Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion

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Peptide synthesis on cellulose using SPOT technology allows the parallel synthesis of large numbers of addressable peptides in small amounts. In addition, the cost per peptide is less than 1% of peptides synthesized conventionally on resin. The SPOT method follows standard fluorenyl-methoxy-carbonyl chemistry on conventional cellulose sheets, and can utilize more than 600 different building blocks. The procedure involves three phases: preparation of the cellulose membrane, stepwise coupling of the amino acids and cleavage of the side-chain protection groups. If necessary, peptides can be cleaved from the membrane for assays performed using soluble peptides. These features make this method an excellent tool for screening large numbers of peptides for many different purposes. Potential applications range from simple binding assays, to more sophisticated enzyme assays and studies with living microbes or cells. The time required to complete the protocol depends on the number and length of the peptides. For example, 400 9-mer peptides can be synthesized within 6 days.

INTRODUCTION

Peptides play a critical role in numerous different functions of living organisms. For example, many of the known hormones regulating physical or mental processes are peptides. In the human brain alone, more than 60 different peptides, carrying out multiple functions, have been discovered[1]. Another example is host defense peptides, a fundamental component of the innate immune system of all organisms. More than 800 host defense peptides have been identified to date (http://www.bbcm.units.it/~tossi/pag1.htm). In addition, many organisms produce peptides that are very potent inhibitors of different enzymes, regulating the effect of many biochemical reactions in cells.

It is possible to synthesize and screen large numbers of peptides using biological techniques such as phage[2], bacterial[3] or ribosome display[4]. One advantage of these techniques is that the peptides are synthesized biologically, and therefore manual synthesis and expensive chemicals are not required. Another advantage is that repetitive rounds of enrichment will increase the chance of discovering highly active peptides. However, the limited numbers of sequences obtained by such methods yield only partial and incomplete information about the interaction of interest. Another disadvantage is that biological peptide libraries are tricky to handle, creating fusion peptides rather than isolated molecules, and containing only gene-encoded amino acids.

Since the introduction of solid phase peptide synthesis by Robert Bruce Merrifield[5] in 1963, this technique has become an integral tool in many biological and chemical fields of science. Two main synthesis strategies using solid phase peptide synthesis have been established, discriminated by their α-amino protection group: the Boc ((tert)-Butyloxy carbonyl)[6–8] and the Fmoc (Fluorenyl methoxy carbonyl)[9] methods. Recently available equipment allows fully automated synthesis and purification of peptides in the range of milligrams to tonnes. However, to synthesize and screen hundreds or thousands of peptides, the standard peptide technology is still relatively slow and expensive. Several different modified peptide synthesis procedures have been developed to address this problem, including tea bag synthesis[10], digital photolithography[11], pin synthesis[12] and SPOT synthesis on cellulose[13]. These modified techniques use standard peptide synthesis chemicals and building blocks in a very efficient way, and avoid purification and analysis of the peptides. Taking the tradeoff into account of unpurified and non-analyzed peptides, the price per peptide drops dramatically compared to standard peptide synthesis. All four different approaches are excellent, but we believe that for a newcomer to this field, SPOT synthesis on cellulose is most suitable, as the technique requires little effort to integrate into the laboratory, is easy to handle, it can be performed either manually, or by using semi- or fully automated robots, the quantity and quality of peptides can be freely modified, and detection of peptide–ligand interactions is simple to perform.

SPOT synthesis was developed by Ronald Frank[13] and co-workers in 1990, and subsequent automation of this method was driven by the Jens Schneider-Mergener group[14]. The SPOT method follows standard Fmoc chemistry, based on the solid phase peptide synthesis, on cellulose filter sheets. The use of cellulose in itself has several advantages over other materials: it is inexpensive and withstands organic solvents and acids used during peptide synthesis. In addition, cellulose is stable in aqueous solutions and, because it is non-toxic, it is appropriate for screening biological samples. Peptides up to a length of about 50 amino acids can be synthesized using SPOT synthesis[15], although the optimal range is between 6 and 18 amino acids. In contrast to biological peptide synthesis, chemical peptide synthesis, including the SPOT technology, is not restricted to gene-encoded amino acids, and more than 600 commercially available

building blocks can be used[16,17]. In addition, SPOT synthesis on cellulose allows peptides to be modified before and after the final side-chain protection group is cleaved. Different types of cyclization strategies can also be performed using this method. This allows peptides that contain one or more disulfide bonds to be studied[18], or identification of more rigid molecules, which might become more resistant to proteolytic cleavage and heat denaturation. Another commonly used modification to enhance the proteolytic stability is acetylation of peptides. For detection purposes, peptides can be labeled by biotinylation or fluorophore coupling. In addition, the SPOT method allows the synthesis of up to approximately 8,000 peptides per 20 × 29 cm cellulose sheet in a highly parallel and addressable manner[19]. The large number of different peptides synthesized on one cellulose sheet means that coupling yields can vary, and therefore the amounts and purity of the peptides also vary. Furthermore, the low amounts of peptide per spot make these peptides relatively difficult to purify. As a consequence, all results obtained by the SPOT method need to be confirmed using peptides synthesized, purified and analyzed according to standard peptide synthesis. Nevertheless, the SPOT technology has been frequently reported to be very reliable[20–22].

The main screening strategies provided by peptide SPOT synthesis include peptide scans, substitution analyses, length analyses, combinatorial libraries and random libraries. These strategies were well known before the SPOT technology was developed[23,24], and have subsequently been adapted for peptide synthesis on cellulose supports. Before use, peptides synthesized via the SPOT technology can be cleaved from the cellulose support by treatment with ammonia gas or other strong bases such as tertiary amines. Alternatively, a trifluoroacetic acid (TFA)-soluble cellulose membrane can be used to obtain soluble peptides[25]. As a consequence of these cleavage procedures, peptides can be used for biological assays either while tethered to the cellulose or as soluble peptides.

A multitude of investigations using peptides are possible, ranging from simple binding assays[19] to more sophisticated enzyme assays[26] and studies with living microbes[27,28] or cells[29]. Peptide–antibody interactions (epitope mapping) are considered a standard method for mapping epitopes of antibodies, and the largest proportion of publications utilizing SPOT synthesis relate to this application. Peptide–antibody interactions with dissociation constants as high as 10^{-3}–10^{-4} M remain detectable[20,30]. Areas where SPOT synthesis has also been successfully applied include peptide–receptor, peptide–enzyme, peptide–microbe, peptide–metal ion and peptide–DNA interactions. In addition, peptides on cellulose have been used to identify phosphorylation sites on proteins, and to study protein–protein interactions. We recently wrote a comprehensive review on cellulose-bound peptide arrays and their preparation and applications, including an overview of several hundred applications[31].

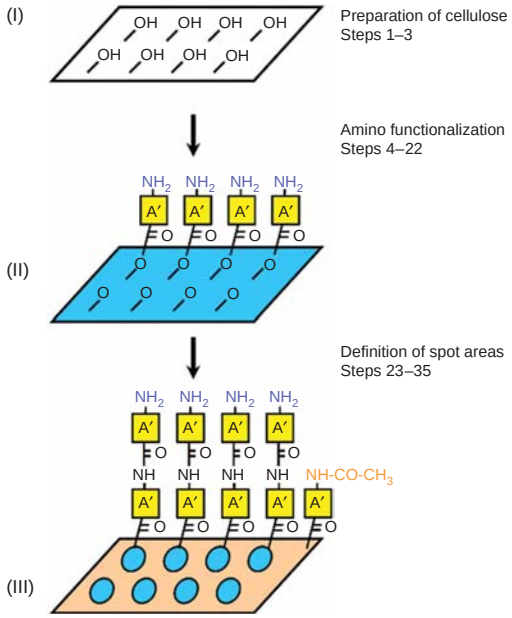


Figure 1 | Preparation of the cellulose membrane. (I) Untreated cellulose membrane with the available reactive hydroxyl groups (OH). (II) Hydroxyl groups have been esterified with the carboxyl groups of Fmoc-protected β-alanine. Following Fmoc deprotection, a layer of β-alanine (A′) is thus bound on the cellulose. The free amino groups are then stained with BPB, as symbolized by the blue color of the cellulose membrane and of the NH_2 groups. (III) At distinct spots, a second Fmoc-protected β-alanine is delivered on the cellulose, defining the areas for the peptide synthesis. Following acetylation of all free amino groups, subsequent Fmoc deprotection and staining with BPB, only those spots where a second β-alanine has been successfully appended display a blue color. The acetylated amino groups are shown in light orange.

Here, we describe a protocol for the manual synthesis of peptides on untreated cellulose sheets. Additional positive and negative control peptides synthesized on the same cellulose membrane can support the process of fine-tuning the corresponding biological assay for individual needs. We recommend first performing the biological assay of choice with control peptides to avoid wasting peptides designated for screening. These additional control peptides can also be used for HPLC and mass spectrometry analysis to assess the quality of the synthesis. A schematic representation of the sequence of reactions for this synthesis is given in **Figures 1–3**. This protocol can be easily adapted to semi- and fully automated peptide synthesis on cellulose, although the specific requirements of the robots would need to be addressed. Such apparati are purchasable through Intavis, Köln, Germany. For manual synthesis, we recommend not exceeding 400 peptides per 20 × 20 cm cellulose sheet, and for synthesis using a robot, a maximum of 6,000 peptides on a cellulose membrane of 20 × 29 cm is recommended, otherwise error probability may increase.

