Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences

Irene Coin, Michael Beyermann & Michael Bienert

Leibniz-Institut für Molekulare Pharmakologie, Department of Peptide Chemistry and Biochemistry, Robert-Rössle-Strasse 10, Berlin 13125, Germany. Correspondence should be addressed to I.C. (coin@fmp-berlin.de) or M.Beyermann (beyermann@fmp-berlin.de).

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This protocol for solid-phase peptide synthesis (SPPS) is based on the widely used Fmoc/tBu strategy, activation of the carboxyl groups by aminium-derived coupling reagents and use of PEG-modified polystyrene resins. A standard protocol is described, which was successfully applied in our lab for the synthesis of the corticotropin-releasing factor (CRF), >400 CRF analogs and a countless number of other peptides. The 41-mer peptide CRF is obtained within ~80 working hours. To achieve the so-called difficult sequences, special techniques have to be applied in order to reduce aggregation of the growing peptide chain, which is the main cause of failure for peptide chemosynthesis. Exemplary application of depsipeptide and pseudoproline units is shown for synthesizing an extremely difficult sequence, the Asn(15) analog of the WW domain FBP28, which is impossible to obtain using the standard protocol.

INTRODUCTION

Peptides play a pivotal role in biological, medical and pharmaceutical research. Therefore, the synthesis of such polyamide structures has been a major focus of organic chemistry for over a century. The first successful coupling of two amino acids was performed via acyl chlorides by Emil Fischer in 1903, but at that time no suitable amino-protecting group was available for synthesizing longer peptides[1]. The introduction of the benzyloxycarbonyl-protecting group by Bergmann and Zervas[2] and other inventions, such as the development of tetraethyl pyrophosphite as a coupling reagent by Anderson *et al.*[3] and the successful protection of the mercapto group of Cys by the benzyl residue[4], as well as the removal of S-benzyl and tosyl groups with sodium in liquid ammonia[4] (for an overview, see ref. 5) allowed, for the first time, the synthesis of the neurohypophysial nonapeptide hormone oxytocin[6], for which du Vigneaud was awarded the Nobel Prize in 1955. Nevertheless, for assembly of longer peptides or small proteins, the repetitive procedures of coupling, deprotection of the N-terminal amino-protecting group, isolation and purification of intermediates were found to be very laborious, when carried out in solution. Moreover, solubility problems often prevented the elongation of the peptide chain. The method conceived by R.B. Merrifield, that is to assemble peptides onto a solid phase[7] (Nobel Prize 1984), had an enormous impact on the further development of peptide synthesis. Solid phase peptide synthesis (SPPS) offers important advantages over the synthesis in solution, in that coupling reactions can be carried out more rapidly and nearly to completion using an excess of the activated amino acid derivative, which is removed at the end of the reaction by simple washing operations. In the beginning, however, application of SPPS presented many pitfalls: more appropriate solid supports and milder chemistries had to be developed, to prevent undesired side-reactions. Although Merrifield's solid support, cross-linked poly(styrene-divinylbenzene) is still in use, more polar resins gave better results[8], and cross-linked poly(dimethylacrylamide) resins[8] were developed, as well as combinations of soft polyamides with rigid, highly permeable matrices, constructed from kieselguhr or highly cross-linked polystyrene[9]. High mechanical stability, in combination with proper solvation behavior, was also successfully achieved by copolymerization of ethylene oxide and polystyrene[10] or by grafting PEG chains onto polystyrene beads[11]. Nowadays, commercially available resins are modified by appropriate handles, which enable anchoring of the protected C-terminal amino acid residue by the formation of ester or amide bonds, thus allowing the synthesis of peptide acids and peptide amides, respectively[12]. For temporary protection of the N-terminal amino group, the Boc-group[13] is ideally suited, because its urethane structure helps to minimize epimerization of activated amino acids, and deprotection can be achieved using various acidic agents under relatively mild conditions. For permanent protection of the side chains during assembly, benzyl-type protecting groups are used, which are cleaved by strong acids, preferably hydrofluoric acid (HF). Although many peptides[14], and even short proteins, have been successfully synthesized using the Boc/Bzl/HF technique, the potential hazards of HF and the requirement for HF-resistant equipment prompted the search for alternative. The introduction of the Fmoc-protecting group—developed by Carpino in 1970 (ref. 15)—into SPPS[16] allowed the entire process of SPPS to be carried out using milder chemistry. The orthogonal Fmoc/tBu chemistry was further improved by extension of the repertoire of novel side-chain protecting groups, such as Asn/Gln(Trt), Lys(Dde), Lys(Aloc), His(Trt), Arg(Pbf), Trp(Boc)[17]. Remarkable achievements have also been made in the chemistry of peptide bond formation. Although more traditional coupling methods, such as diimide-based activation[18], anhydride-mediated couplings[19] and preactivated esters[20] have been successfully applied, coupling reagents such as phosphonium[21]- or uronium/guanidinium (aminium)[22]-based structures are the most widely used today, especially in automated SPPS.

