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Using hydroxymethylphenoxy derivates with the SPOT technology to generate peptides with authentic C-termini

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ABSTRACT

The SPOT technology can fulfill most requirements for highly parallel, multiple peptide synthesis of soluble peptides within the upper microgram range. Here, we report on an improved method using hydroxymethylphenoxyacetic acid (HMPA) for 19 amino acids and 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) for proline as acidic labile linkers in SPOT synthesis. Using this approach we could reduce side-chain reactions normally occurring during conventional alkaline peptide cleavage from cellulose membranes. All synthesis steps were adapted to fully-automated SPOT synthesis and therefore represent a time- and cost-saving procedure. Furthermore, the improved cleavage and washing steps resulted in peptides with authentic C-termini in a purity range of 60–95%. Our improved method is ideal for synthesizing many thousand different peptides subsequently used directly for different biological assays requiring authentic C-termini, such as CD8 T-cell epitope screening, vaccine immunization, or tumor imaging.

Discovering new targets for vaccines and novel vaccination methods are current research goals in immunology.[1,2] Besides the well-known combinatorial approaches,[3] huge arrays of sequence-based peptides (e.g., virus proteome-based) are powerful tools for proteome-wide T-cell epitope mapping studies seeking to deliver new targets for vaccine development. Recently, we explicitly demonstrated such a knowledge-based approach, revealing novel peptide vaccines even in the case of CD8 T-cell responses.[4] A modified SPOT technology synthesis protocol enabled the synthesis of thousands of human cytomegalovirus virus-derived peptides with free and authentic C-termini, a prerequisite for a CD8 T-cell response.[5,6]

In addition to its original use—the synthesis and screening of cellulose membrane-bound peptide arrays (for a review see[7–9])—SPOT technology[10] has emerged as an efficient micro-scale synthesis method. Thousands of soluble peptides can be synthesized in a short time with microgram yields and purities sufficient for biological screening assays[11–14]—one advantage compared with commonly used large-scale SPPS. Usually, peptides are cleaved from the cellulose support by alkaline hydrolysis or amminolysis[11,13]: the former providing peptides with a carboxylated C-termini, the latter producing the corresponding carboxyl-amide. However, alkaline conditions lead to several unwanted side reactions, primarily oxidation, to different extents, of cysteine and methionine sulfur atoms. Cysteine is especially affected by strong oxidation (up to 50%)[4] and can be decomposed to the dehydroalanine moiety.[4,15] Another drawback is the necessity to neutralize the alkaline peptide solutions, which result in high salt concentration impurities. Time-consuming desalting steps are essential not only for correctly determining the peptide concentration but also for salt-free biological assays.[4]

To overcome these problems, we present here an improved method to generate cleavable peptides with free C-termini following the SPOT procedure, but using acid labile-linker molecules (hydroxymethylphenoxy derivates). It was reported that these linkers are used for SPPS on spherical cellulose resin,[16] but to our surprise we found no report on the use of hydroxymethylphenoxyacetic acid (HMPA) in SPOT technologies. We synthesized peptide sequences containing 19 different C-terminal amino acids directly on the HMPA-linker modified cellulose membrane with efficient yields and purity using 1,1′-carbonyl-di-imidazole (CDI) or 1,1′-carbonyl-di-(1,2,4-triazole) (CDT) as activators. For proline we additionally tested 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) to avoid diketopiperazine formation. The synthesis protocol was further adapted to the fully-automated SPOT synthesis technology, allowing fast and efficient synthesis of soluble peptides with authentic C-termini. Finally, the novel synthesis approach was successfully validated in a biological assay that determined the stimulatory activity of the CD8 T-cell epitope VTEHDTLLY (VPAP protein, SWISS-PROT Accession No. P16790) from human cytomegalovirus (CMV).[4]

The well-known linker molecule HMPA is an ideal anchor for the SPOT synthesis technology due to its stability against