MATERIALS

REAGENTS

- Dimethylformamide, DMF (ACS/synthesis quality; VWR #CADX1730-3) **! CAUTION** Flammable.
- DMF (amine and water free; VWR #CADX1729-1) **! CAUTION** Flammable.
- Methanol (ACS/synthesis quality; VWR #CAMX0485-5) **! CAUTION** Flammable/toxic)
- Ethanol (ACS/synthesis quality; VWR/Malinckrodt #7019-08) **! CAUTION** Flammable.
- *N*-methylpyrrolidone, NMP (amine and water free; Fluka #69117) **! CAUTION** Irritant.
- Diethylether (ACS/synthesis quality; VWR #CAEX0190-4) **! CAUTION** Highly flammable.
- Dichloromethane (DCM), methylene chloride (ACS/synthesis quality; VWR #CADX0835-3) **! CAUTION** Harmful/irritant.

▲ CRITICAL Unless explicitly described, the solvents used were ACS or synthesis quality (see Troubleshooting section).

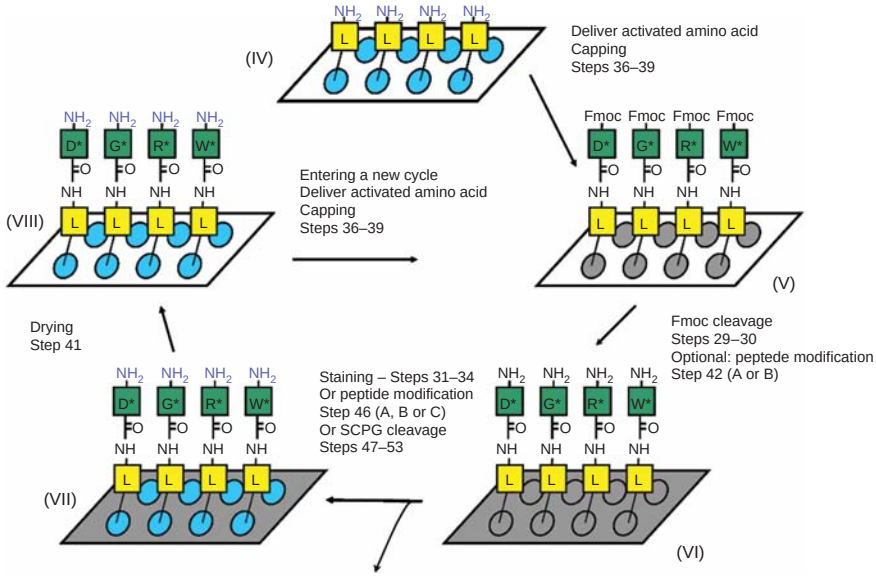
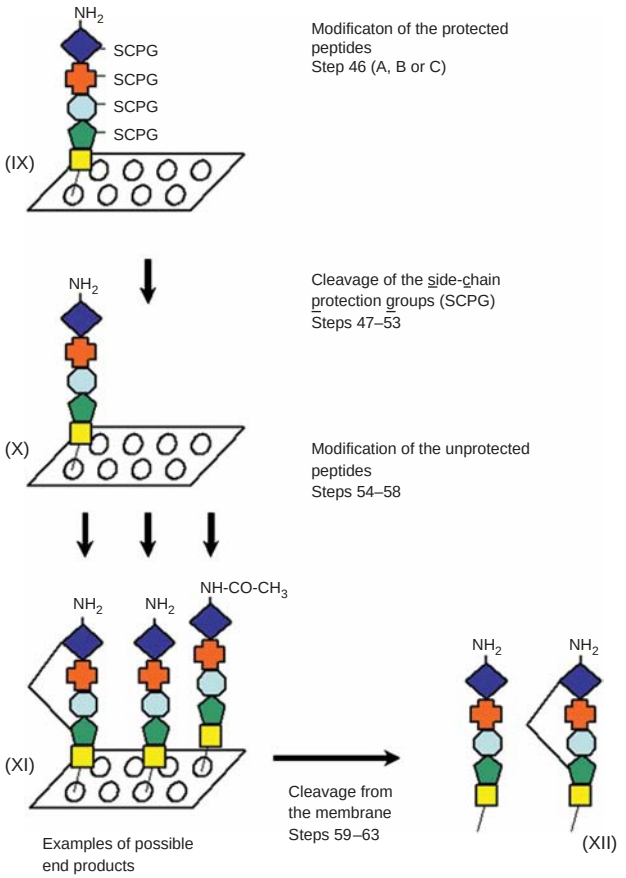


Figure 2 | Amino acid coupling cycle. (IV) The linker on the membrane, consisting of two β-alanine, is shown as "L" within a yellow colored box. (V) On each spot, the required Fmoc-protected amino acid is delivered, as symbolized by a green colored box with the one-letter code for the amino acid. The star refers to the side-chain protection groups of the amino acids. The gray spot areas indicate that solutions of the amino acids are used (wet spots on a dry membrane). (VI) Fmoc deprotection with piperidine frees amino groups for the next coupling. The gray color indicates that the membrane is treated with solvents. (VII) The free amino groups are stained with BPB (indicated by the blue dots). (VIII) The cellulose membrane is dried (indicated by the white color) and the spots are left stained.

- Cellulose: Whatman 50, Whatman 540 or Chr1 (Whatman). ▲ **CRITICAL** Note that premodified cellulose is also commercially available (e.g., from AIMS Scientific Products and Sigma-Genosys/Sigma-Aldrich); however, for these materials, preparation and cleavage conditions may be different compared to our procedure described below. When using pretreated cellulose, follow the recommendations of the manufacturer.
- Diisopropylcarbodiimide (DIC, DIPC; purum >98%; Fluka #38370) ! **CAUTION** Flammable/highly toxic
- 1,1′-carbonyl-diimidazole, CDI (EMD Biosciences #01-62-0002)
- 1-hydroxybenzotriazole hydrate, HOBt (p.a.; Aldrich #157260) ! **CAUTION** Harmful/irritant.
- 2-(1*H*-azabenzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate, (HATU, >99%; GL Biochem #00703) ! **CAUTION** Harmful/irritant.



- 1-methylimidazole (99%; Alfa Aesar #A12575) ! **CAUTION** Corrosive/highly flammable.
- Diisopropylethylamine (DIPEA, DIEA; p.a.; Aldrich #550043) ! **CAUTION** Corrosive/highly flammable.
- *N*-methylmorpholine (NMM, 99%; Alfa Aesar #A12158) ! **CAUTION** Corrosive/highly flammable.
- TFA (99%; Aldrich #T62200) ! **CAUTION** Corrosive/toxic, wear appropriate safety clothing.
- Acetic anhydride (p.a.; Fluka #45830) ! **CAUTION** Corrosive.
- Piperidine (ACS/synthesis quality; Sigma #80642-2) ! **CAUTION** Toxic/flammable.
- Triisopropylsilane (TIPS, 99%; Aldrich #233781 ! **CAUTION** Irritant/flammable)
- Triisobutylsilane (TIBS, p.a.; Fluka ! **CAUTION** Irritant/flammable)
- Phenol (>99%; Fisher Chemicals #A92-100) ! **CAUTION** Corrosive/toxic.
- DMSO (99.9%; Fisher Chemicals #D128-1)
- Sodium hydroxide, NaOH (>99%; EMD #B10252-34)
- Bromophenol blue, BPB (ACS/synthesis quality; Sigma-Aldrich #114391)
- Fmoc-β-alanine (EMD Biosciences #04-12-1044 or GL Biochem #35003)
- Non-preactivated amino acids with protection groups according to the Fmoc protection strategy (EMD Biosciences or GL Biochem)
- Amino-acid pentafluorophenyl ester derivatives with protection groups according to the Fmoc protection strategy[32] (EMD Biosciences or Bachem) ! **CAUTION** Harmful/irritant.
- Ammonia gas (Sigma) ! **CAUTION** Highly corrosive/flammable.

! **CAUTION** Most of the reagents are dangerous (toxic, flammable, corrosive and harmful); please check the Material Safety Data Sheets. All steps using organic solvents should be performed under a fume hood.

EQUIPMENT

- Rocking shaker (VWR)
- Set of pipettors (200–1,000, 20–200 and 2–20 µl) (VWR)
- Multistep pipettor (0.5–10 µl) (VWR)
- Sealable PP centrifuge tubes (0.5, 1.0 and 1.5; VWR)
- Parafilm
- Stainless steel box or PP box at least 15 cm × 10 cm (for a membrane in the format of a 96-well plate) with sharp edges and a lid
- Graduated cylinder (100 and 1,000 ml)
- Zip lock bag (PP), large enough to contain the box
- Tweezers
- Glass desiccator (ID >18 cm)

Figure 3 | Modifications and final deprotection of the peptide. (IX) The different shapes represent different amino acids of one peptide. The side-chain protection groups are represented by the abbreviation SCPG. (X) The side-chain protection groups are cleaved from the peptide. (XI) Examples of different products of the method are given (from right to left): a cyclized peptide, an unmodified peptide and an amino-terminal acetylated peptide. (XII) The two peptides without cellulose represent the possibility to cleave peptides from the support after synthesis.