As a result of this fruitful chemical research, nowadays the synthesis of many medium-sized 30–50-mer peptides can be smoothly accomplished by manual or automate-assisted SPPS and even longer protein-like peptides can be synthesized by coupling protected segments (for reviews see refs. 23–30) or more efficiently by chemical ligation[31] of nonprotected purified sequences. SPPS has also been successfully applied to large-scale production of peptide pharmaceutics[32]. Although for certain peptides chemical synthesis may still remain not convenient, for most of the sequences appropriate

synthesis protocols will be found, making it possible to obtain crude products that can be purified via HPLC (**Fig. 1**). The standard protocol reported in the PROCEDURE was optimized for the synthesis of the 41-mer peptide human/rat corticotropin-releasing factor (CRF)[33], SEEPP ISLDL TFHLL REFLE MARAE QLAQQ AHSNR KLMEI I-NH_2, which is the principal neuroregulator of the basal and stress-induced secretion of ACTH, β-endorphin and other peptides from the anterior pituitary[34]. According to this protocol, several hundreds of CRF analogs have been prepared, in the context of ligand–receptor interaction studies[35–37]. (An alternative synthesis of CRF using the Boc/Bzl/HF strategy at elevated temperature is reported in ref. 38.) Although peptide synthesis is often performed using a peptide synthesizer, we describe here the manual procedure. Manual synthesis (double couplings), purification and characterization of the 41-mer peptide can be completed within ~80 working hours (3–4 d for automate-assisted synthesis). The assembly can, in principle, be sped up using single couplings, but often to the expense of the quality of the crude peptide, which can be more difficult to purify. In the manual synthesis, the number of (expensive) second couplings can be minimized by checking the completeness of the first coupling step at each cycle (see also **Box 1**) using Kaiser tests[39].

Although this protocol allowed smooth syntheses of a countless number of medium-sized peptides, it led to very poor raw products when applied to two classes of peptides: the first category comprises sequences that contain sterically hindered amino acid residues like Cα- and Nα-alkylated amino acids[22,40,41], whereas the second category consists of sequences that show a strong tendency to aggregate under conditions of SPPS. The latter often contain domains prone to form β-sheet-like structures, which cause a collapse of the peptide-resin matrix. Under such conditions, the diffusion of reagents into the matrix is limited, coupling and deprotection reactions are often slow and incomplete, and the Kaiser test may give false negative results (reviewed in ref. 14). Those so-called 'difficult sequences'[7,42] might be more easily assembled by the Boc/Bzl than Fmoc/tBu strategy, because trifluoroacetic acid (TFA), which is used for removing the temporary Nα-protecting group in the first case, can destroy aggregates, in contrast to piperidine/DMF, which is mostly used for Fmoc-

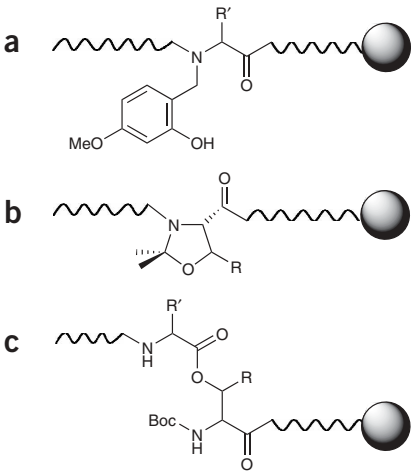

Figure 2 | Special units used during assembly to prevent peptide chain aggregation. R: H/CH_3 (Ser/Thr). (**a**) Hmb-amino acid. (**b**) Pseudoproline. (**c**) Depsidipeptide unit.

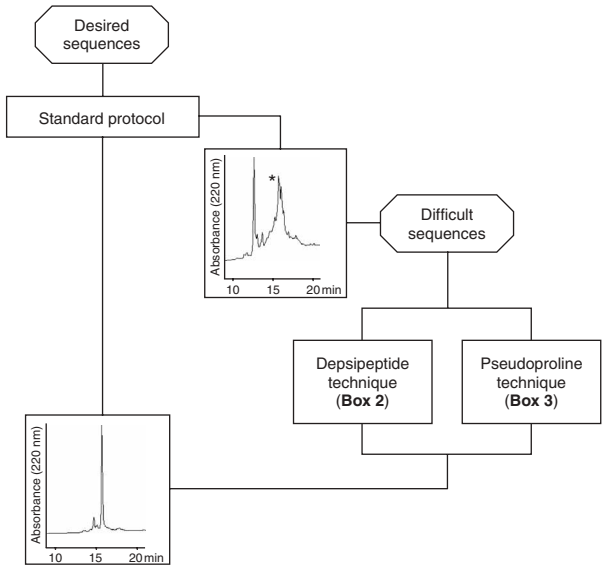

Figure 1 | Nowadays, most of the desired peptide sequences can be obtained by chemosynthesis, using appropriate protocols.

removal. The synthesis of 'difficult sequences' may be improved by using polar solvents[43] and intermediate acid washing steps[44], but better results are obtained by applying reversible modifications to the peptide backbone. The observation that sequences containing Nα-alkyl-amino acids and Pro are often synthesized without difficulties[45] led to the development of reversibly Nα-alkylated amino acids[46], to be incorporated instead of the corresponding nonalkylated amino acids into the peptide chain (preferentially (Hmb)Gly-derivatives, commercially available; **Fig. 2a**) and reconverted into the native residue by TFA treatment. Using this strategy, syntheses of various difficult sequences, such as β-amyloid-derived peptides[47], have been improved. To avoid difficulties associated with acylation of Nα-alkylated residues, dipeptide blocks containing Hmb-amino acids (some of them commercially available) can be used.

The structure of Pro is mimed in the so-called 'pseudoprolines'[48,49], residues of Ser or Thr in which the β-hydroxyl function is reversibly bound through an alkyl bridge to the α-amino group (**Fig. 2b**). Pseudoprolines, introduced into a peptide by coupling-preformed dipeptide derivatives (commercially available), destabilize peptide folding in β-sheets[50] and efficiently reduce the formation of aggregates. Pseudoprolines are converted into the native Ser/Thr residues by treatment with TFA.

