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Microwave-assisted synthesis of triple-helical, collagen-mimetic lipopeptides

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Abstract

Collagen-mimetic peptides and lipopeptides are widely used as substrates for matrix degrading enzymes, as new biomaterials for tissue engineering, as drug delivery systems and so on. However, the preparation and subsequent purification of these peptides and their fatty-acid conjugates are really challenging. Herein, we report a rapid microwave-assisted, solid-phase synthetic protocol to prepare the fatty-acid conjugated, triple-helical peptides containing the cleavage site for the enzyme matrix metalloproteinase-9 (MMP-9). We employed a PEG-based resin as the solid support and the amino acids were protected with Fmoc- and tert-butyl groups. The amino acids were coupled at 50 °C (25 W of microwave power) for 5 min. The deprotection reactions were carried out at 75 °C (35 W of microwave power) for 3 min. Using this protocol, a peptide containing 23 amino acids was synthesized and then conjugated to stearic acid in 14 h.

INTRODUCTION

Collagens are the most abundant proteins in mammals and are the major component of the extracellular matrix (ECM)¹. Structurally, collagen is composed of three polypeptide strands of ~1,000 amino acids each with polyproline II structures, forming a right-handed triple helix.¹ Repeats of the amino acid sequence glycine–proline–hydroxyproline (G–P–O (O is 4-(R)-hydroxyproline)) are responsible for the triple helical structure and stability of the collagens^{1,2}. Defects in the biosynthesis or post-translational modification of these proteins lead to several diseases³. Collagens are used in various medical procedures, as scaffolds for tissue engineering and hydrolyzed collagen (i.e., gelatin) is used in many pharmaceutical

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Supplementary information

The detailed operational steps that the CEM Liberty peptide synthesizer performs for the single coupling, double coupling and cleavage from the resin are included in the Supplementary Methods.

AUTHOR CONTRIBUTIONS S.M. conceived the idea and supervised the project; J.B. and A.J.H. carried out the lipopeptide synthesis; J.B.S. and W.W.M. recorded the mass spectra; and S.M. and J.B. wrote the manuscript.

Note: Supplementary information is available via the HTML version of this article.

and food preparations³. Collagen-mimetic peptides are widely used as model systems to study the structure–function relationships of these proteins, cell attachment of the ECM and so on^{4,5}.

Degradation of the ECM collagen and gelatin signals the onset and progression of various diseases including cancer and rheumatoid arthritis⁶. Matrix metalloproteinases (MMPs) are a family of Zn^{2+} containing enzymes, which hydrolyzes these fibrous proteins under physiological conditions. MMP-2 and -9 are responsible for the progression and metastasis of many types of cancers^{6–8}. In addition, these two enzymes are involved in Alzheimer's disease, acute pancreatitis, chronic obstructive pulmonary disease, atherosclerotic plaque rupture, stroke and multiple sclerosis^{9–11}. Collagen-mimetic substrate peptides for MMP-2 and -9 have been used to determine the selectivities of various MMPs and to conduct mechanistic studies on the enzymatic reactions^{12,13}.

Collagen-mimetic peptides and their fatty acid conjugates have been studied for their structural and cellular adhesion properties^{14–18}. A collagen-mimetic peptide conjugated with various fatty acids has, e.g., been demonstrated to bind selectively to the CD44 receptors overexpressed in the surface of metastatic melanoma cells¹⁹. Liposomes incorporating these peptides target the metastatic melanoma cells and after internalization, deliver the liposomal contents to the cell cytosol^{17,19}. For these reported studies, the collagen-mimetic peptides and their fatty-acid conjugates were synthesized employing conventional solid-phase peptide synthesis. Typically, the amino acid coupling requires 1–2 h and each Fmoc-group deprotection reaction is conducted for 1 h^{14–19}. Consequently, the total synthesis times are fairly long^{14–21}. For example, we have previously prepared a stearic acid conjugated triple helical peptide containing 23 amino acid residues²⁰. The synthesis required more than 7 d to complete employing a conventional automatic solid-phase peptide synthesizer²⁰. In addition, the purification of the crude peptides by HPLC are often challenging, requiring more than one purification steps^{18,20}.