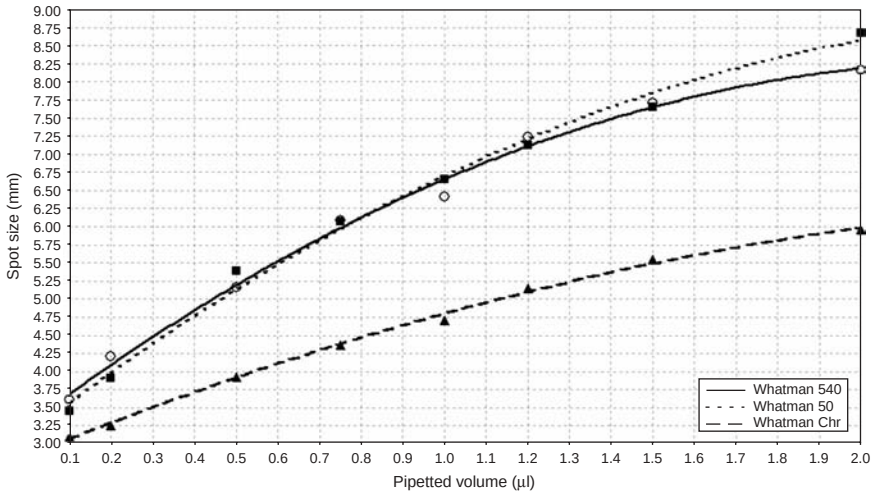


Figure 4 | Dependency of the spot size from the pipetted volume of DMSO on different filter papers (○ Whatman 540; ■ Whatman 50; ▲ Whatman Chr). For each volume, at least ten data points were analyzed. Observed error was smaller than 10%.

- Pencil
- Ruler
- Scissors

REAGENT SETUP

- Capping solution A: 2% (v/v) acetic anhydride in DMF
- Capping solution B: 2% (v/v) acetic anhydride + 2% (v/v) diisopropylethylamine in DMF
- Piperidine solution: 20% (v/v) piperidine in DMF
- Staining solution: 0.002–0.005% (w/v) (=20–50 mg in 1 liter) BPB in methanol[33]
- Cleavage solution A: 90% (v/v) TFA + 3% (v/v) TIPS or TIBS + 2% (v/v) distilled water + 1% (w/v) phenol + 4% (v/v) DCM[27]
- Cleavage solution B: 50% (v/v) TFA + 3% (v/v) TIPS or TIBS + 2% (v/v) distilled water + 1% (w/v) phenol + 44% (v/v) DCM
- 10 mM phosphate-buffered saline (PBS): 9 mM (1.278 g) Na_2HPO_4 (or 1.6 g $Na_2HPO_4 \cdot 2H_2O$) + 1 mM (120 mg) NaH_2PO_4 (or 138 mg $NaH_2PO_4 \cdot H_2O$) + 150 mM (8.766 g) NaCl in 1 liter; adjust with HCl/NaOH to pH 7.4
- 20% (w/v) aq. NaOH solution (=5 M) or diluted aq. ammonia solution

PROCEDURE

Preparation for synthesis ● TIMING about 30 min

1| Cut a piece of filter paper to the size of a microtiter plate (10 × 12 cm), which will result in 96 peptide spots. When calculating the number of spots necessary for the assay, include in the computation at least three control peptides, one for HPLC and/or mass spectroscopy and, if necessary, at least two peptides for testing the response in the corresponding biological assay. For a different number of required peptides, please adapt membrane size to accommodate all peptides to be synthesized, including controls. Be sure to leave room for labeling (use **Fig. 4** to determine the size of the peptide spots depending on the pipetted volume. A frequently used volume is 1 µl). The volumes of solutions described in this protocol are for a cellulose sheet of the size of 10 × 12 cm. Please adapt these volumes when using a different membrane size.
▲ CRITICAL STEP Please note that all steps should be performed at room temperature (~ 20 °C).

2| Mark the synthesis positions with a pencil as dots on the filter paper (see **Fig. 5**). The dots represent the center of the peptide spot, and will support the delivery of the reaction mixture to the correct position. Place the spots in the same scheme as the wells on a microtiter plate (8 × 12 spots).
▲ CRITICAL STEP The distance between the edges of the spots must be at least 1.5 mm (see **Fig. 5**). For example, using 1 µl of reaction solution on Whatman 50 or Whatman 540, the resulting diameter of a spot is 7 mm, therefore the distance between the spots' centers should be at least 8.5 mm.

3| Position and arbitrarily label or number the spots for each peptide with a pencil (not necessarily specifying the peptide sequences). It is also possible to mark the membrane using a chessboard pattern, where the *x* and *y* coordinates determine each distinct peptide (see **Fig. 5**).

Calculation of the desired amounts of amino acids ● TIMING about 2 h

4| Prepare two tables in MS Word or Excel, using **Table 1a** and **b** as templates.

5| Select the longest of the peptides chosen for the synthesis. The number of amino acids in this peptide, plus 1,

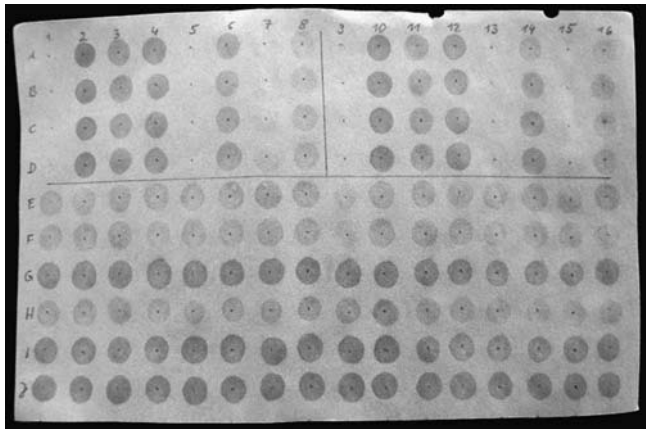


Figure 5 | Picture of a cellulose sheet where 160 peptides were synthesized. The peptide spot centers are marked with a pencil dot, and the peptide positions defined in a checkerboard manner. The membrane was stained with BPB. All peptides that have a free N-terminal α-amino group are stained. The intensity of staining depends on the acidity of the synthesized sequence, in particular, of the last coupled amino acid. Uncolored spots in columns 1, 5, 9 and 13 show peptides with blocked N-terminal α-amino groups.

1. NH_2 - WEFGHC - COOH
2. NH_2 - YCRNMA - COOH
3. NH_2 - QWERKM - COOH
4. NH_2 - SSEVYT - COOH
5. NH_2 - LRTGFF - COOH
6. NH_2 - HDRSV - COOH
7. NH_2 - NVGTE - COOH
8. NH_2 - RTEGS - COOH
9. NH_2 - AMNGH - COOH
10. NH_2 - RFDT - COOH
11. NH_2 - VCRH - COOH
12. NH_2 - HFKS - COOH
13. NH_2 - LKWQ - COOH

Figure 6 | List of 13 different peptide sequences. These sequences were transferred to **Table 1a** to determine the amino acids that are necessary for synthesis cycle.

7| In the first right-hand column of the second table, fill in the amino acids to be used during the synthesis (**Table 1b**).

8| Count each utilized amino acid separately in each column of **Table 1b** (synthesis cycle), and write this number in the corresponding field of the second table for the amino acid (row) and synthesis cycle (column) (compare **Table 1a** and **b**).

9| As a control, calculate the sum of all the numbers in each column (entered in the bottom row of **Table 1b**) to obtain the total number of amino acids needed per cycle. This number must correspond to the number of peptides that undergo the corresponding cycle (compare **Table 1a** and **b**).

10| To calculate the desired volume of amino-acid solutions per cycle, per day or per synthesis, use the following equation:

$$V_d = N_{aa} \times V_s \times N_c \times 1.25$$

V_d – desired volume of amino-acid solution (in µl)

N_{aa} – number of amino acids needed for the desired cycles (see **Table 1b**)

V_s – delivered volume used per spot (in µl; we recommend 1 µl for manual synthesis)

N_c – number of couplings per cycle (e.g., double coupling = 2)

1.25 – factor to create excess/safety volume.

The desired amounts of the amino acids can be calculated using the data according to **Tables 2** and **3**. To calculate the amounts of non-preactivated unusual or modified amino acids (e.g., D-amino acids, *N*-substituted amino acids) or other building blocks (e.g., bromoacetic acid for synthesis of peptoidic elements), use the following formula:

$$m_d = MW_{bb}\, V_d \times 0.45/1,000$$

m_d – desired amount of a building block (in mg)

determines the number of columns in both tables. In the second column from the right, label the header "1", and consecutively label all columns going from right to left. These numbers represent the number of coupling cycles.

6| In the subsequent rows of this first table, fill in the amino-acid sequences of the desired peptides. Start with the C-terminal amino acid in the second right column (cycle 1), and continue to fill in the sequence toward the left (compare **Table 1a** with peptide sequences in **Figure 6**). In the first right-hand column of the first table, write the sequential number of the peptides or the coordinates of the peptides on the membrane (**Table 1a**). We recommended ordering the peptides according to their length.

TABLE 1 | Example of tables constructed for an easy overview and calculation of the necessary amino acids per synthesis cycle.