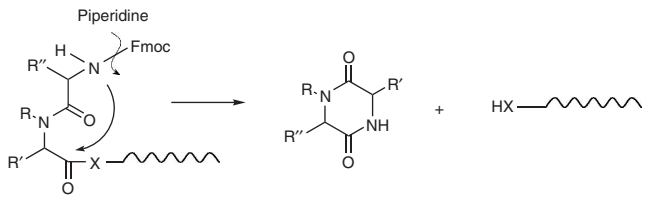

Figure 3 | Diketopiperazine (DKP) formation promoted by piperidine during Fmoc removal. For common peptide chains, for X = NH, DKP formation occurs mostly during deprotection of the amino acid following either a Pro or an Nα-alkylated residue (R = alkyl), and preferably when R″ = H. By assembly of depsipeptides (X = O), DKP formation can always occur during deprotection of the second residue following the ester bond, in an extent which is strongly dependent on the sequence[54].

BOX 1 | MONITORING SOLID-PHASE PEPTIDE SYNTHESIS

Fmoc determination

Monitoring the resin loading during synthesis gives useful information about the progress of the assembly. This can easily be done by quantifying the amount of Fmoc removed at each deprotection/coupling cycle using spectrophotometry.

EQUIPMENT

UV spectrometer, UV cell (10 mm), small flasks or glass vials, measuring pipette.

PROCEDURE

1. Collect neatly the piperidine solution (12 ml mg^{-1}) used for deprotection (Steps 4 and 5 of the standard protocol) in a small flask or glass vial.
2. Dilute 1/20 with 20% piperidine in DMF (in a small vial: 100 µl collected solution + 1.9 ml 20% piperidine) and mix.
3. Fill the UV cell with 2.7 ml of 20% piperidine in DMF (reference solution), place the cell into the spectrophotometer and zero at $\lambda = 301$ nm.
4. Add 300 µl of the solution prepared at Step 2 into the cell, mix and measure the absorbance.
5. Calculate the loading using the following equation:

Loading (mmol g^{-1}) = $Abs_{sample} \times 0.4^a$

[a]based on $\varepsilon_{301} = 6{,}000\ M^{-1}\ cm^{-1}$ (ε depends also on the specifications of the spectrometer); 3 ml deblocking solution, 1/200 dilution, 250 mg resin.

6. Constance (or slight progressive decrease) of loading is an indication of good progress of the synthesis. If the loading values vary irregularly or decrease drastically, a microcleavage test (described below) should be performed.

Kaiser test

The Kaiser test[39] is a qualitative test for the presence or absence of free primary amino groups, and it can be a useful indication about the completeness of a coupling step. The test is based on the reaction of ninhydrin with primary amines, which gives a characteristic dark blue color. The test requires minimal amounts of analyte and is completed within a few minutes.

REAGENTS

- 0.5 g ninhydrin in 10 ml ethanol (EtOH)
- 0.4 ml of 0.001 M KCN_{aq} in 20 ml pyridine

PROCEDURE

1. Transfer a few resin beads to a small glass tube and wash several times with ethanol.
2. Add 100 µl of each of the solutions mentioned above (see REAGENTS in this box).
3. Mix well and place the tube in a preheated oven (115 °C) for 5 min.

▲ **CRITICAL STEP** To reduce the incidence of false negative results, it is recommended to carry out a parallel positive control. The test is not applicable to N-terminal Pro residues (secondary amine) and N-alkyl amino acids. The test may give false negative results when applied to aggregate sequences.

Microcleavage

When Fmoc removal data show anomalies, before performing a step that requires the use of particularly expensive materials, or after a critical step, and in general when assembling longer sequences, it may be useful to cleave and analyze a small amount of intermediate product.

PROCEDURE

1. Transfer a sample containing ~1–2 mg dry peptide-resin to a small syringe (2 ml).
2. Add 300 µl of the cleavage cocktail (trifluoroacetic acid/H_2O/phenol/triisopropylsilane 8.5/0.5/0.5/0.5) to the dried peptide resin, stir gently for 30 s and wait for 3 h (stir gently in-between).
3. Collect the solution in a small HPLC vial, dilute with 400 µl acetonitrile/water 1/1 and mix.
4. At this point, the solution can be analyzed in an analytical HPLC system (inject 20 µl) and/or further diluted (1/10) to be injected (2 µl) in liquid chromatograph-mass spectrometer.

Alternatively, difficult peptides may be obtained by the synthesis of depsipeptide (also named O-peptide, or O-acyl isopeptide) analogs[51–53]. In a depsipeptide, the tendency toward aggregation is reduced by interrupting the regular pattern of amide bonds with ester bonds, which are introduced at the level of Ser/Thr residues by extending the peptide chain via the β-hydroxyl function (**Fig. 2c**). Depsipeptide units are assembled via O-acylation directly onto the resin-bound peptide, or more conveniently incorporated by coupling preformed depsidipeptide blocks[54,55], some of which are commercially available. In this case, coupling is best performed via carbodiimide in nonpolar solvents[56,57]. During depsipeptide assembly, care must be taken during Fmoc removal from the second amino acid residue following the ester bond at the N-terminal side, where diketopiperazine (DKP) formation[58] can occur[54] (**Fig. 3**). The use of Bsmoc[59] for Nα-protection at this position can prevent DKP formation, because this group is removed faster and under less basic conditions than Fmoc. At the end of the synthesis, depsipeptides are cleaved intact from the solid support with TFA. Compared with the target peptide, the corresponding depsipeptide isomer is more soluble in aqueous media, due to the presence of an additional ionizable moiety provided by the depsipeptide unit, and therefore can be more easily purified, as reported for the Alzheimer Aβ (1–42) peptide[51–53]. Final conversion of the depsi into the amide form (**Fig. 4**) is smoothly achieved after peptide purification through an *O,N*-acyl shift[60] under weakly alkaline conditions.