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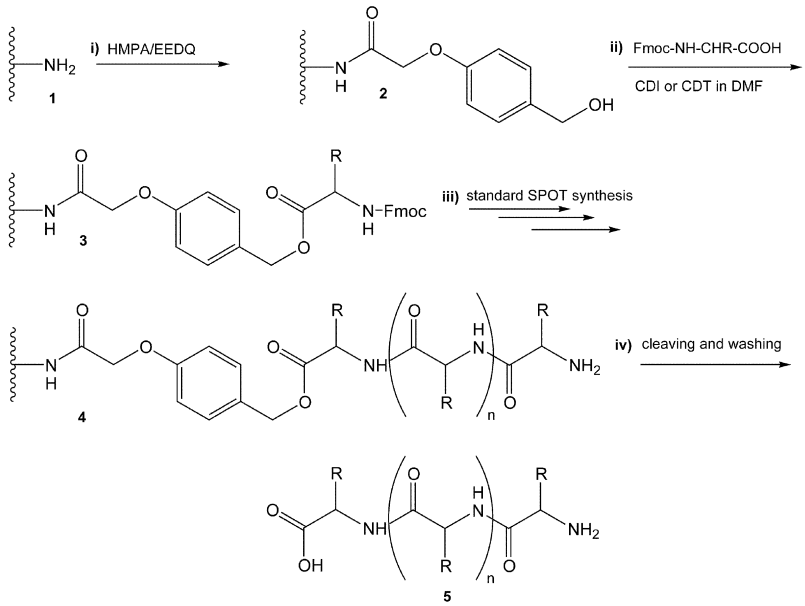
piperidine and sensitivity to acidic conditions, for example, >50% trifluoroacetic acid (TFA).[18] Scheme 1 describes the modification steps applied to a cellulose membrane (detailed protocol is given in Supplementary material). First, the membrane was treated with Fmoc-β-alanine as described[17] resulting in the amino-functionalized support, called matrix **1** (800–1000 nmol/cm^2). Second, the bifunctional HMPA molecule (0.6 M in DMF) was attached spotwise to the surface of **1**. Several activation reagents were tested; finally 1.1 equiv of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) was chosen as the most favored candidate due to its long-lived active ester (Scheme 1, step i). Figure 1A shows that a 3- or 4-times coupling approach is sufficient for HMPA attachment. Third, in analogy to our recent publication[14] the ester bound between the first L-amino acid (0.4 M, in DMF) and cellulose membrane-bound HMPA (**2**) was formed with the aid of 3 equiv of CDI or 3 equiv of CDT (Scheme 1, step ii). CDT was used for the amino acids glutamic acid, proline, and tyrosine. The coupling efficiency was determined by measuring the Fmoc-piperidine adduct concentration as described previously.[17] Figure 1B summarizes the coupling efficiency of all 20 Fmoc-L-amino acids. We obtained coupling yields between 140 and 450 nmol/cm^2 generally using 4-times coupling, but specifically 8-times coupling for glutamic acid, proline, and tyrosine using freshly prepared reagents. Fourthly, after an additional acetylating step (Scheme 1, step iii) peptides were synthesized according to the standard SPOT synthesis protocol.[17] Finally, the spots were punched out and transferred into eppendorf tubes or deep-well plates. The peptides were released from the support and side chains were deprotected by the follow-