(a)

Amino acids per cycle

6	5	4	3	2	1	Spot number
W	E	F	G	H	C	**1**
Y	C	R	N	M	A	**2**
Q	W	E	R	K	M	**3**
S	S	E	V	Y	T	**4**
L	R	T	G	F	F	**5**
	H	D	R	S	V	**6**
	N	V	G	T	E	**7**
	R	T	E	G	S	**8**
	A	M	N	G	H	**9**
		R	F	D	T	**10**
		V	C	R	H	**11**
		H	F	K	S	**12**
		L	K	W	Q	**13**

(b)

Number of amino acids needed per cycle

6	5	4	3	2	1	Amino acids
—	1	—	—	—	1	A
—	1	—	1	—	1	C
—	—	1	—	1	—	D
—	1	2	1	—	1	E
—	—	1	2	1	1	F
—	—	—	3	2	—	G
—	1	1	—	1	2	H
—	—	—	1	2	—	K
1	—	1	—	—	—	L
—	—	1	—	1	1	M
—	1	—	2	—	—	N
1	—	—	—	—	1	Q
—	2	2	2	1	—	R
1	1	—	—	1	2	S
—	—	2	—	1	2	T
—	—	2	1	—	1	V
1	1	—	—	1	—	W
1	—	—	—	1	—	Y
5	9	13	13	13	13	Total

(**a**) List of sequences: the spot numbers in the right column could also contain the coordinates (chessboard) of the spots on the membrane. (**b**) List of numbers of amino acids used per cycle.

MW_{bb} — molecular weight of the building block

V_d — desired volume of amino-acid solution (in µl)

0.45 — concentration of the stock solution in M.

For the calculation of the amounts of preactivated amino acids (e.g., amino-acid pentafluorophenyl esters), use the following formula:

$m_d = MW_{bb}\, V_d \times 0.3/1{,}000$

m_d — desired amount of the preactivated amino acid (in mg)

MW_{bb} — molecular weight of the preactivated amino acid

V_d — desired volume of amino-acid solution (in µl)

0.30 — concentration of the final solution in M.

TABLE 2 | Calculated amounts (in mg) of common Fmoc amino acids for the preparation of *in situ* activated coupling mixtures.

(a)

Amino acid in the sequence (OLC)	Protected amino acid	MW of protected amino acid (g/mol)	Amounts of protected amino acid in mg according to the desired total volume (volume of amino acid/HOBT solution)			
			0.75 ml (0.563 ml)	1.0 ml (0.75 ml)	1.5 ml (1.125 ml)	2.5 ml (1.875 ml)
	Fmoc-β-Ala-OH	311.3	105.1	140.1	210.1	350.2
A	Fmoc-Ala-OH	311.3	105.1	140.1	210.1	350.2
C	Fmoc-Cys(Trt)-OH	585.7	197.7	263.6	395.3	658.9
D	Fmoc-Asp(OtBu)-OH	411.5	138.9	185.2	277.8	462.9
E	Fmoc-Glu(OtBu)-OH	425.5	143.6	191.5	287.2	478.7
F	Fmoc-Phe-OH	387.4	130.7	174.3	261.5	435.8
G	Fmoc-Gly-OH	297.3	100.3	133.8	200.7	334.5
H	Fmoc-His(Trt)-OH	619.7	209.1	278.9	418.3	697.2
I	Fmoc-Ile-OH	353.4	119.3	159.0	238.5	397.6
K	Fmoc-Lys(Boc)-OH	468.5	158.1	210.8	316.2	527.1
L	Fmoc-Leu-OH	353.4	119.3	159.0	238.5	397.6
M	Fmoc-Met-OH	371.5	125.4	167.2	250.8	417.9
N	Fmoc-Asn-OH	354.4	119.6	159.5	239.2	398.7
N	Fmoc-Asn(Trt)-OH	596.7	201.4	268.5	402.8	671.3
P	Fmoc-Pro-OH	337.4	113.9	151.8	227.7	379.6
Q	Fmoc-Gln-OH	368.4	124.3	165.8	248.7	414.5
Q	Fmoc-Gln(Trt)-OH	610.7	206.1	274.8	412.2	687.0
R	Fmoc-Arg(Pbf)-OH	648.8	219.0	292.0	437.9	729.9
S	Fmoc-Ser(tBu)-OH	383.4	129.4	172.5	258.8	431.3
T	Fmoc-Thr(tBu)-OH	397.5	134.2	178.9	268.3	447.2
V	Fmoc-Val-OH	339.4	114.5	152.7	229.1	381.8
W	Fmoc-Trp-OH	426.5	143.9	191.9	287.9	479.8
W	Fmoc-Trp(Boc)-OH	526.6	177.7	237.0	355.5	592.4
Y	Fmoc-Tyr(tBu)-OH	459.6	155.1	206.8	310.2	517.1

(b)

Activator/coupling reagent	MW of reagent (g/mol)	Amounts of reagent in mg or µl (volume of reagent solution in ml) according to the desired total volume of activated solution			
		0.75 ml	1.0 ml	1.5 ml	2.5 ml
0.9 M HOBt	153.1	51.7 (0.375)	68.9 (0.5)	103.4 (0.75)	172.3 (1.25)
1.35 M DIC	126.2	37.6 (0.188)	50.1 (0.25)	75.2 (0.375)	125.3 (0.625)

(a) The amounts of amino acids were calculated in relation to the desired volume (formulas in Step 10). To prepare a 0.3 M reaction mixture, the appropriate volume of 0.9 M HOBt (in amine-free NMP) must first be added to the weighed amount of amino acid. The resulting volume of the amino acid/HOBt solution must be adjusted to the correct volume by adding more amine-free NMP. This mixture is stable at –20 °C for at least 1 week. To activate this solution, one volume of 1.35 M DIC (mixed with amine-free NMP) is added to three volumes of the previously prepared amino acid/HOBt mixture. This activated solution is stable for 1 day. Owing to the low stability of the activated arginine mixture, we recommend to prepare this solution fresh before each coupling step. **(b)** The amounts of coupling reagents were calculated in relation to the desired total volume (formula in Step 11). The calculated amount of HOBt is in mg of DIC in µl (includes the physical density). Dissolve the amino acids in the corresponding volume of HOBt (in parentheses), then adjust with NMP, as in **(a)**. Add the corresponding solution of the DIC the same day the *in situ* activated mixture will be used.

TABLE 3 | Calculated amounts (in mg) of common amino-acid pentafluorophenyl ester.

Amino acid in the sequence (OLC)	Protected preactivated amino acid	MW of protected amino acid (g/mol)	Amounts of protected preactivated amino acid in mg according to total volume			
			0.5 ml	1.0 ml	1.5 ml	2.0 ml
	Fmoc-β-Ala-OPfp	477.39	71.6	143.2	214.8	286.4
A	Fmoc-Ala-OPfp	477.39	71.6	143.2	214.8	286.4
C	Fmoc-Cys(Trt)-OPfp	751.79	112.8	225.5	338.3	451.1
D	Fmoc-Asp(OtBu)-OPfp	577.5	86.6	173.3	259.9	346.5
E	Fmoc-Glu(OtBu)-OPfp	591.50	88.7	177.5	266.2	354.9
F	Fmoc-Phe-OPfp	553.49	83.0	166.0	249.1	332.1
G	Fmoc-Gly-OPfp	463.36	69.5	139.0	208.5	278.0
H	Fmoc-His(Trt)-OPfp	785.78	117.9	235.7	353.6	471.5
I	Fmoc-Ile-OPfp	519.47	77.9	155.8	233.8	311.7
K	Fmoc-Lys(Boc)-OPfp	634.60	95.2	190.4	285.6	380.8
L	Fmoc-Leu-OPfp	519.47	77.9	155.8	233.8	311.7
M	Fmoc-Met-OPfp	537.51	80.6	161.3	241.9	322.5
N	Fmoc-Asn-OPfp	520.42	78.1	156.1	234.2	312.3
N	Fmoc-Asn(Trt)-OPfp	762.74	114.4	228.8	343.2	457.6
P	Fmoc-Pro-OPfp	503.43	75.5	151.0	226.5	302.1
Q	Fmoc-Gln-OPfp	534.50	80.2	160.4	240.5	320.7
Q	Fmoc-Gln(Trt)-OPfp	776.77	116.5	233.0	349.5	466.1
R	Fmoc-Arg(Pbf)-OPfp	814.84	122.2	244.5	366.7	488.9
S	Fmoc-Ser(tBu)-OPfp	549.5	82.4	164.9	247.3	329.7
T	Fmoc-Thr(tBu)-OPfp	563.52	84.5	169.1	253.6	338.1
V	Fmoc-Val-OPfp	505.44	75.8	151.6	227.4	303.3
W	Fmoc-Trp-OPfp	592.52	88.9	177.8	266.6	355.5
W	Fmoc-Trp(Boc)-OPfp	692.70	103.9	207.8	311.7	415.6
Y	Fmoc-Tyr(tBu)-OPfp	625.60	93.8	187.7	281.5	375.4

The amounts were calculated in relation to the desired volume. To prepare a 0.3 M solution, the weighed amount must be dissolved in the appropriate volume of amine-free DMF (except derivatives of serine, threonine, proline and tryptophan that must be dissolved in amine-free NMP). Fmoc-β-Ala-OPfp is spotted as a solution in anhydrous DMSO.

11| For the *in situ* activation of the non-preactivated amino acids and building blocks, activators are necessary. Normally, a combination of DIC and HOBt is used as an activator. The desired volume of HOBt and DIC solutions can be calculated using the following formula:

$$V_a = N_t \times V_s \times N_c \times F_r$$

V_a – desired volume of activation reagents (HOBt or DIC; in μl)

N_t – total number of couplings for the cycles concerned (see the last row of **Table 1b**)

V_s – delivered volume used per spot (in μl; we recommended 1 μl for manual synthesis)

N_c – number of couplings per cycle (e.g., double coupling = 2)

F_r – reagent-specific factor (for activators like HOBt = 0.5; for coupling reagents like DIC = 0.25).