In **Box 2** we describe the application of the depsipeptide, and in **Box 3** of the pseudoproline method to the assembly of an extremely difficult sequence, the Asn(15)-amide analog of the WW domain FBP28, a small, 37-residue peptide, GATAV SEWTE YKTAD GKTYY YNNRT LESTW EKPQE LK, recently used as a model system in studies about β-sheet stability and folding[61,62], and which is impossible to synthesize using standard protocols[54,62]. We synthesized Asn(15)-analogs, because a considerable piperidine-catalyzed aspartimide[63,64] formation (**Fig. 5**) was observed at position Asp(15)-Gly(16) for the assembly of the wild type. The

wild type was successfully synthesized using (Hmb)Gly at position 16, which prevents aspartimide formation and helps also to reduce the aggregation tendency of the growing peptide chain[47,65]. The depsipeptide strategy was compared with the pseudoproline method and was shown to be equally well suited for SPPS[54].

MATERIALS

REAGENTS

- Solid support, Fmoc-SRam-PEG-PS resin, capacity 0.25 mmol g^{-1} (RAPP Polymere GmbH, cat. no. 124.889)
- DMF (Fluka, cat. no. 40250 or another peptide synthesis grade quality) ! CAUTION Toxic.
- Methylene chloride (DCM; Fluka, cat. no. 66738) ! CAUTION Harmful.
- Fmoc-Xxx-OH (Orpegen) ! CAUTION Irritant.
- Fmoc-(Fmoc-Hmb)-Gly-OH (Novabiochem, cat. no. 04-12-1135)
- Pseudoproline dipeptides: Fmoc-Glu(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH, Fmoc-Lys(Boc)-Thr($\Psi^{Me,Me}$pro)-OH, Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH (Novabiochem, cat. nos. 05-20-1002, 05-20-1116, 05-20-1001)
- Bsmoc-Xxx-OH (Morre-Tec Ind. Inc) ! CAUTION Irritant.
- 1-[Bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide (HBTU; Iris Biotech GmbH, cat. no. RL-1030) ! CAUTION Irritant/harmful.
- 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b] pyridinium hexafluorophosphate 3-oxide (HATU; GL Biochem) ! CAUTION Irritant/harmful.
- *N,N*-diisopropylethylamine (DIEA; Fluka, cat. no. 03440) ! CAUTION Corrosive/highly flammable.
- Piperidine (Acros, cat. no. 14718 0025) ! CAUTION Higly flammable/toxic.
- *N,N′*-Diisopropylcarbodiimide (DIC; Fluka, cat. no. 38370) ! CAUTION Extremely flammable/toxic.
- 1-Hydroxybenzotriazole (HOBt; Fluka, cat. no. 54802) ! CAUTION Highly flammable/harmful/irritant.
- *N*-Methylimidazole (NMI; Fluka, cat. no. 67560) ! CAUTION Corrosive/highly flammable.
- Acetic anhydride (Fluka, cat. no. 45830) ! CAUTION Corrosive.
- TFA (Acros, cat. no. 13972 0010) ! CAUTION Corrosive/toxic.
- Triisopropylsilane (TIPS; Fluka, cat. no. 92095) ! CAUTION Irritant/flammable.
- Phenol (Riedel-de Haën, cat. no. 33517) ! CAUTION Toxic/corrosive.
- Water (mQ grade)
- Diethyl ether (Acros, cat. no. 12399 0050) ! CAUTION Extremely flammable/harmful.

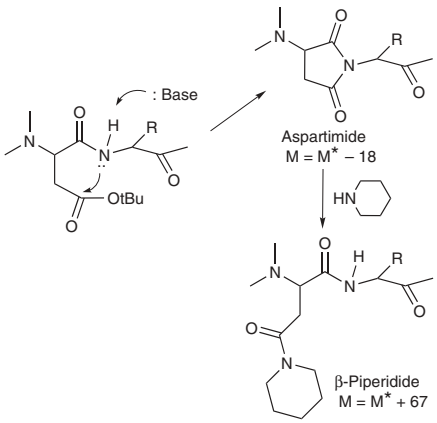

Figure 5 | Base-catalyzed aspartimide formation on an O*t*Bu-protected aspartic acid residue and subsequent aminolysis by piperidine, yielding the corresponding piperidide; in dimethyl formamide (DMF) the β-piperidide may preferably be formed. The mass of the aspartimide peptide corresponds to the mass of the target peptide (M*) –18, whereas the piperidide peptide shows a mass difference of +67.

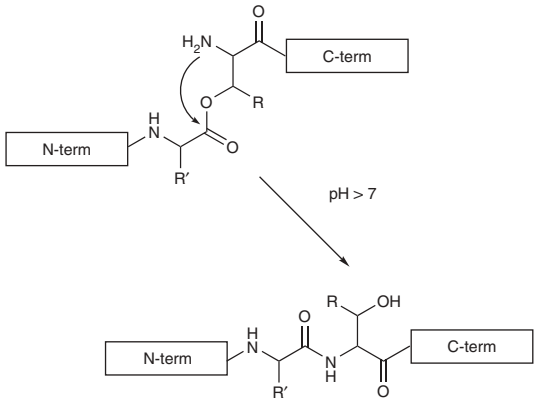

Figure 4 | Depsipeptides are converted into the all-amide form through an *O,N*-acyl shift, which occurs quantitatively under mildly basic conditions over a short period. R: H/CH_3 (Ser/Thr).

- Ninhydrin (Aldrich, cat. no. 454044) ! CAUTION Harmful.
- Pyridine (Fluka, cat. no. 82704) ! CAUTION Extremely flammable/harmful.
- Potassium cyanide (KCN; Fluka, cat. no. 60180) ! CAUTION Very toxic and dangerous for the environment.
- Ethanol (99.9% vol; Prolabo, cat. no. 20 065.362) ! CAUTION Extremely flammable/irritant.