Scheme 1. Reaction scheme for the synthesis of cleavable peptides with authentic C-termini using acid-label HMPA on cellulose membrane. Reaction conditions: (i) 0.6 M HMPA in DMF activated with 1.1 equiv of EEDQ was directly coupled (3–4 times) onto the β-alanine-modified cellulose membrane (**1**) resulting in **2**. (ii) After acetylation [2% Ac_2O in DMF] of the remaining amino-group of the β-alanine, 0.4 M Fmoc-L-amino acid-OH in DMF activated with 3 equiv of CDI/CDT was coupled (4–8 times) to generate the cleavable site (**3**). (iii) The remaining OH-groups of HMPA were acetylated [2% Ac_2O, 1% DIPEA in DMA]. Thereafter, peptide elongation was carried out by standard SPOT synthesis[17] (**4**). (iv) Using our improved cleavage/washing step procedure [2.5 h 60% TFA, 3% triisobutylsilane (TIBS), 2% H_2O in DCM; vacuum dry; 1.0 h 90% TFA, 3% TIBS, 2% H_2O in DCM; vacuum dry; 1× H_2O wash, 3× *tert*-butylmethylether wash, vacuum dry and dissolve in degassed acetonirile/H_2O (1:1)] we obtained peptides with authentic C-termini.

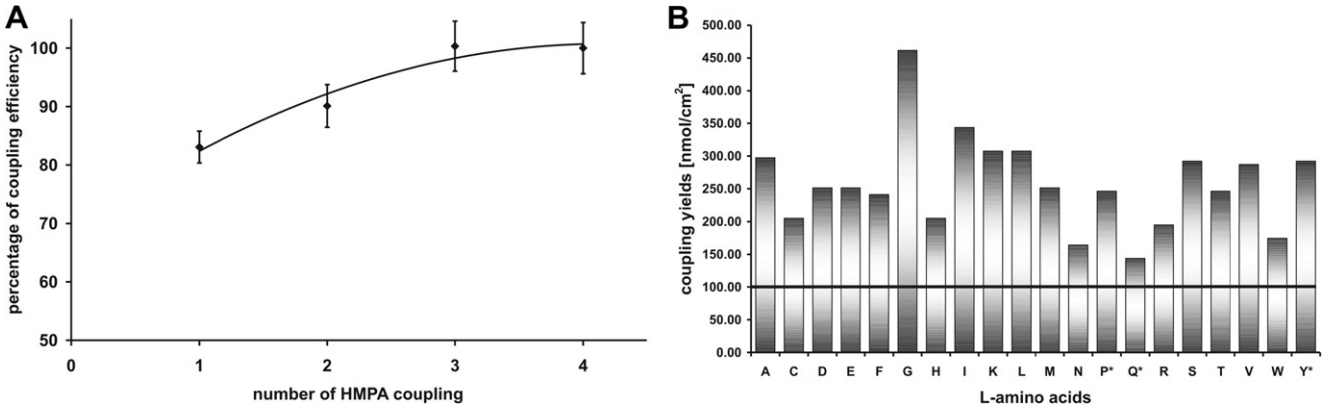


Figure 1. Determining the coupling efficiencies of the first two steps during SPOT synthesis with HMPA. (A) 0.6 M HMPA in DMF activated with 1.1 equiv of EEDQ was coupled 1–4 times on a β-alanine-modified cellulose membrane. Thereafter, 0.4 M Fmoc-L-glycine-OH in DMF activated with 3 equiv of CDI was coupled 4-times. Quantification was achieved by measuring the Fmoc-piperidine complex[17] cleaved from one spot (0.25 cm^2). (B) 0.4 M Fmoc-L-amino acid-OH in DMF activated with 3 equiv of CDI, coupled 4 times. Amino acids denoted with * were coupled 8 times and were activated with 3 equiv of CDT. Quantification was achieved by measuring the Fmoc-piperidine complex[17] cleaved from one spot (0.25 cm^2). The horizontal line determines the threshold of 100 nmol/cm^2.