12| Prepare an outlay describing the positions for the amino acids coupled per cycle. This outlay system can be based on option A or B: option A uses a table for each synthesis step, whereas option B uses a map-like overview for each synthesis step.

(A) Outlay system using a table for each synthesis step

(i) Prepare a table similar to **Table 1b**. Change the column heading from "Number of amino acids needed per cycle" to "Positions of amino acids delivered per cycle". For each single cycle, according to the sequence of each peptide (see **Table 1a**), fill out the fields of the corresponding amino acids using the sequential number or coordinates of the peptides on the membrane. Repeat this for each cycle.

(B) Outlay system using a map–like overview for each synthesis step

(i) Copy the labeled membrane (Steps 2 and 3) to a transparent foil or sheet of paper. One copy is required for each cycle, and is labeled with the distinct coupling cycle number.

(ii) Label each spot synthesis position on the copy with the one letter symbol of the required amino acid to be coupled to the specific spot at this particular coupling cycle. Repeat this procedure until all the positions for all cycles are complete.

Amino functionalization of the filter paper ● TIMING **About 3 h/overnight**

13| Prepare the reaction mix for modification of the cellulose. For the 10 × 12 cm cellulose membrane, prepare the following reaction mix: dissolve 0.96 g Fmoc-β-alanine in 15 ml amine-free DMF; add 560 µl DIC and 475 µl 1-methylimidazole and mix well. Transfer this solution into a metal box with a lid, which can withstand the organic solvents used, and is large enough to accommodate the cellulose membrane. Alternatively, if higher coupling yields are required (see ANTICIPATED RESULTS), use 0.87 g Fmoc-glycine instead of Fmoc-β-alanine, and 612 µl CDI instead of DIC[29]. For other sizes of cellulose sheet, adapt the required chemicals and volumes.

▲ CRITICAL STEP Reagents must be protected from moisture. Reagent bottles stored in the fridge or freezer should be warmed up to room temperature for approximately 30 min before use.

14| Carefully transfer the prepared cellulose membrane (Steps 1–3), with the help of tweezers (do not touch the membrane with bare hands), into the box (Step 13). Close the box with the lid.

▲ CRITICAL STEP Avoid air bubbles under the membrane; they will cause an incomplete or non-homogeneous modification of the membrane. Keep the cellulose membrane surface covered by the solution prepared in Step 13.

15| Incubate the reaction mixture without shaking for at least 2 h.

■ PAUSE POINT An overnight reaction time is also possible.

16| Discard solution from Step 15 and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 to obtain three wash steps.

■ PAUSE POINT It is possible to store the membrane at −80 °C for at least 1 month. Before storage, perform washing steps 19–22, and place the dried membrane in a sealable polypropylene bag. Before use, equilibrate the membrane to room temperature, and subsequently place the membrane in the box and add 30 ml DMF. Place the box on a shaker for at least 10 min, then proceed with Step 17. To obtain the best results, we do not recommend storing the membrane, as it is always better to use freshly prepared membranes.

17| Discard solution from Step 16 and fill the box with 30 ml 20% (v/v) piperidine in DMF. Place box on a shaker and shake for 5 min. Discard used solution, refill with new 20% (v/v) piperidine in DMF solution and shake for 5 min more.

18| Discard solution from Step 17 and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

19| Discard solution from the previous step and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2).

20| Discard solution from Step 19 and fill the box with 30 ml staining solution (see REAGENTS). Place the box on a shaker and shake for at least 2 min, until the filter paper shows a homogeneous blue color.

▲ CRITICAL STEP The filter paper must display a homogeneous blue color, indicating homogeneous modification with amino groups. Otherwise, the peptide density may vary.

? TROUBLESHOOTING

21| Discard solution from Step 20 and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2). Repeat the procedure of wash step 2 until the wash solution remains colorless.

22| Dry the membrane in the air stream of a fume hood, or with a hair dryer set on cold.

Preparation of amino-acid solutions and definition of spot areas ● TIMING **About 5–6 h**

23| Depending on the availability of the preactivated amino acids and building blocks, option A or B can be performed. Option A provides a protocol for non-preactivated amino acids and all other building blocks, whereas option B presents a protocol for already activated amino acids[14,22,34].

! CAUTION All steps using organic solvents should be performed under a fume hood.

▲ CRITICAL STEP Reagents must be protected from moisture. Reagent bottles stored in the fridge or freezer should be warmed up to room temperature for approximately 30 min before use.

(A) Preparation of solutions with *in situ* activated amino acids (about 3 h)

(i) Calculate the desired amounts of amino acid for synthesis cycles over an entire week (2–4 cycles per 8-h working day are possible; see section Calculation of the desired amounts of amino acids and **Table 2**), and weigh the calculated amount of amino acids into sealable 0.5, 1.0, 1.5 or 2 ml centrifuge tubes.

(ii) Prepare an adequate volume of HOBt solution (see Step 11).

(iii) Add the corresponding volume of HOBt solution to the protected amino acid or building block. In case the required total volume is not gained by addition of HOBt solution, adjust the volume to the corresponding total volume by adding amine-free NMP (see **Table 2**). Mix the solution well.
? TROUBLESHOOTING

(iv) Remove the desired volume for the current day from these amino acid/HOBt mixtures. Store the remaining solutions at −20 °C or below.
■ PAUSE POINT The solutions of Fmoc-protected amino acids in amine-free NMP mixed with the appropriate volume of HOBt solution can be stored at −20 °C for at least a week.
▲ CRITICAL STEP Owing to the low stability of protected arginine, it is recommended to prepare a fresh solution of this compound when needed for each coupling step. Furthermore, to obtain the activated mixtures, DIC solution needs to be added every day (see next step).

(v) Prepare the desired volume of DIC/NMP mixture for the current day (refer to **Table 2b** and Step 23A(vi)).

(vi) Add the corresponding amount of the DIC/NMP mixture to the protected amino acid/HOBt mixtures in a ratio of 1:3. Mix them well.
? TROUBLESHOOTING

(B) Preparation of solutions with preactivated amino-acid derivatives ● TIMING **About 3 h**

(i) Calculate the desired amount for synthesis cycles over an entire week (2–4 cycles per 8 h working day are possible; see section Calculation of the desired amounts of amino acids and **Table 2**) and weigh the calculated amount in sealable 0.5, 1.0, 1.5 or 2 ml centrifuge tubes.

(ii) Add amine-free NMP to the corresponding volume and dissolve the amino acid. Owing to their poor solubility or their tendency to precipitate in crystalline form, the derivatives of serine, threonine, tryptophan and proline must be dissolved in amine-free DMF instead of NMP. Check the volume after dissolving, and if necessary adjust the volume by adding more NMP or DMF. Label the tubes with a permanent marker.
? TROUBLESHOOTING

(iii) Remove the desired volume from these solutions for the current day. Store the remaining solutions at −20 °C or below.
■ PAUSE POINT The solutions of preactivated protected amino acids can be stored at −20 °C for at least a week.
▲ CRITICAL STEP An exception is the preactivated derivative of arginine, for which fresh solutions must be prepared every day. Discard used amino acid solutions the next day morning.

24| Place the dried membrane on an inert surface (e.g., glass, stainless steel or PP).

25| Add the required volume of activated Fmoc-β-alanine solution to all spot positions; we recommended using a multistep pipettor. Reaction time after delivery is at least 20 min.
▲ CRITICAL STEP The size of the spot at this step determines the final size of the spots!

26| (OPTIONAL) We recommend a double coupling by repeating Step 25, to gain more reproducible results.

27| Add 20 ml of capping solution A into the box. Place the membrane face down in this solution. Take care that there are no air bubbles under the surface. Allow for a reaction time of 5 min.
▲ CRITICAL STEP Despite the added capping solution, the spotted activated amino acids are still reactive. To avoid merging of the spotted activated amino acids, do not shake during this step!

28| Remove the liquid. Add 20 ml of capping solution B into the box. Place the membrane face up in this solution. Keep the membrane in the solution for 5–20 min.

29| Discard solution from the previous step and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat the procedure of wash step 2 twice to obtain four wash steps in total.

30| Discard solution from Step 29 and fill the box with 30 ml 20% (v/v) piperidine in DMF. Place the box on shaker and shake for 5 min. Discard used solution, refill with new 20% (v/v) piperidine in DMF solution and shake for 5 min more.

31| Discard solution from the previous step and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

32| Discard solution from Step 31 and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard used methanol or ethanol, refill with 30 ml new methanol or ethanol and shake for at least 30 s more (wash step 2).

33| To indicate the spot locations, the membrane should be treated with staining solution. Shake the membrane with 25–30 ml staining solution in the box. Let it react for at least 15 s, until most of the spots show a blue color. The staining solution might need to be renewed if the solution changes its yellow color to blue very rapidly. Some amino acids show little or no staining when coupled on the corresponding spot; therefore, the coupling success cannot be determined accurately.
? TROUBLESHOOTING

34| Discard solution from Step 33 and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2). Repeat wash step 2 until no more stain comes off the membrane. If faster drying is necessary, use two additional washing steps, by using 30 ml diethylether each time.

