). C-terminal elongation is limited to a dimer (compound **VI**), which is reduced to approximately 15% of the applied Fmoc-glycine-OH adduct (all compounds here refer to Fig. 2B). Although this percentage still seems to be too high, we assumed that by spotting the activated amino acids onto the cellulose membrane we could reach a displacement of the equilibrium compared to the pure activated solution (for more details see above).

After optimization of the amino acids and activator amount, we determined the coupling efficiency of all 20 L-amino acids by Fmoc-cleavage[15] (Fig. 1, grey bars). To avoid precipitation of Fmoc-glutamine-, Fmoc-proline- and Fmoc-tyrosine-imidazolide adducts we used 3 equiv of CDT instead of CDI. With these improved conditions we obtained coupling yields of at least 50 μmol/cm^2 generally using four-time coupling, but

eight-time coupling for proline, glutamine, arginine and tyrosine, of freshly prepared reagents (Fig. 1). Altogether, we obtained similar coupling yields for all 20 L-amino acids, as is required for the homogeneous synthesis of peptides with different C-terminal amino acids on one cellulose membrane (for more details, see Fig. 1). A basic membrane loading of ~50 nmol/cm^2 ensures a peptide amount of ~50 μg/cm^2 (nonamer estimated with 1000 g/mol), which results in an adequate peptide amount for stock solutions for subsequent biological assays.

In order to prove the reaction efficiency of our ameliorated C-terminal amino acid coupling conditions, we synthesized 400 different and randomly chosen nonamer peptides deduced from the CMV (AD169) proteome.[19] All 400 peptides were synthesized using the improved coupling method for C-terminal amino acids reported here, followed by the standard Fmoc strategy[7] for the remaining peptide synthesis. The peptides were cleaved from the membrane using a 0.125 M NaOH solution as described previously,[8] and analyzed by HPLC and ESI mass spectrometries. The dimer formation average for peptide sequences with the same C-terminal amino acid was calculated using the peak intensities of the different mass spectra (as in Fig. 2). As shown in Figure 3, we obtained negligible dimerization in most cases and dimerization of alanine, glycine and threonine, as observed under the previous conditions, although now at less than 5%. This impurity (below 5%) is in our opinion negligible for subsequent potential biological assays.

To determine the possibility of racemization during loading procedure of the first amino acid, we synthesized di-peptides (L)L–(L)X and (L)L–(D)X on resin using the standard Fmoc-protocol. We chose as X the amino acid alanine, aspartic acid, methionine, lysine and tryptophan, respectively. By HPLC we analyzed the retention time of each pure di-peptide and each mixture (L/L and L/D). Thereafter, we synthesized the same L–X di-peptide on cellulose membrane under our improved conditions and cleaved them as described above. We analyzed them likewise by HPLC to determine the percentage of racemization (linear solvent gradient: A, 0.05% TFA in water; B, 0.05% TFA in acetonitrile; gradient 5–60% B over 30 min). As shown in Table 1, we determined only small amount of the diastereomeric L/D-peptide between 1% and 8%. We are notably beneath the racemization found with the coupling reagents MSNT and HOBt-ester or the symmetric anhydride.[10]

Furthermore, we compared all HPLC chromatograms of the 400 cleaved peptide sequences to estimate the purity of the improved method. HPLC chromatogram of synthesized peptides with all 20 different C-terminal amino acids demonstrated purities in the range between 60% and 95% (Figs. 1 and 2 of the Supplementary data). These purities are generally sufficient for CD8 T-cell epitope screening using flow cytometry analysis.

The main advantage of our improved method for the synthesis of cleavable peptides with free C-termini is its adaptation to fully automated SPOT synthesis. The MultiPep robot contained an integrated washing unit, and furthermore, a program to freshly pre-activate the Fmoc-amino acid-OH. Compared to β-alanine or glycine membranes, our improved method allows us to synthesize peptides with different C-terminal amino acids on one membrane, which could be used directly for biological screening. This is a time-saving step: (i) avoiding any sorting of the dissected peptide sequences resulting from a virus proteome and (ii) therefore allowing protein-wise screening.

Using both the ameliorated protocol and the automated synthesizer, it is now possible to generate huge peptide libraries (approximately 5000 peptides) within one week for the proteomic screening of T-cell epitopes or other biological assays.

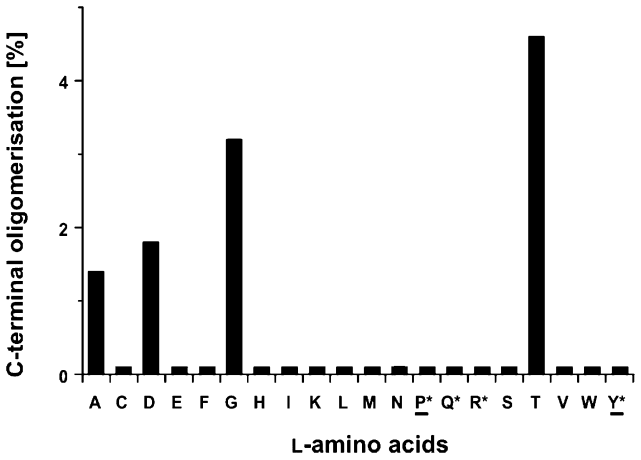

Figure 3. Quantification of the C-terminal elongation of the 20 L-amino acids. The percentages of oligomerization of the 20 C-terminal amino acids are calculated based on the intensities of the mass peaks of all respective peptide sequences with the same C-terminal amino acid. The C-terminal amino acids were normally coupled four times, but those denoted with an * were coupled eight times. Underlined amino acids were activated with 3 equiv of CDT. All amino acids were pre-activated for 30 min and coupled within 30 min.