EQUIPMENT

- 10-ml plastic syringe equipped with a frit column plate
- 2-ml plastic syringe equipped with a frit column plate
- Vacuum membrane pump
- Magnetic rod
- Centrifuge
- Centrifuge tubes
- Small glass tubes
- Analytical HPLC (see EQUIPMENT SETUP)
- HPLC vials
- Preparative HPLC (see EQUIPMENT SETUP)
- Liquid chromatograph-mass spectrometer
- Rotary evaporator
- UV cell (light path 10 mm)

EQUIPMENT SETUP

Analytical HPLC Use an HPLC-gradient system equipped with a detector (220 nm) and a PolyenCap-A 300 column (250 × 4 mm^2). Run a gradient of 5–95% B in 40 min (flow 1 ml min^{-1}; solvent A: 0.1% TFA, solvent B: 80% acetonitrile (ACN)/0.1% TFA) as shown below.

Time (min)	Eluent B (%)
0	5
40	95
41	99
46	99
48	5
55	5

Preparative HPLC Use an HPLC-gradient system equipped with a detector (220 nm) and a PolyenCap-A 300 column (250 × 20 mm^2). Run a gradient of 30–70% B in 70 min (flow 10 ml min^{-1}; solvent A: 0.1% TFA, solvent B: 80% ACN/0.1% TFA) as shown below.

Time (min)	Eluent B (%)
0	30
70	70
73	99
78	99
79	30
88	30

PROCEDURE

Resin preparation

1| Weigh 250 mg SRam resin (0.25 mmol g^{-1}) into a plastic syringe with a frit column plate, and connect the outlet to a membrane pump via a collecting flask.

2| Add 2.0 ml DCM to the dried resin for resin swelling, stir gently for 1 min, wait for 15 min, and then remove the solvent by vacuum filtration.

3| Add 1.5 ml DMF to the resin (washing step), stir gently for 1 min and then remove the solvent via vacuum filtration.

Deprotection/coupling cycle

4| Add 1.5 ml of 20% piperidine/DMF (vol/vol), stir gently for 1 min (first Fmoc removal step), and then remove the solvent by vacuum filtration.

5| Add 1.5 ml of 20% piperidine/DMF (vol/vol), stir gently for 10 min (second Fmoc removal step), and then remove the solvent by vacuum filtration.

6| Add 1.5 ml DMF (washing Step 1), stir for 30 s, and then remove the solvent by vacuum filtration.

7| Add 2.0 ml DMF (washing step), stir for 30 s, and remove the solvent by vacuum filtration.

8| Repeat Step 7 four times.

9| Dissolve 0.375 mmol Fmoc-Ile-OH and HBTU (142 mg) in 1.5 ml DMF. Add the solution to the resin, and stir gently for 30 s.

10| Add 0.75 µmol DIEA (131 µl). Stir gently for 30 s and wait for 5 min. Repeat five times (total reaction time is 30 min, coupling step), and then remove the solvent by vacuum filtration.
▲ **CRITICAL STEP** Intermittent stirring is preferred than continuous stirring because of the fragility of the PEG-PS resin, in order to minimize the fragmentation of the resin in small-sized particles which can lead to difficulties during filtration. As an alternative, continuous gentle shaking or nitrogen bubbling through the reaction vessel can be applied.

11| Repeat Steps 9 and 10.

12| Add 2.0 ml DMF (washing step), stir for 30 s and remove the solvent by vacuum filtration.

13| Repeat Step 12 three times.

14| Repeat the cycle starting from Step 4 going to Step 13 for each of the subsequent amino acids according to the h/r CRF sequence using the following Fmoc-amino acid derivatives: Fmoc-Ile-OH (133 mg), Fmoc-Glu(OtBu)-OH (160 mg), Fmoc-Met-OH (140 mg), Fmoc-Leu-OH (133 mg), Fmoc-Lys(Boc)-OH (176 mg), Fmoc-Arg(Pbf)-OH (243 mg), Fmoc-Asn(Trt)-OH (224 mg), Fmoc-Ser(tBu)-OH (144 mg), Fmoc-Ala-OH (117 mg), Fmoc-His(Trt)-OH (233 mg), Fmoc-Gln(Trt)-OH (229 mg), Fmoc-Val-OH 127 mg), Fmoc-Phe-OH (145 mg), Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Pro-OH (127 mg) and Fmoc-Asp(OtBu)-OH (154 mg).
▲ **CRITICAL STEP** The progress of the synthesis can be followed at every cycle by monitoring Fmoc removal, and if necessary, using microcleavage test (see **Box 1**). To speed up the assembly, single couplings can be performed instead of double couplings. The requirement for the repetition of the coupling step can be derived from the result of the Kaiser test (see **Box 1**).
■ **PAUSE POINT** The synthesis can, in principle, be interrupted at the end of every coupling cycle, when the N-terminal amino group is protected. To avoid undesired removal of the Fmoc group during storage in DMF, wash the peptide-resin five times with DCM and let it dry at room temperature (18–22 °C). Close the syringe with its plunger and cap, and store at <4 °C. Before resuming the synthesis, let the sample reach room temperature, and swell the dry resin as described in Steps 2 and 3.

Removal of the N-terminal Fmoc-group and drying of the peptide resin

15| Add 1.5 ml of 20% piperidine/DMF (vol/vol), stir gently for 1 min (first Fmoc removal step), and then remove the solvent by vacuum filtration.

16| Add 1.5 ml of 20% piperidine/DMF (vol/vol), stir gently for 10 min (second Fmoc removal step), and then remove the solvent by vacuum filtration.

17| Add 1.5 ml DMF (washing Step 1), stir for 30 s, remove the solvent by vacuum filtration.

18| Add 2.0 ml DMF (washing step), stir for 30 s, remove the solvent by vacuum filtration.