ing protocol (Scheme 1, step iv): (a) Peptides were cleaved from the support by treatment with a mixture of 60% TFA for 2.5 h. (b) The cellulose spots were then removed and the solution was subsequently dried in a vacuum. (c) The remaining residuum was once again treated with a mixture of 90% TFA for 1 h and again dried in a vacuum. (d) Remaining TFA–salt complexes were removed with H_2O by azeotropic distillation. (e) The residue was then washed 3 times with *tert*-butylmethylether and dried. It should be mentioned that following exactly the order of the TFA-cleavage steps is important to obtain optimal yields. During the 60% TFA solution procedure the ester linkage between HMPA and the peptide is cleaved, but without releasing the HMPA moiety from the cellulose membrane. After separation from the cellulose spot we further ensure complete peptide side-chain deprotection with 90% TFA. Separation of the cellulose spot from the first cleavage mixture (60% in DCM) significantly reduces the amount of HMPA complex impurities[18], while the washing steps are necessary to remove byproducts and TFA–salt complexes. This is confirmed in Figure 2A, which shows the HPLC elution profile and ESI mass spectra of the synthesized peptide WKL-G. The HPLC chromatogram depicts the peptide's excellent purity, while its integrity is demonstrated by mass spectrometry. Greater amounts of impurities are not detected in either HPLC or mass spectrometry.

One known problem of amino acid carboxy-group activation is the resulting racemization. To determine racemization levels during coupling of the first amino acid to the HMPA molecule, we synthesized di-peptides (L)L–(L)X and (L)L–(D)X on resin using the standard Fmoc-protocol.[17] To enable comparison with our previously reported method[14], we sequentially choose as X, the amino acids alanine, aspartic acid, methionine, lysine, and tryptophan. We analyzed the HPLC retention time of each pure di-peptide and each mixture (L/L and L/D). Then we synthesized the same L–X di-peptide on a HMPA/β-alanine-modified cellulose membrane using our improved conditions. These di-peptides were subsequently cleaved using standard TFA cleavage conditions[17] and also analyzed by HPLC. We determined the percentage of racemization by peak area integration, and only detected a small amount, between 1% and 8%, of the diastereomeric L/D-peptide (Supplementary material Table 1). This is clearly below the racemization found with the coupling reagents MSNT and HOBt-ester or the symmetric anhydride[19] and within the range of the directed cellulose coupling method.[4,14]

The improved protocol was used to synthesize the peptides WKL-X, where X represents all 20 gene-encoded amino acids. Measured and expected masses of these peptides, putative byproducts, as well as peptide purities are presented in Table 1. Our analyses revealed that purities ranged between 60% and >95%. As expected, our results demonstrate that the formation of dehydroalanine in the cysteine-containing peptide[4,15] was totally suppressed by the novel protocol, and that oxidation was reduced to 10%. Methionine oxidation, which occurs during standard solid-phase synthesis,[20] was reduced to less than 50% (Table 1). Furthermore, we could exclude any C-terminal oligomerization of the synthesized peptides.