We have recently demonstrated that fatty-acid conjugates of triple-helical, substrate peptides for MMP-2 and -9 are efficiently hydrolyzed by these enzymes²¹. We have prepared liposomes incorporating these lipopeptides and have demonstrated that the liposomal contents are efficiently released in the presence of recombinant as well as cancer cell-secreted MMP-2 and -9, and not in the presence of MMP-7 and -10 (refs. 21,22). These peptides and the corresponding fatty-acid conjugates were synthesized employing a microwave-assisted, automatic, solid-phase peptide synthesizer (Liberty and Discover from CEM, Matthews, North Carolina)^{21,22}. The coupling and the deprotection conditions were optimized for the synthesis of the collagen-mimetic peptides. For all the amino acids (except Arg), we used 25 W of microwave power at 50 °C and 5 min of coupling time during the synthesis. The Arg residues were subjected to double coupling. The initial coupling was carried out without using microwave power at 25 °C for 25 min. In the second stage, we used 25 W microwave power for coupling at 25 °C for 5 min. The use of less or no microwave power and low temperature minimizes γ -lactam formation²³. Low temperature should also be maintained for amino acids like Cys and His, which are very prone to racemization²³. A PEG-based resin (CLEAR Acid resin from Peptides International, Louisville, Kentucky) was used as the solid support during the synthesis. Fmoc-deprotection

reactions were carried out employing 35 W of microwave power at 75 °C for 3 min. After removal of the N-terminal Fmoc group from the synthesized peptide, several carboxylic acids (e.g., stearic acid, oleic acid, linoleic acid, hexynoic acid and so on) were conjugated using 25 W of microwave power at 50 °C for 5 min. Following the same synthetic protocol, we have systematically varied the number of G–P–O repeat units in the peptides to alter the melting temperatures of the resultant triple-helical lipopeptides²². In addition, we have recently reported the conjugation of a synthesized pyrene-containing fatty acid to the triple-helical peptides employing the microwave-assisted automatic peptide synthesizer²².

In this protocol, we describe the details for the synthesis of one of these triple-helical lipopeptides containing the cleavage site for MMP-9 as an illustrative example. The complete amino acid sequence of this peptide is:

CH₃(CH₂)₁₆CONHGPQGIAGQR(GPO)₄GG (the MMP-9 cleavage site is between the amino acids glycine and isoleucine). Compared with the time requirements of the conventional solid-phase peptide synthesis (7–10 d)^{18–20}, the microwave-assisted protocol requires 14 h to prepare this lipopeptide containing 23 amino acids^{21,22}. The yield and the purity of the crude product was considerably improved in the microwave-assisted synthesis (45–50% yield, >60% purity) compared with the conventional synthesis (< 30% yield, < 30% purity)^{20–23}. In addition to increasing the reaction temperature, the microwave irradiation causes de-aggregation of the growing peptide chains on the solid support^{24,25}. The protected amino acids and the coupling reagents in solution then access the chain termini, resulting in higher yield and purity of the synthesized peptides^{24,25}.