19| Repeat Step 18 four times with DMF.

20| Repeat Step 18 four times with DCM.

21| Air-dry the peptide resin.

BOX 2 | SYNTHESIS OF A DIFFICULT SEQUENCE VIA DEPSIPEPTIDE UNITS

Assembly of N(15)-FBP28 WW domain GATAV SEWTE YKTAN GKTYY YNNRT LESTW EKPQE LK-amide. O-acylation steps at the underlined positions

1. Prepare the resin as described in the standard protocol (Steps 1–3).
2. Couple Fmoc-Lys(Boc)-OH (176 mg) as described in the standard protocol (Steps 4–13).
3. Acetylation in the presence of *N*-methylimidazole (NMI): to the peptide-resin add 2.0 ml methylene chloride (DCM), 30 μl NMI (0.375 mmol) and 35 μl acetic anhydride (0.375 mmol). Stir gently for 30 s and let the mixture react for 90 min, stirring 30 s every 10 min. Remove the solvent by vacuum filtration. Repeat the whole step.
4. Wash the resin as described in the standard protocol (Steps 12 and 13).

▲ CRITICAL STEP In following steps, O-acylations onto Ser/Thr residues are performed. To ensure that no free hydroxyl or amino groups are left available on the solid support after linkage, it is necessary to perform a capping step under conditions similar to those of O-acylation (that is in the presence of NMI)[54].

5. Couple in the exact order following steps Steps 4–13 of the standard protocol: Fmoc-Leu-OH (133 mg), Fmoc-Glu(OtBu)-OH (160 mg), Fmoc-Gln(Trt)-OH (229 mg), Fmoc-Pro-OH (127 mg), Fmoc-Lys(Boc)-OH (176 mg), Fmoc-Glu(OtBu)-OH (160 mg), Fmoc-Trp(Boc)-OH (197 mg), Fmoc-Thr(tBu)-OH (149 mg) and Boc-Ser-OH (77 mg).
6. *O-acylation*: In a small vial, suspend 160 mg Fmoc-Glu(OtBu)-OH (0.375 mmol) in 2.0 ml dry DCM, add 58 μl *N,N'*-diisopropylcarbodiimide (0.375 mmol) and 27 μl NMI (0.337 mmol), and stir for 1 min. Add the solution to the resin. Stir gently for 30 s, and let the mixture react for 2 h, stirring 30 s every 10 min. Remove the solvent by vacuum filtration.
7. Wash the peptide-resin as described in the standard protocol using dry DMF (Step 12, repeated two times).
8. Repeat Step 6.
9. Wash the peptide-resin as described in the standard protocol (Steps 12 and 13).

▲ CRITICAL STEP O-acylation reactions onto solid phase might happen to be slow[54], therefore a microcleavage before going on with the capping step is recommended. Further repetition of Step 6 can be evaluated (see ref. 54). General control of assembly via Fmoc removal monitoring (**Box 1**) is recommended in the course of the whole synthesis.

10. *Capping*: Repeat Step 3.
11. Wash the resin as described in the standard protocol (Steps 12 and 13).
12. Couple in the exact order following the standard protocol (Steps 4–13): Fmoc-Leu-OH (133 mg), Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Arg(Pbf)-OH (243 mg), 2 × Fmoc-Asn(Trt)-OH (224 mg), 3 × Fmoc-Tyr(tBu)-OH (172 mg) and Boc-Thr-OH (82 mg).
13. *O-acylation and capping*: repeat Steps 6–11 using Fmoc-Lys(Boc)-OH (176 mg) instead of Fmoc-Glu(OtBu)-OH.

▲ CRITICAL STEP Before capping, check completeness of O-acylation using microcleavage.

14. Couple Bsmoc-Gly-OH (111 mg) as described in the standard protocol (Steps 4–13).
15. *Bsmoc removal*: Add 1.5 ml piperidine 2% in DMF, stir gently for 1 min and remove the solvent by vacuum filtration.
16. Repeat Step 15 two times.
17. Add 1.5 ml DMF, stir for 20 s and remove the solvent by vacuum filtration.
18. Add 2.0 ml DMF, stir for 20 s and remove the solvent by vacuum filtration (two times).

▲ CRITICAL STEP The use of the Bsmoc group instead of Fmoc for N-protection at this position is required to minimize diketopiperazine (DKP) formation (see INTRODUCTION and **Fig. 3**). Note that DKP formation is sequence dependent: for this peptide, no DKP formation was observed during standard deprotection of Fmoc from Leu^{26}, whereas the use of Bsmoc was necessary for Tyr^{11} and Ala^{4}.

19. Couple immediately Fmoc-Asn(Trt)-OH (224 mg) following the standard protocol (Steps 9–13).
20. Couple in the exact order following the standard protocol (Steps 4–13): Fmoc-Ala-OH (117 mg), Boc-Thr-OH (82 mg).
21. *O-acylation and capping*: repeat Steps 6–11 using Fmoc-Lys(Boc)-OH (176 mg) instead of Fmoc-Glu(OtBu)-OH.

▲ CRITICAL STEP Before capping, check completeness of O-acylation using microcleavage.

22. Couple Bsmoc-Tyr(tBu)-OH (172 mg) as described in Steps 4–13 of the standard protocol.
23. *Bsmoc removal*: Repeat Steps 14–17.
24. Couple immediately Fmoc-Glu(OtBu)-OH (160 mg) following the standard protocol (Steps 9–13).
25. Couple in the exact order following the standard protocol (Steps 4–13): Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Trp(Boc)-OH (197 mg), Fmoc-Glu(OtBu)-OH (160 mg) and Boc-Ser-OH (77 mg).
26. *O-acylation and capping*: Repeat Steps 6–11 using Fmoc-Val-OH (127 mg) instead of Fmoc-Glu(OtBu)-OH.