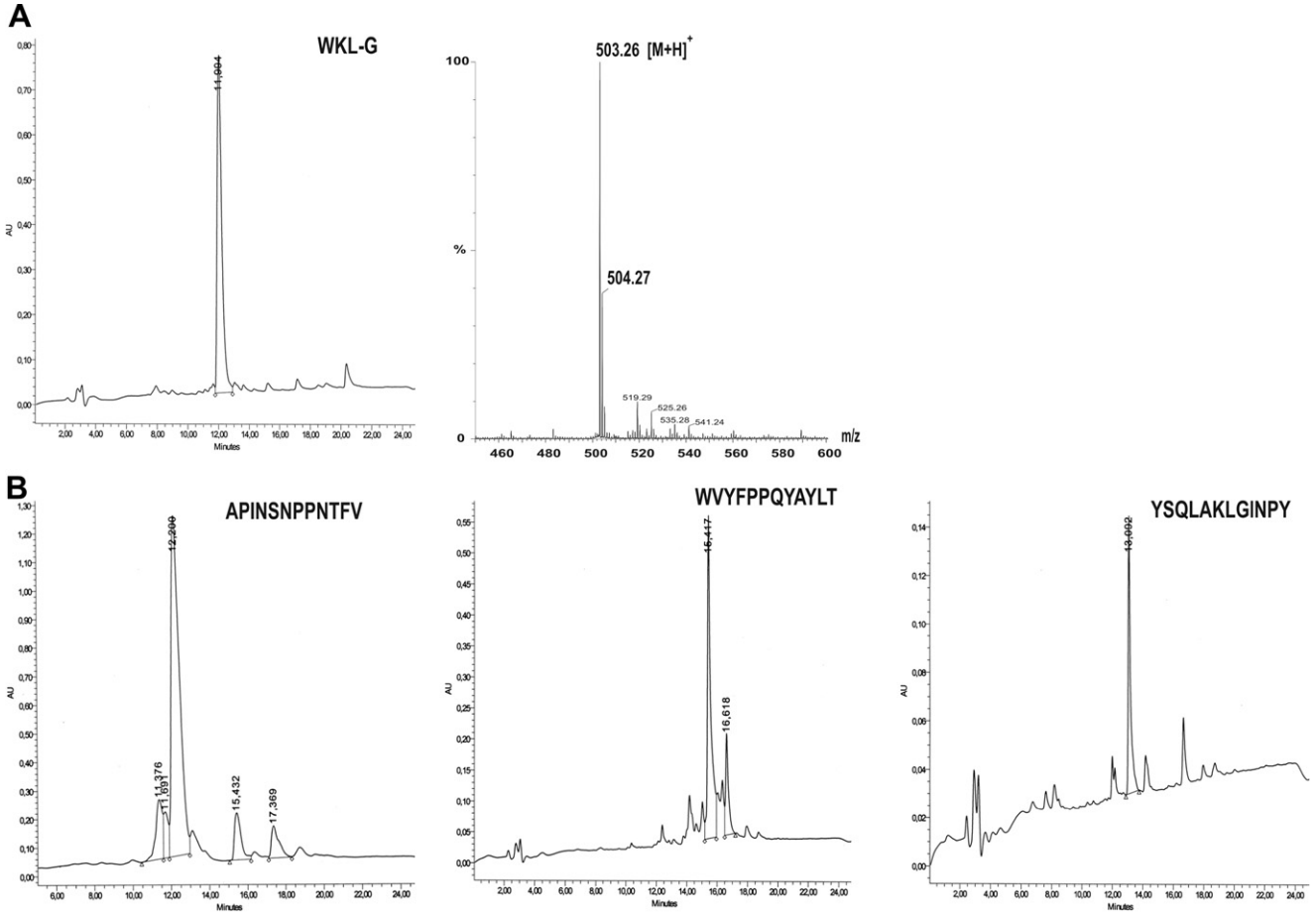


Figure 2. Model peptides synthesized using the improved method. (A) HPLC chromatogram and ESI mass spectra of the model peptide WKL-G revealed no C-terminal elongation during CDI-coupling. For details of peptide synthesis see Scheme 1. (B) HPLC chromatograms of the peptides APINSNPPNTFV, WVYFPPQYAYLT and YSQLAKLGINPY showing purities >60%.

Table 1
Characterization of model peptides by ESI mass spectrometry and analytical HPLC

Sequences	Expected [M]	Measured $[M+H^+]^+$	Purity[a] (%)	Byproducts[b]
WKL-A	516.30	517.28	>95	<1
WKL-C	548.28	549.22	80	15
WKL-D	560.29	561.24	>95	<1
WKL-E	574.30	575.22	>95	n.d.
WKL-F	592.33	593.27	>95	<1
WKL-G	502.28	503.26	>95	<1
WKL-H	582.32	583.28	>95	n.d.
WKL-I	558.34	559.28	>95	<1
WKL-K	573.35	574.28	>95	n.d.
WKL-L	558.34	559.27	>95	<1
WKL-M	576.30	577.28	65	10
WKL-N	559.30	560.29	>95	n.d.
WKL-Q	573.32	574.29	80	n.d.
WKL-R	601.36	602.35	>95	n.d.
WKL-S	532.29	533.22	>95	n.d.
WKL-T	546.31	547.29	>95	<1
WKL-V	544.33	545.28	>95	<1
WKL-W	631.34	632.32	80	n.d.
WKL-Y	608.32	609.30	90	<1
APINSNPPNTFV	1269.62	1270.57	70	n.d.
WVYFPPQYAYLT	1546.74	1547.83	79	n.d.
YSQLAKLGINPY	1365.71	1366.79	68	n.d.