Microwave irradiation is demonstrated to be one of the most efficient methods to accelerate reaction rates^{24–26}. The rapid dielectric polarization of the solvent and the reactant molecules in the presence of microwave radiation leads to dramatically short reaction times, improved yields and purity of the products²⁵. Microwave irradiation is frequently used in organic syntheses to conduct reactions that are not possible using conventional heating²⁴. This approach has been used in solid-phase peptide synthesis to improve the purity of the crude peptides and to shorten the total synthesis time^{27–32}. Gellman³³ has reported a protocol for microwave-assisted preparation of β-peptide libraries. These peptides are difficult to prepare under conventional peptide synthesis conditions. Kappe³⁴ has reported a protocol to synthesize a calmodulin-binding peptide using microwave irradiation. However, these reported protocols are for the *manual* synthesis of short peptides (less than ten amino acids). In principle, these protocols can be used to synthesize the collagen-mimetic peptides containing more than 20 amino acids. However, the repetitive nature of the protocol will render the manual synthesis of the triple helical peptides extremely tedious and labor-intensive. The problem will be exacerbated if more GPO repeat units are needed in the triple helical peptides to impart the desired structural stability and biological property of the resultant triple-helical peptides^{17–19}. The automatic, microwave-assisted protocol reported herein does not suffer from these limitations. To the best of our knowledge, there is no previous protocol reported for automatic, microwave-assisted synthesis of triple-helical, collagen-mimetic lipopeptides.

In this protocol, details are provided to synthesize and purify the stearic acid conjugate of the triple-helical peptide in 100-μmole scale using the CEM Liberty Automated Microwave

Assisted Peptide Synthesiser and associated PepDriver software. The general synthesis protocol for the microwave-assisted peptide synthesis is shown in Figure 1. To the best of our knowledge, CEM Corporation is the sole supplier of microwave-assisted, automatic, solid-phase peptide synthesizers.

We have followed the same procedure to prepare these lipopeptides in 10–100- μ mole scale without any loss of purity and efficiency. In addition to the conjugation of fatty acids, other fluorophores containing carboxylic acid group can be conjugated to the peptides employing this protocol. We observed that the temperature during the coupling of these fatty acids and other carboxylic acids need to be optimized to improve the purity of the crude lipopeptide. For example, the saturated fatty acids (e.g., stearic acid and palmitic acid) can be conjugated at 50 °C without any detectable loss of purity. However, for the conjugation of unsaturated fatty acids (e.g., oleic, linoleic and so on), the conjugation reaction needs to be conducted at 40 °C or less to maintain sufficient purity of the crude lipopeptides. If a peptide sequence contains high amounts of Arg, His or Cys residues, the conditions for the microwave-assisted peptide synthesis needs to be carefully optimized to reduce the formation of γ -lactams and racemization²³.

MATERIALS

REAGENTS

- Fmoc-Gly-Clear-Acid Resin (Peptides International, cat. no. CFG-1233-PI, substitution: 0.44 meq g⁻¹)
- Fmoc-Gly-OH (Novabiochem, cat. no. 14-12-1001)
- Fmoc-Ile-OH (Peptides International, cat. no. FL1-1724-PI)
- Fmoc-Pro-OH (Chem Pep, cat. no. 71989-31-6)
- Fmoc-Gln(Trt)-OH (Chem Pep, cat. no. 13232780-1)
- Fmoc-Ala-OH (Chem Pep, cat. no. 35661-39-3)
- Fmoc-Arg(Pbf)-OH (Peptides International, cat. no. FLR-1746-PI)
- Fmoc-Hyp(tBu)-OH (Hyp = 4-(*R*)-hydroxyproline) (Chem Pep, cat. no. 122996-47-8)
- Stearic acid (Alfa Aesar, cat. no. R33, R36/37/38)
- 1-Hydroxybenzotriazole (HOBt) (AK Scientific, cat. no. 30G5E431) ▲
CRITICAL Store in a dry and well ventilated place.
- 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) (Chem Pep, cat. no. 94790-37-1)
- 1-Methyl-2-pyrrolidone (NMP) (TCI America, cat. no. MO418) ! **CAUTION**
Hygroscopic, keep container tightly sealed or store under nitrogen.
- Piperazine, anhydrous (TCI America, cat. no. PO446)