35| Dry the membrane in the air stream of the fume hood or with a hair dryer set at cold. The membrane is now ready for the first coupling cycle.

Coupling cycle ● TIMING About 2–3 h

36| Deliver the activated protected amino-acid solutions to the corresponding positions on the membrane appropriate for the particular cycle. Use the overview prepared according to Step 12A. It is recommended to use a multistep pipettor. Reaction time after delivery is at least 20 min.
▲ CRITICAL STEP To cover the whole area of large spots, we recommend using at least 20% more amino-acid solution volume than the initial Fmoc-β-alanine solution volume in Step 25. This excess volume is already accounted for in the formula of desired volume of amino-acid solution V_d in Step 10.

37| (OPTIONAL) We recommend a double coupling by repeating Step 36, to gain more reproducible results.

38| Add 20 ml of capping solution A into the box. Place the membrane face down in this solution. Reaction time is 2–5 min.
▲ CRITICAL STEP To avoid incomplete capping, take care that there are no air bubbles under the surface. Despite the added capping solution, the spotted activated Fmoc-β-alanine is still reactive. To avoid merging of the spotted activated amino acid, do not shake during this step!

39| Remove the liquid. Add 20 ml of capping solution B into the box. Place the membrane (face up) in this solution. Reaction time is 2–5 min.

40| Except after the last coupling cycle, repeat Steps 29–34 for each cycle, and then continue with Step 41.
▲ CRITICAL STEP After the last coupling cycle, carry out only Step 31 and proceed with Step 43, that is, no Fmoc deprotection and no staining! If the peptide should be modified according to Step 42, perform also Steps 32 and 41.

41| Dry membrane in the air stream of the fume hood or with a hair dryer set at cold. The membrane is now ready for the next coupling cycle. Except after the last coupling cycle, restart at Step 36.

Modification of the peptide before final Fmoc and side-chain deprotection (OPTIONAL)

42| This step can be carried out for several purposes. Here, we describe two applications for peptide modification before final Fmoc and side-chain deprotection. Please find in the section of Step A the directions to perform the cyclization of the nascent peptide. A generalized approach to modify the side chains in the nascent peptide chain is given in Step B.

(A) Cyclization of the peptides is carried out by their side-chain amino and carboxy groups ● TIMING About 3 h

(i) To perform side-chain to side-chain cyclization, orthogonally protect the amino acids to undergo cyclization: The carboxyl side chain of glutamic acid or aspartic acid should be protected as a 2-phenylisopropyl ester (O-2-PhiPr; EMD Biosciences). Orthogonal protection of the amino side-chain group of lysine, ornithine, diaminobutyric acid or diaminopropionic acid should be carried out using 4-methyltrityl derivatives (Mtt; EMD Biosciences). These orthogonal protected amino acids should be coupled during the synthesis like other non-preactivated amino acids (*in situ* activated amino acids).
▲ CRITICAL STEP To avoid undesired side reactions, amino acids that are not involved in the cyclization must not be protected with these orthogonal protecting groups.

(ii) Shake the membrane three times, for 2 min, in a mixture of 3% (v/v) TIPS (or TIBS) and 2% (v/v) TFA in DCM. Use a fresh mixture each time. During the treatment, close the box with the lid.
! CAUTION TFA is highly corrosive!
? TROUBLESHOOTING

(iii) Discard solution from Step (ii) and fill the box with 30 ml DCM. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard used DCM, refill with 30 ml new DCM and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(iv) (OPTIONAL; staining is not required for successful cyclization.) Discard solution from Step (iii) and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure to obtain three wash steps in total. Discard methanol or ethanol and add 30 ml of staining solution onto the membrane. Place the box on a shaker and shake for at least 15 s, until the spots show a blue color.

(v) Discard solution from Step (iv) and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(vi) Discard solution from Step (iv) and add 20 ml of a solution consisting of 1.9 g HATU and 0.6 ml NMM in amine-free DMF; allow a reaction time of 2 h. Note that to increase coupling yields, a double coupling is recommended.

(vii) Discard solution from Step (vi) and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(viii) (Optional) To estimate cyclization success, wash the membrane three times with methanol or ethanol (wash time is at least 30 s each). Afterwards, stain the membrane with staining solution by shaking for at least 15 s. Owing to the reacted side-chain amino groups, the spots with successfully cyclized peptides should show a lower intensity of blue color compared to that before cyclization.

? TROUBLESHOOTING

(B) A side-chain amino group can be modified by coupling a building block (e.g., biotin, fluorescein or protected amino acid ● TIMING About 2–3 h/overnight

(i) To perform modification of the peptide via amino side-chain groups, orthogonally protect the corresponding amino acids. Orthogonal protection of the amino side-chain group of lysine, ornithine, diaminobutyric acid or diaminopropionic acid should be carried out using commercially available 4-methyltrityl derivatives (EMD Biosciences). If two different modifications via amino side-chain groups are necessary, the peptide must have two different orthogonal protecting groups (see **Table 4**). These orthogonal protected amino acids should be coupled during the synthesis like other non-preactivated amino acids (*in situ* activated amino acids).

▲ CRITICAL STEP Amino acids that are not involved in the modification must not be protected with these orthogonal protecting groups! Otherwise, these groups could also react and lead to undesired side products.

(ii) To remove the Mtt orthogonal side-chain protection group, shake the membrane three times, for 2 min each, in a mixture of 3% (v/v) TIPS (or TIBS) and 2% (v/v) TFA in DCM. Use a fresh mixture each time. During the treatment, close the box with the lid.

! CAUTION TFA is highly corrosive!

? TROUBLESHOOTING

(iii) Discard solution from Step (ii) and fill the box with 30 ml DCM. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard used DCM, refill with 30 ml new DCM and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(iv) (OPTIONAL; staining is not required for successful modifications.) Discard solution from the previous step and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure to obtain three wash steps in total. Discard methanol or ethanol and add 30 ml of staining solution onto the membrane. Place the box on a shaker for at least 15 s, until the spots show a blue color.

TABLE 4 | Possible pairs of orthogonal protecting groups for selective deprotection of carboxyl and amino groups for amide cyclization.

Amino protection	Carboxy protection	Cleavage condition	Remarks
Mtt	O-2-PhiPr	2% (v/v) TFA + 3% (v/v) TIPS in DCM	Highly recommended
Aloc	O-All	3 eq. $(Ph_3P)_4Pd$ in DCM/acetic acid/NMM (37:2:1)	Remaining Pd will stain the membrane black during TFA treatment; very important is the high quality of the amine-free NMM; incomplete deprotection was observed several times
Dde or iv-Dde	O-Dmab	2% (v/v) hydrazine/DMF	This strong basic treatment will lead to a loss of peptide from the membrane and to Fmoc cleavage; incomplete deprotection was observed several times
Teoc	O-Tmse (=O-TMSEt)	1 M TBAF/DMF	This strong basic treatment will lead to a loss of peptide from the membrane

(v) Discard solution from the previous step and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(vi) Treat with a solution of 6 mmol of the modification building block (e.g., biotin, fluorescein) and 2.3 g HATU and 0.725 ml NMM in 20 ml amine-free DMF; reaction time 20 min; a repeat of this coupling is also recommended. Alternatively, it is possible to use a 0.3 M solution of preactivated derivatives (i.e., *N*-hydroxysuccinimidyl ester, pentafluorophenyl ester, etc.). Calculate the amount in grams of a non-preactivated building block as follows:

$$m = 6 \times MW/1000$$

$$m - \text{amount in g}$$

$$MW - \text{molecular weight}$$

(vii) (OPTIONAL) Instead of coupling on the whole membrane, it is also possible to couple on selective spots by pipetting the solutions. For this purpose, follow Steps 23, 36 and 37.

(viii) Discard solution from Step (vi) or (vii) and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(ix) (OPTIONAL) Wash three times with methanol or ethanol, wash time is at least 30 s each; then stain the membrane with staining solution. Shake the membrane in the box. Let it react for least 15 s and then wash twice with methanol. Due to the reduction of the number of free amino groups, the spots with successfully modified peptides should show a lower intensity of blue color compared to that prior to modification.

Final Fmoc deprotection ● TIMING About 30 min

43| Discard solution from Step 42 or 40, depending on whether or not optional steps 41 and 42 have been implemented, and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

44| Discard solution from Step 43 and fill the box with 30 ml 20% (v/v) piperidine in DMF. Place box on shaker and shake for 5 min. Discard used solution, refill with new 20% (v/v) piperidine in DMF solution and shake for 5 min more.

45| Discard solution from Step 44 and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

Modification of peptides involving the N-terminal α-amino group before side-chain deprotection (OPTIONAL)

46| Here we describe some applications for peptide modifications of the N-terminal α-amino group before the side-chain deprotection step. Option A gives directions as to how to acetylate the N terminus of the peptide, option B deals with peptide cyclization through the N terminus and a carboxylate from a side chain, and option C gives general directions as to how to modify the N terminus of the protected final peptide. All of these approaches can be easily adapted to other modifications required for the individual needs.