[a] Purities were determined by HPLC peak area integration.
[b] Byproducts represented C-terminal elongations and/or oxidation of cysteine/methionine; n.d., not detectable.

Such a side reaction was previously described as a problem during CDI/CDT activation.[14]

In addition, these results could be reproduced with three different, randomly chosen 12-mer peptide sequences, whose purities are also >65% (Fig. 2B and Table 1) and many other sequences (data not shown).

Synthesis of the WKL-P sequence turned out to be more complicated due to the well-known formation of diketopiperazine. The extent of this side reaction is generally limited, but increases markedly when the *cis*-conformation of the peptide bond is favored, for example, in peptides with a C-terminal proline.[21,22]

To prevent diketopiperazine formation we analyzed another acid-cleavable linker: 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB). First, we determined the best activation reagent, which proved to be 1.1 equiv of EEDQ for HMPB, just as for HMPA.

Thereafter, we synthesized the sequences WKL-G (as a control), KDT-P (the protected T should avoid cleavage), and WKL-P. By measuring the Fmoc-piperidine adducts of the first C-terminal amino acid, we could follow the coupling efficiencies of the first amino acid on the linker (data not shown). MS measurements revealed the expected mass for WKL-G and KDT-P (although peak intensities were very low) using both linkers. The mass peak of WKL-P could only be detected using HMPB.

Nevertheless, there should still be enough peptide for a biological screen. To prove this hypothesis, we synthesized a His-tag on the three sequences using SPOT synthesis. The cleaved peptides were then printed on a solid support (e.g., PVDF-membrane) and detected by antibody incubation (Fig. 3). The signal intensities were measured as Böhringer Light Units (BLU in Table 2). In all cases, we could measure different signal intensities that approximately correspond to peptide yields. Based on these results, for automated SPOT synthesis we recommend using a HMPB linker if proline is the C-terminal amino acid (HMPB is 2-fold more expensive than HMPA).

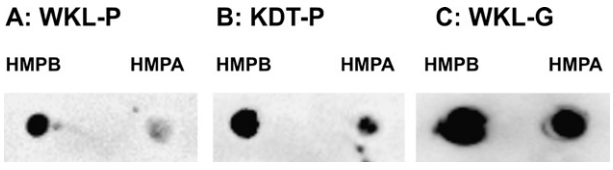


Figure 3. Test for biological activity. His-tagged peptides as listed in Table 2 were spotted on PFDV-membranes and detected using anti-polyHis/anti-mouse-HRP antibodies. (For details see Supplementary material.)

To assess the quality of SPOT synthesized peptides using the HMPA-linker approach, we used the peptide VTEHDTLLY, known as a CMV-derived CD8 T-cell epitope (VPAP protein), as a model. T-cell stimulation by this peptide was determined by flow cytometry (detailed protocol is given in Supplementary material). First, the peptide sequence VTEHDTLLY was synthesized by conventional Fmoc-chemistry on resin and purified by HPLC. Then the same peptide sequence was synthesized by our novel HMPA-linker-aided SPOT technology. Subsequently, both peptides were tested in parallel for T-cell stimulation measured by flow cytometry.