▲ CRITICAL STEP Before capping, check completeness of O-acylation using microcleavage.

27. Couple Bsmoc-Ala-OH (117 mg) as described in Steps 4–13.
28. *Bsmoc removal*: Repeat Steps 15–18.
29. Couple immediately Fmoc-Thr(tBu)-OH (149 mg) following the standard protocol (Steps 9–13).
30. Couple in the exact order following the standard protocol (Steps 4–13): Fmoc-Ala-OH (117 mg), Fmoc-Gly-OH (111 mg).
31. *Removal of the N-terminal Fmoc-group, final cleavage of side-chain protecting groups and cleavage from the resin*: Follow the standard protocol (Steps 15–25).
32. *O,N-shift*: The final conversion of the depsi-analog to the desired all-amide peptide can be performed either before or after purification. For this, the peptide is dissolved in 0.1 M NH_4CO_3 buffer at pH 8 ($\sim$1 mg ml^{-1}). After 1 h, the solution is neutralized with trifluoroacetic acid (TFA) and lyophilized.
33. *Purification of the crude product and characterization*: Follow Steps 26 and 27 of standard protocol.

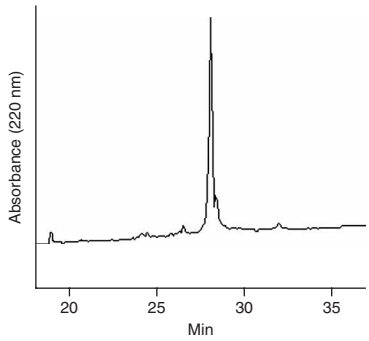

Figure 6 | HPLC profile of the crude corticotropin-releasing factor (CRF) synthesized according to the standard protocol.

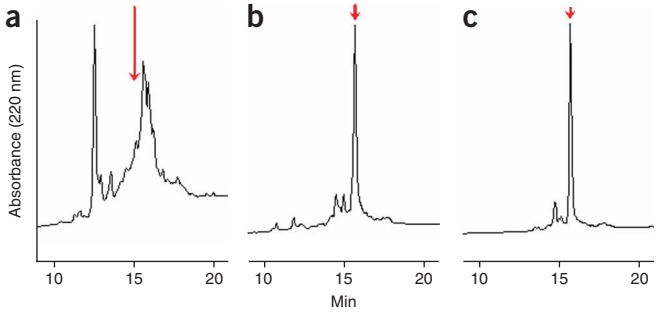

Figure 7 | HPLC profiles of the crude N(15)FBP28WW. The red arrows indicate the target product. (**a**) Product synthesized following a standard protocol, too complex to be purified. (**b**) Product obtained by synthesis of a depsipeptide analog, as described in **Box 2**. (**c**) Product synthesized using pseudoprolines, as described in **Box 3**. The improvement in product quality from A to B or C is impressive.

Final cleavage of side-chain protecting groups and cleavage from the resin

22| Add 2.0 ml of the cleavage cocktail [TFA/H_2O/phenol/TIPS 8.5/0.5/0.5/0.5] to the dried peptide resin, stir gently for 30 s and wait for 3 h (stir gently in-between).

23| Filtrate the cleavage mixture and add it dropwise to 20 ml cold diethyl ether in a centrifuge tube.
▲ CRITICAL STEP When working with highly hydrophobic sequences or short peptides, it may be necessary to concentrate the TFA solution before precipitation.

24| After standing for 30 min, isolate the precipitate by centrifugation (2,200*g*, for 5 min).

25| Wash the crude peptide with 10 ml diethyl ether and then centrifuge. Air-dry the crude product overnight at room temperature: 258 mg (yield: 87%).

BOX 3 | SYNTHESIS OF A DIFFICULT SEQUENCE VIA PSEUDOPROLINE UNITS

Assembly of N(15)-FBP28 WW domain, GATAV SEWTE YKTAN GKTYY YNNRT LESTW EKPQE LK-amide using three pseudoprolines units (underlined position)

1. Prepare the resin as described in the standard protocol (Steps 1–3).
2. Couple in the exact order following Steps 4–13: Fmoc-Lys(Boc)-OH (176 mg), Fmoc-Leu-OH (133 mg), Fmoc-Glu(OtBu)-OH (160 mg), Fmoc-Gln(Trt)-OH (229 mg), Fmoc-Pro-OH (127 mg), Fmoc-Lys(Boc)-OH (176 mg), Fmoc-Glu(OtBu)-OH (160 mg), Fmoc-Trp(Boc)-OH (197 mg), Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Glu(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH (207 mg), Fmoc-Leu-OH (133 mg), Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Arg(Pbf)-OH (243 mg), 2 × Fmoc-Asn(Trt)-OH (224 mg), 3 × Fmoc-Tyr(tBu)-OH (172 mg), Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Lys(Boc)-OH (176 mg), Fmoc-Gly-OH (111 mg), Fmoc-Asn(Trt)-OH (224 mg) and Fmoc-Ala-OH (117 mg).
▲ CRITICAL STEP The progress of the assembly can be followed by monitoring Fmoc removal and, if necessary, using microcleavage tests (see **Box 1**) in particular before coupling the expensive pseudoproline block. Single coupling can be used, with Kaiser test control (**Box 1**).
3. *Fmoc removal*: repeat Steps 4–8 of the standard protocol.
4. Coupling via 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide (HATU). Dissolve 0.375 mmol Fmoc-Lys(Boc)-Thr($\Psi^{Me,Me}$pro)-OH (229 mg) and 142 mg HATU in 1.5 ml DMF. Add the solution to the resin, and stir gently for 10 s.
5. Add 0.75 mmol *N,N*-diisopropylethylamine (DIEA) (131 µl), stir gently for 30 s, and let the mixture react for 2 h, stirring 30 s every 10 min. Remove the solvent by vacuum filtration.
6. Repeat Steps 4 and 5.
7. Wash the peptide-resin as described in the standard protocol (Steps 12 and 13).
8. *Capping*: add to the peptide resin 1.4 ml DMF, 200 µl DIEA and 200 µl acetic anhydride. Stir gently 30 s, wait for 3 min, stir gently for 30 s, wait for 3 min, stir gently for 30 s, wait for 3 min and remove the solvent by vacuum filtration.
9. Wash the peptide-resin as described in the standard protocol (Steps 12 and 13).
▲ CRITICAL STEP Coupling at this position was found to be difficult, in that a standard procedure gave ~40% of incomplete coupling. Therefore, a more efficient coupling method is necessary, followed by a capping step.
10. Couple in the exact order following the standard protocol. Fmoc-Tyr(tBu)-OH (172 mg), Fmoc-Glu(OtBu)-OH (160 mg), Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Trp(Boc)-OH (197 mg), Fmoc-Glu(OtBu)-OH (160 mg), Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH (175 mg), Fmoc-Ala-OH (117 mg), Fmoc-Thr(tBu)-OH (149 mg), Fmoc-Ala-OH (117 mg) and Fmoc-Gly-OH (111 mg).
11. *Removal of the N-terminal Fmoc-group, final cleavage*: follow the standard protocol (Steps 15–25).
12. *Purification of the crude product and characterization*: follow the standard protocol (Steps 26 and 27).