Table 1.

Di-peptide[a]	Resin[b]		Resin mix[c]		Spot[d] % L/D
	L/L	L/D	L/L	L/D	
L–A	9.2	10.5	8.7	10.0	3.7
L–D	8.2	7.0	8.1	8.4	<1.0
L–K	7.7	8.0	7.7	7.8	<1.0
L–M	12.0	13.2	11.7	12.9	7.8
L–W	15.0	15.5	14.9	15.4	5.3

[a] Di-peptide sequences.
[b] HPLC retention times of the resin synthesized di-peptides in the L/L and L/D conformation.
[c] HPLC retention times of the resin synthesized di-peptides as mixtures of L/L and L/D to deduce their mutually influences.
[d] Percentage of the L/D-diastereomer of the di-peptide sequences synthesized on cellulose membrane and cleaved from one spot (0.25 cm^2). Percentage was determined by HPLC peak area integration.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.11.093.

References and notes

1. De Groot, A. S. *Drug Discovery Today* **2006**, *11*, 203–209.
2. Paston, S. J.; Dodi, I. A.; Madrigal, J. A. *Hum. Immunol.* **2004**, *65*, 544–549.
3. Kiecker, F.; Streitz, M.; Ay, B.; Cherepnev, G.; Volk, H. D.; Volkmer-Engert, R.; Kern, F. *Hum. Immunol.* **2004**, *65*, 523–536.
4. Kern, F.; Surel, I. P.; Brock, C.; Freistedt, B.; Radtke, H.; Scheffold, A.; Blasczyk, R.; Reinke, P.; Schneider-Mergener, J.; Radbruch, A.; Walden, P.; Volk, H. D. *Nat. Med.* **1998**, *4*, 975–978.
5. Kamradt, T.; Volkmer-Engert, R. *Mol. Divers.* **2004**, *8*, 271–280.
6. Hilpert, K.; Volkmer-Engert, R.; Walter, T.; Hancock, R. E. *Nat. Biotechnol.* **2005**, *23*, 1008–1012.
7. Frank, R. *Tetrahedron* **1992**, *48*, 9217–9232.
8. Licha, K.; Bhargava, S.; Rheinländer, C.; Becker, A.; Schneider-Mergener, J.; Volkmer-Engert, R. *Tetrahedon Lett.* **2000**, *41*, 1711–1715.
9. Sieber, P. *Tetrahedon Lett.* **1987**, *28*, 6147–6150.
10. Blankemeyer-Menge, B.; Nimtz, M.; Frank, R. *Tetrahedon Lett.* **1990**, *31*, 1701–1704.
11. Barlos, K.; Dimitrios, G.; Kallitsis, J.; Papaioannou, D.; Sotiriou, P.; Schaefer, W. *Liebigs Ann. Chem.* **1987**, *1987*, 1031–1035.
12. Pilawa, S. Ph.D. Thesis, Universität Carolo-Wilhelmina, 2001.
13. Wenschuh, H.; Hoffmann, B.; Schaller, S.; Germeroth, L.; Schneider-Mergener, J.; Volkmer-Engert, R. Efficient Parallel Synthesis of Cellulose Bound and Cleavable Peptides via Direct Anchoring of Fmoc-amino Acid Fluorides onto Cellulose. In *Peptides 1998*; Bajusz, S., Hudecz, F., Eds.; Akademiai Kiado: Budapest, 1999.
14. Kern, F.; Faulhaber, N.; Frommel, C.; Khatamzas, E.; Prosch, S.; Schonemann, C.; Kretzschmar, I.; Volkmer-Engert, R.; Volk, H. D.; Reinke, P. *Eur. J. Immunol.* **2000**, *30*, 1676–1682.
15. Wenschuh, H.; Gausepohl, H.; Germeroth, L.; Ulbricht, M.; Matuschewski, H.; Kramer, A.; Volkmer-Engert, R.; Heine, N.; Ast, T.; Scharn, D.; Schneider-Mergener, J. Positionally Addressable Parallel Synthesis on Continuous Membranes. In *Combinatorial Chemistry*; Fenniri, H., Ed.; Oxford: NY, 2000; pp 95–116.
16. Atherton, E.; Bury, C.; Sheppard, R. C. *Tetrahedon Lett.* **1979**, *20*, 3041–3042.
17. Otterson, K. M.; Harrison, J. L.; Ligutom, A.; Ashcroft, P. Solid Phase Peptide Synthesis with *N*-Methylpyrrolidone as the Solvent for Both Fmoc and Boc Synthesis. Eleventh American Peptide Symposium, 1989, California.
18. Carpino, L. A.; Han, G. Y. *J. Am. Chem. Soc.* **1970**, *92*, 5748–5749.
19. Paston, S. J.; Dodi, I. A.; Madrigal, J. A. *Hum. Immunol.* **2004**, *65*, 544–549.