Peripheral blood mononuclear cells (PBMC) were obtained from CMV (AD169) sero-positive healthy donors (HLA 0101*). The PBMC assay (5 million cells, stimulated overnight) was performed as described previously[4] using a peptide concentration of 1 μg/ml. A positive signal corresponds to CD8 and interferon γ (INFγ) signals over a threshold of 0.03% of the total CD8 T-cells. Figure 4 presents the stimulation effect of both synthesized peptides (Fig. 4C and D) compared to the controls, DMSO (as a solvent, Fig. 4A), and HMPA (as a potential impurity, Fig. 4B). Neither control had any impact on T-cells (both signals below <0.03%). Both the peptide prepared by standard resin-based Fmoc-chemistry (Fig. 4C) and the peptide synthesized according to the HMPA-linker-aided SPOT technology (Fig. 4D) resulted in high T-cell stimulation. Furthermore, the stimulation values of 0.270% and 0.210% for these peptides are within the same range, thus directly validating our synthesis strategy.

Taken together, using both the hydroxymethylphenoxy derived linkers and the automated synthesizer, it is now possible to generate huge sets of soluble peptides without restriction, in the upper microgram range (approximately 5000 peptides) within one week, for example, for screening CD8 T-cell epitopes. This represents a time- and cost-saving advantage to the conventional large-scale SPPS.

Here, we demonstrate in detail that introducing the HMPA linker during the SPOT synthesis procedure, combined with an optimized cleavage/washing procedure, results in peptides with authentic C-termini without racemization or oligomerization of the C-terminal amino acid, and with excellent purity. For peptides with proline as C-terminal amino acid, we advise using HMPB to

Table 2
Characterization of proline sequences in comparison to glycine sequences

Linker	Seq.	Tag	$Mass_{exp.}$	$Mass_{found}$	BLU
HMPA	WKL-P	—	542.3	—	
HMPA	WKL-P	HHHHH	1227.6	—	16,111
HMPB	WKL-P	—	542.3	543.31	
HMPB	WKL-P	HHHHH	1227.6	410.22**	73,588
HMPA	KDT-P	—	459.2	460.22	
HMPA	KDT-P	HHHHH	1144.5	382.53**	29,574
HMPB	KDT-P	—	459.2	460.23	
HMPB	KDT-P	HHHHH	1144.5	573.30*	171,811
HMPA	WKL-G	—	502.3	503.21	
HMPA	WKL-G	HHHHH	1187.6	594.8*	617,774
HMPB	WKL-G	—	502.3	503.28	
HMPB	WKL-G	HHHHH	1187.6	594.8*	1,455,500

$Mass_{exp.}$, expected mass; $Mass_{found}$, measured mass using ESI mass spectrometry $[M+H^+]^+$; BLU, Boehringer Light Units detected by blot analysis and measured using LuniImiger.
* $[M+2H^+]^{++}$.
** $[M+3H^+]^{+++}$.