(A) Acetylation of the N-terminal α-amino group ● TIMING About 40 min

(i) (OPTIONAL; staining is not required for successful acetylation.) Wash at least two times with methanol or ethanol. Then stain the membrane with staining solution. Shake the membrane in the box. Let it react for at least 15 s until the spots show a blue color. The staining solution might need to be renewed if the solution changes its yellow color to blue very rapidly. After staining, wash three times with DMF for at least 30 s each.
? TROUBLESHOOTING

(ii) If the optional step (i) has been implemented, discard first the solution from Step (i), otherwise simply place the membrane from Step 45 in the box and fill it with 20 ml of capping solution B. Place the box on a shaker and shake for at least 20 min. This step can also be carried out on selected spots by pipetting. In preparation for the selective acetylation, after staining, wash the membrane with methanol or ethanol instead of DMF (as per Step 46A(i)) and dry the membrane (according to Steps 34 and 35). It is recommended to deliver the capping solution B twice to the selected spots with a duration of about 20 min.

(iii) Discard solution from Step (ii) and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(iv) (OPTIONAL) Discard solution from Step (iii) and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure to obtain three wash steps in total. Discard methanol

or ethanol and add 30 ml of staining solution onto the membrane. Place the box on a shaker and shake for at least 15 s, until the spots show a blue color. Owing to blocked amino groups, the spots with acetylated peptides should be colorless. After checking the pattern of staining, remove the dye by washing twice for 5 min with 20% (v/v) piperidine/DMF and subsequently washing five times with DMF for at least 1 min each time.

(B) Cyclization of peptides via a side-chain carboxy group and the N-terminal α-amino group ● TIMING 2–3 h/overnight

(i) To perform this cyclization, orthogonally protect the corresponding amino acids. The carboxyl side chain of glutamic or aspartic acid should be protected as commercially available 2-phenylisopropyl ester (*O*-2-PhiPr; EMD Biosciences). These orthogonally protected amino acids are coupled beforehand on the desired positions during the synthesis similar to what is done with common protected amino acids.

▲ **CRITICAL STEP** Amino acids that are not involved in the cyclization should not be protected with these orthogonal protecting groups (see above).

(ii) Discard solution from Step (i) and fill the box with 30 ml DCM. Place the box on a shaker and shake for at least 2 min (wash step 1). Discard used DCM, refill with 30 ml new DCM and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(iii) Discard solution from Step (ii) and fill the box with 30 ml of a mixture of 3% (v/v) TIPS (or TIBS) and 2% (v/v) TFA in DCM. During the treatment, close the box with the lid. Place the box on a shaker and shake for at least 2 min. Discard used solution, refill with 30 ml new solution and shake for at least 2 min more. Repeat this once more.

! CAUTION TFA is highly corrosive!

? TROUBLESHOOTING

(iv) Discard solution from Step (iii) and fill the box with 30 ml DCM. Place the box on a shaker and shake for at least 2 min (wash step 1). Discard used DCM, refill with 30 ml new DCM and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(v) (OPTIONAL; staining is not required for successful modifications.) Discard solution from Step (iv) and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure to obtain three wash steps in total. Discard methanol or ethanol and add 30 ml of staining solution onto the membrane. Place the box on a shaker and shake for at least 15 s, until the spots show a blue color.

(vi) Discard solution from Step (v) and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(vii) Discard solution from Step (vi) and add 20 ml of a solution consisting of 1.9 g HATU and 0.6 ml NMM in amine-free DMF; allow a reaction time of 2 h. Please note that to increase coupling yields, a double coupling or overnight coupling is recommended.

(viii) Discard solution from Step (v) and fill the box with 30 ml DMF. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard DMF, refill with 30 ml DMF and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

(ix) (OPTIONAL) Discard solution from Step (iv) and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 30 s (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure to obtain three wash steps in total. Discard methanol or ethanol and add 30 ml of staining solution onto the membrane. Place the box on a shaker and shake for at least 15 s, until the spots show a blue color. The spots with successfully cyclized peptides should show a lower intensity of blue color compared to before the cyclization (due to the blocked amino groups). After checking the staining pattern, remove the dye by washing twice for 5 min with 20% (v/v) piperidine/DMF and subsequently washing five times with DMF.

? TROUBLESHOOTING

(C) Other modifications of the N-terminal α-amino group of the peptides ● TIMING About 2–3 h

(i) (OPTIONAL) Implement Step 42B(iv).

(ii) Perform as described in Step 42B(v).

(iii) Perform as described in Step 42B(vi).

(iv) (OPTIONAL) Perform as described in Step 42B(vii).

(v) Perform as described in Step 42B(viii).

(vi) (OPTIONAL) Perform as described in Step 42B(ix).

Final side-chain deprotection ● TIMING About 5 h

47| Discard solution from Step 46 or 45, depending on whether or not optional step 46 has been implemented, and fill the box with 30 ml DCM. Place the box on a shaker and shake for at least 2 min (wash step 1). Discard used DCM, refill with 30 ml new DCM and shake for at least 30 s more (wash step 2).

48| Discard solution from Step 47 and fill the box with at least 50 ml of cleavage solution A. The surface of the membrane must be well covered by the cleavage solution. Reaction time is 30 min. Keep the box closed. The texture of cellulose membrane may become unstable (very soft) owing to the TFA treatment; therefore, do not shake.
! CAUTION Cleavage solution A contains TFA, which is highly corrosive!
▲ CRITICAL STEP Do not exceed the advised reaction time of 30 min. The membrane will lose stability and could be degraded.

49| Discard the solution very carefully. Fill the box with 40 ml DCM. Place the box on a shaker and shake for at least 2 min (wash step 1). Discard used DCM, refill with 40 ml new DCM and shake for at least 30 s more (wash step 2). Repeat wash step 2 procedure twice to obtain four wash steps in total.

50| Discard solution from Step 49 and fill the box with at least 50 ml of cleavage solution B. The surface of the membrane must be well covered by the cleavage solution. Reaction time is 3 h. Keep the box closed and do not shake.
! CAUTION Cleavage solution B contains TFA, which is highly corrosive!

51| Discard the solution very carefully. Fill the box with 40 ml DCM. Place the box on a shaker and shake for at least 2 min (wash step 1). Discard used DCM, refill with 40 ml new DCM and shake for at least 30 s (wash step 2). Repeat wash step 2 procedure twice to obtain five wash steps in total.
▲ CRITICAL STEP After TFA treatment, the membrane may become very fragile. Do not try to lift the membrane before it becomes more robust during the washing steps (at around the last DCM or first methanol washing step).

52| Discard solution from Step 51 and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 1 min (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at 1 min more (wash step 2). Repeat wash step 2 procedure to obtain five wash steps in total.

53| Dry the membrane in the air stream of a fume hood or with a hair dryer set at cold.
■ PAUSE POINT The membrane can be stored at −80 °C for several months until needed.

Modification of the unprotected peptides—cysteine cyclization (optional ● TIMING About 3 h or overnight)
54| Even though the present procedure can be carried out for several purposes, here we describe only the most common type of modification of the unprotected peptide bound to the cellulose membrane: the cyclization via disulfide bridges. For a defined single cyclization, the peptide sequences must contain exactly two cysteines. If the peptide should have more than one defined disulfide bridge, the peptide sequence requires a corresponding number of paired orthogonal side-chain protected cysteines (see **Table 5**). In addition, for peptides that require more than one cyclization step, the different pairs of orthogonal side-chain protection groups can be selectively cleaved according to cleavage mixtures listed in **Table 5**. The newly unprotected cysteines will be cyclized according to Step 55.

55| To build the disulfide bridge by oxidizing the cysteines, treat the membrane by shaking with 10% (v/v) DMSO in 10 mM PBS (pH 7.2–7.4). Check the pH 30 min after adding the reaction solution. If the pH has decreased, use a new DMSO/PBS solution or readjust the pH by adding 20% (w/v) aq. NaOH or diluted aq. ammonia solution dropwise. Incubate the reaction for at least 2 h.
■ PAUSE POINT Overnight treatment is recommended.

56| Discard solution from Step 55 and fill the box with 30 ml of 10 mM PBS (pH 7.2–7.4). Place the box on a shaker and shake for at least 2 min (wash step 1). Discard PBS buffer, refill with 30 ml buffer and shake for at least 2 min more (wash step 2). Repeat wash procedure of Step 2, but with distilled water to obtain five wash steps in total.

TABLE 5 | Possible orthogonal protecting groups for selective deprotection of cysteine residues for disulfide cyclization on cellulose membranes.

Protecting group	Cleavage condition	Remarks
Trt	Final side-chain deprotection	Common for single cyclizations after final deprotection of the peptides
Mmt	2% (v/v) TFA + 3% (v/v) TIPS in DCM	Highly recommended for selective cyclizations before final deprotection of the peptides
tBuS	0.6 M Bu_3P/propanol	The treatment should be performed under argon; fill a blanket of argon in the box and close it tightly with the lid
Acm	1 M HCl + 0.1 M I_2 in methanol/acetic acid/water (1:1:2)	This procedure leads directly to the cyclized peptide (Step 55 is unnecessary)

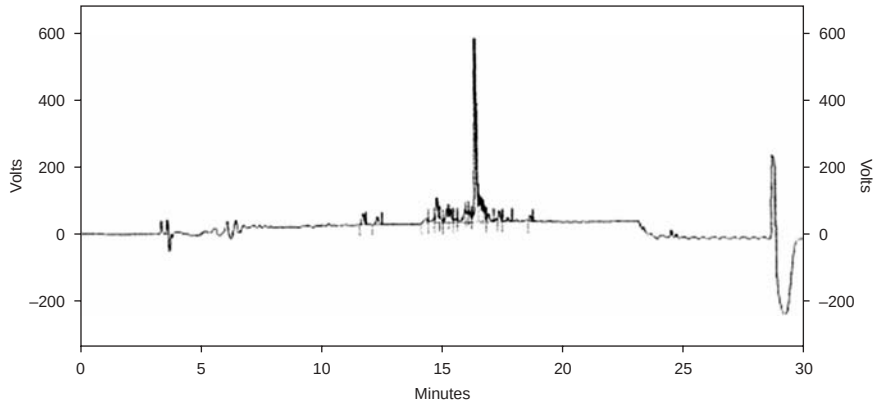


Figure 7 | A typical HPLC profile of a crude 9-mer peptide with the D-amino-acid sequence NH_2–mdeaffd–A′-A′-$CONH_2$ containing two β-Ala (A′) as a spacer. This peptide was synthesized manually on cellulose and cleaved from the membrane with ammonia vapor according to the protocol described here.