Purification of the crude product and characterization

26| Dissolve 100 mg crude product in 0.5 ml ACN and 1.8 ml of 0.1% aqueous TFA, and inject this on a semi-preparative HPLC system. Collect the fractions (each 10 ml) corresponding to the main peak (elute at ~50% B) and remove the ACN by evaporation at reduced pressure (in a rotary evaporator at 40 °C). The aqueous solution is finally lyophilized.

27| Dissolve 0.7 mg of the purified peptide in 0.7 ml of 0.1% aqueous TFA and apply 20 µl of the sample solution to an analytical HPLC system.

? TROUBLESHOOTING

● TIMING

Steps 1–3: 20 min
Steps 4–13: 100 min
Step 14: number of amino acid residues of the sequence × 100 min
Steps 15–21: 40 min + drying (overnight)
Steps 22–25: 4 h
Step 26: 2 h + lyophilization (overnight)
Step 27: 70 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Presence of deletion/truncation sequences in the crude product	Removal of the Fmoc group is incomplete	(i) Add 2% 1,8-diazabicyclo[5.4.0]undec-7-ene to the deblocking solution[66,67]. (ii) Prolong the deblocking times. (iii) Perform acidic washes between the deblocking steps[44]
	Coupling is incomplete due to sterical hindrance at the activated amino acid	Use a stronger activation method, like one of the following: (i) in a normal coupling cycle replace 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide with 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide[22,41] (see **Box 3**, Steps 4–6), (ii) use Fmoc-amino acid fluorides[40], especially for coupling of Cα-alkylamino acids The number of deletion sequences can be reduced by performing capping steps after each coupling cycle (15 min treatment with a solution of acetic anhydride/*N,N*-diisopropylethylamine/DMF 1/2/7 vol/vol/vol)
	Coupling is incomplete due to aggregation of the growing peptide chains	Improve peptide chain solvation by (i) using special solvents[43] (tetrafluoroethylene/NMP), (ii) adding chaotropic salts[68], (iii) increasing the temperature[38], (iv) reducing the capacity on the resin[69], (v) or using amino acid derivatives with bulky side-chain protecting groups (reviewed in ref. 70) Diminish association of the growing chain by inserting (i) reversibly Nα-alkylated amino acids (e.g., Hmb-Gly), (ii) pseudoproline units (**Box 3**), or (iii) depsipeptide units (**Box 2**). For references see INTRODUCTION
For sequences containing aspartic acid residues, presence of side-products showing a mass difference of either –18 or +67	Formation of aspartimide and/or piperidide at aspartic acid residues during Fmoc removal steps. See **Figure 5**	Add 0.1 M 1-hydroxybenzotriazole (HOBt) or 0.1 M 2,4-dinitrophenol to the piperidine solution used for Fmoc-removal[64]. Alternatively, use piperazine containing 0.1 M HOBt[71] For peptides containing the sequence –Asp-Gly–, the introduction of the Gly residue as Hmb protected may be necessary[65]
Presence of side-products missing two adjacent amino acids (most probably Xxx-Pro or Pro-Xxx)	Diketopiperazine formation. See **Figure 3**	(i) Use acid-sensitive Nα-protection at the appropriate position[72] and perform the following coupling by *in situ* activation/neutralization coupling[72,73]. (ii) Use more base-labile Nα-protection (Bsmoc) at the appropriate position[54,59] (see **Box 2**, Steps 14–18)
Presence of side-products showing a mass difference of +16	S-oxidation of Met	Reduction by the use of dimethylsulfide/hydrochloric acid/trifluoroacetic acid[74]

M* = Mass of the target peptide

ANTICIPATED RESULTS

The yield of purified peptide (purity >95%) depends on the specific sequence and the equipment used: CRF (standard protocol: ~25%) and FBP-28 WW (depsipeptide technique ~15%, pseudoproline technique ~25%). **Figures 6** and **7** show representative HPLC profiles for the corresponding crude products. The identity of the peptides was confirmed by correct mass (matrix-assisted laser desorption/ionization–time of flight): h/r CRF; M: 4753.51, found $[M+H]^+$: 4754.23, N(15)-FBP 28 WW; M: 4357.70, found $[M+H]^+$: 4358.61.

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