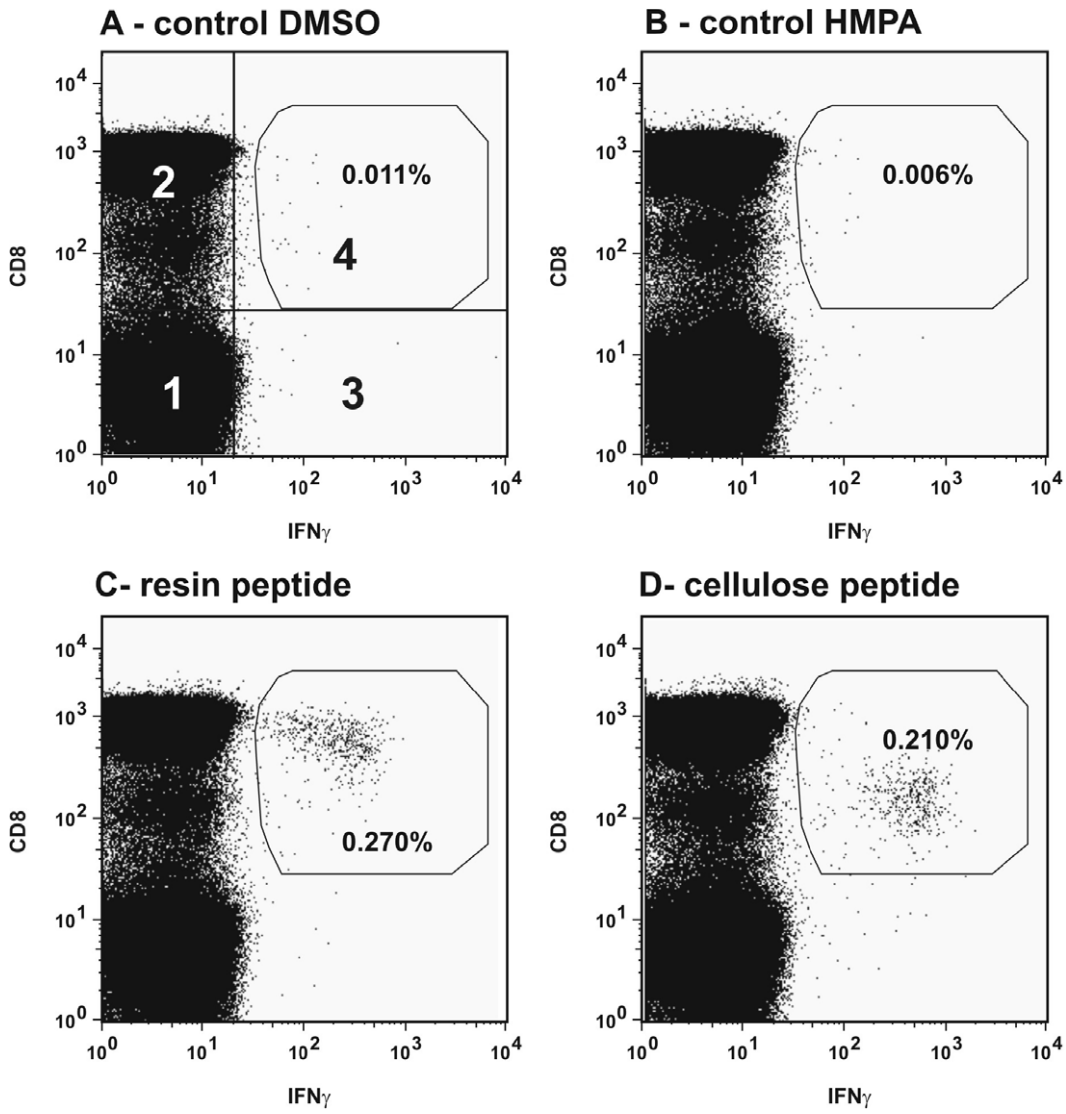


Figure 4. Validating the biological activity of the peptide VTEHDTLLY. The plots of the FACS measurements show the interferon γ (IFNγ) signal versus the CD8 signal. As depicted on the top left panel, we could divide the graph into four parts: (1) lymphocytes without INFγ or CD8 signals; (2) T-cells without INFγ but with CD8 signals; (3) lymphocytes with INFγ but without CD8 signals and (4) lymphocytes with both INFγ and CD8 signals. A signal is significant if more than 0.03% of the total measured T-cells gave a positive signal for INFγ and CD8. (A) T-cell stimulation with DMSO as a negative control (0.011%). (B) T-cell stimulation with HMPA (0.006%) as a second negative control. (C) T-cell stimulation with the resin synthesized peptide as a positive control (0.270%) is in same range as the cellulose synthesized peptide generated using our improved conditions (0.210%) (D).

decrease diketopiperazine formation and increase peptide yields. Additionally, the acidic cleavage conditions decrease cysteine and methionine oxidation and also represent a time-saving step due to simultaneous cleavage and side-chain deprotection of the peptides. These advantages extend the SPOT technique to an ideal peptide synthesis tool for any biological assay.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.116.

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