57| Discard solution from Step 56 and fill the box with 30 ml methanol or ethanol. Place the box on a shaker and shake for at least 1 min (wash step 1). Discard methanol or ethanol, refill with 30 ml methanol or ethanol and shake for at least 1 min more (wash step 2). Repeat wash procedure of Step 2 to obtain three wash steps in total.

58| Dry the membrane in the air stream of a fume hood or with a hair dryer set at cold. The membrane is now ready to use for biological assays. To perform the assays, the membrane can be used directly, or the peptides can be punched out and transferred into tubes or microtiter plates. Note that at this point the peptides are tethered on the cellulose. If soluble peptides are required, proceed to Step 59.

Cleavage of the peptides from the membrane (optional ● TIMING overnight)

59| Place the membrane in a glass desiccator.

60| Generate a vacuum in the desiccator.

61| Use gaseous ammonia to cleave the peptide from the cellulose support. To fill the desiccator with ammonia gas, it is recommended not to connect the desiccator directly to the ammonia pressure container. Instead, fill ammonia gas into a chemically resistant balloon, and then connect the balloon to the valve of the desiccator to transfer the gas into the desiccator. This allows better adjustment for excess pressure.

! CAUTION All steps using ammonia gas must be performed under a fume hood. Ammonia gas is corrosive and highly irritating!

■ **PAUSE POINT** Allow the reaction mixture to incubate in the desiccator overnight.

62| After venting, remove the lid of desiccator under a fume hood and release the gas from the membrane over a period of 30 min. The peptides are now cleaved from the support and only absorbed onto the cellulose; they can be released from the membrane using appropriate solvents. The spots can be punched out using a single-hole punch (e.g., Stables) and placed in small centrifuge tubes or microtiter plates.

▲ CRITICAL STEP Be aware of electrostatic interactions between the cellulose and 96-well plate that may cause cellulose spots to be ejected from the well. Because of the higher likelihood that incorrect peptides are present at the margin of the spots, it is advisable to use a puncher that punches holes just a bit smaller than the diameter of the peptide spots. Carefully punch out the peptide spots using the labeled center in the middle of the resulting spot. If peptides are punched out off-center, spots may contain cellulose areas containing peptide, as well as areas that do not contain peptides. This may result in decreased peptide concentration and false conclusions.

■ **PAUSE POINT** The membrane or punched out spots can be stored at −80 °C for several months until needed.

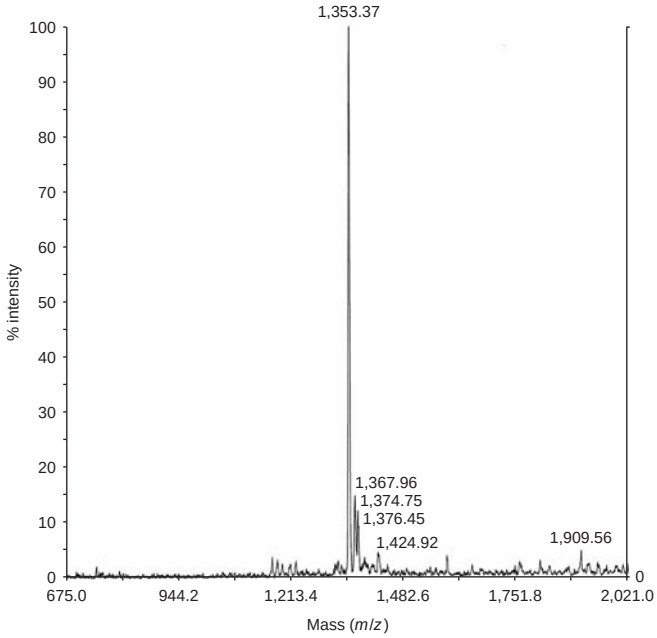


Figure 8 | A MALDI-ToF-MS image of a biotinylated and cystein-cyclized crude 10-mer peptide with the sequence biotin-CSHFNDYC-A′A′-$CONH_2$ containing two β-Ala (A′) as a spacer (calc. MW = 1,353.47). This peptide was synthesized and modified manually on cellulose, and cleaved from the membrane with ammonia vapor according to the protocol described here.

● TIMING

This timing does not include optional modification steps (above).

First day (preparation), Steps 1–23: about 8 h

Next days (coupling), Steps 24–41: about 2–3 h per coupling cycle
Last day (final deprotections), Steps 43–45 and 47–53: about 6 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 6**.

TABLE 6 | Troubleshooting table.

Problem (step no.)	Possible reason	Solution
Reagents: formation of amines in solvents during storage (REAGENTS)	Exposure to light	Store in the dark; for rarely used solvents, storage of small amounts are recommended; additionally, NMP can be stored over molecular sieve beads; to check the amine content, prepare a 1% (w/v) methanolic BPB stock solution and add 10 µl of that to 1 ml of the solvent; if the solution remains yellow or has a yellow-green color, the quality is good for dissolving reagents[34]. If the color changes to green or blue, the solvent amine content is too high, and the solvent can only be used for washing before Fmoc deprotection (Steps 16, 29, 43, 46A(iii) and 46B(viii))
The membrane does not show a homogeneous color after staining (Step 20)	Not enough staining or no homogeneous modification of the filter paper	Try to repeat the staining procedure with more staining solution and a longer incubation time. If after that the membrane does not show a homogeneous blue color again, there are strong indications that the modification of the filter paper is not homogeneous. The preparation of a new membrane is recommended
Over a day crystals form in the amino-acid solutions activated with DIC/HOBt (Step 23A(vi))	Formation of poorly soluble urea	The crystals have no influence on the coupling yield. To avoid massive crystallization, shake the mixtures after adding DIC to the amino acid/HOBt solutions for about 30 min and separate solid material by centrifugation
Labels written with marker pen are washed away by the reagent solutions (Steps 23A(iii) and 23B(ii))	Color of marker pens not usually stable against organic solvents	Protect the label by covering with transparent tape
The spots do not show a homogeneous color (Steps 33 and 46A(i))	Different acidity of the coupled amino acids	This is normal and does not affect the synthesis; however, an uncolored spot could also indicate an uncoupled amino acid and blocked synthesis. Mark this position and observe it over the next cycles. If it was only because of the acidity of the coupled amino acid, during the next cycles the spot should once again become colored. Otherwise, this peptide should probably be discarded
After modification and staining, the dye remains the same color as compared to that before modification (Step 42A(viii))	Coupling was not completed	Repeat the coupling step
During treatment with TFA, the color of the spots changes to yellow (Steps 42A(ii), 42B(ii) and 46B(iii))	Formation of the trityl-cation	The dye should disappear after refreshing the cleavage solution and after longer exposure time
The bromophenol blue dye of the spots cannot be completely removed (Step 46B(ix))	Insertion of the dye into the peptide or peptide structure due to too prolonged treatment with BPB or too high BPB concentration	Wash the membrane 20 min with 20% (v/v) piperidine/DMF. Occasionally, it is not possible to completely remove inserted bromophenol blue

ANTICIPATED RESULTS

Each spot can be seen as a separate reaction vessel, and the amount of peptide per spot can be altered by changing the pipetted amino-acid volume and the amino functionalization used. It is possible to synthesize up to 10 μmol of peptide per cm^2 cellulose[31,35]. In this protocol, we described the esterification of β-alanine with DIC to obtain a cellulose membrane derivative, and employed an additional coupling of a second β-alanine as a spacer[13] This is the easiest and most commonly used method to obtain a cellulose membrane derivative, which is used to synthesize peptides on the membrane. Peptide yields typically gained are from 0.4 to 0.6 μmol of peptide per cm^2 cellulose. Yields can be increased to 0.8–1.9 μmol cm^{-2} by esterification of the cellulose with glycine, particularly if CDI is used as a reagent instead of DIC[29]. This higher functionalization, and potentially higher yield of peptides, is of special interest for applications where the peptides are to be cleaved from the membrane and used for solution assays[27]. In some cases, however, a high peptide density can cause difficulties in binding assays when assessing interactions with the molecules of interest. One typical observation is that only the edge around a peptide spot shows a binding signal (e.g., "ring spot effect")[20]. In this case, lowering the peptide concentration on the peptide spot apparently resolves this problem[20]. If such difficulties occur, or if the peptide concentration dependence of selected interactions is investigated, a reduction in amino group loading may be necessary. The purity of peptides synthesized on cellulose was reported by Takahashi *et al.*[36] to be higher than 92%; however, several authors described lower purities[20]. A typical HPLC chromatogram and a MALDI-ToF-MS of peptides synthesized manually on cellulose according to this protocol are shown in **Figures 7** and **8**.

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