- N-ethyl-diisopropylamine (DIEA) (Alfa Aesar, cat. no. A11001) ! **CAUTION** highly flammable and corrosive; work in the hood with proper protection.
- Trifluoroacetic acid (TFA) (EMD Chemicals, cat. no. TX1276-7) ! **CAUTION** Corrosive, causes severe burns.
- Tri-isopropylsilane (TIS) (TCI America, cat. no. T1533)
- N,N-dimethylformamide (DMF) (Mallinkrodt Chemicals, cat. no. 4929-08) ▲ **CRITICAL** Store over ion-exchange resin to remove any traces of dimethylamine to reduce the amount of Fmoc-group removal under microwave conditions.
- Dichloromethane (DCM) (Mallinkrodt Chemicals, cat. no. 4879-10) ! **CAUTION** Skin irritant.
- Acetonitrile (J.T. Baker, cat. no. 9017-03) ! **CAUTION** Flammable.
- Deionized water

EQUIPMENT

- Reagent bottles (HDPE, 125 ml; VWR International, cat. no. 414004-156)
- 50 and 25 ml polypropylene centrifuge tubes (VWR International, cat. nos. 21008-089 and 21008-240)
- Liberty automated microwave-assisted peptide synthesizer (CEM) ▲ **CRITICAL** Regular maintenance, which includes back flushing, volume calibration and sensor calibration should be carried out every 15 and 30 d, respectively.
- Screw line secondary filters (Liberty, cat. no. 172150) (These filters are used in three locations on the liberty) ▲ **CRITICAL** Screw filters should be replaced after each synthesis to prevent clogging.
- Polypropylene line filters used on all chemical reagent lines (Liberty, cat. no. 167485-M) ▲ **CRITICAL** Line filters should be replaced after each synthesis to prevent clogging.
- 30 ml Teflon reaction vessel for standard 0.1–1.0 mmol scale synthesis (Liberty, cat. no. 167260) ▲ **CRITICAL** Change reaction vessel every three months; but this will depend on the frequency of usage. Check for clogs from time to time.
- 1 liter glass bottle with safety coating (Liberty, cat. no. 167550). This is used for storing the deprotection solution
- 250 ml Amber color glass bottle used for storing the activator solution
- Two 250 ml clear glass bottles (these bottles are used for activator base and cleavage)
- 20 liter waste carboy (Liberty)

- 4 liter Protective secondary containers for 4 liter glass bottles containing DMF and DCM (VWR International, cat. no. 56609-222)
- Adjustable speed large capacity centrifuge (VWR International, cat. no. 82107-654)
- Vacuum pump (Welch 8905; VWR International, cat. no. 54970-515)
- HPLC (Hitachi High Technologies America) analytical and semi prep HPLC with a fraction collector
- Semi-preparatory HPLC column (GRACE VYDAC, cat. no. 219TP510, 10 mm × 250 mm)
- HPLC analytical column (GRACE VYDAC, cat. no. 219TP5415, 4.6 mm × 150 mm)
- 2 ml Disposable conical micro tubes with silicone O-rings (VWR International, cat. no. 89004-300)
- Round bottom flask (250 ml)
- Rotary evaporator (Yamato, cat. no. RE 540), water bath (Yamato, cat. no. BM 100), vacuum pump (BUCHI Vac, cat. no. V-500)
- Lyophilizer (Labconco)
- Mass spectrometry (matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF))
- CD spectrometer (Jasco, cat. no. J 815)

EQUIPMENT SETUP

Microwave synthesizer—CEM Liberty system is a microwave-assisted automated peptide synthesizer (Fig. 2). The actual synthesis is carried out in a teflon reaction vessel housed within the microwave chamber (CEM Discover). A fiber-optic temperature probe is directly inserted into the reaction vessel. The system is equipped with 20 positions dedicated to 20 naturally occurring amino acids. Separate manifold containing five slots to the right are allocated for external reagents or non-natural amino acids. The resin and the product manifolds each contain 12 slots numbered 1–12. Three separate positions on the left manifold are designated for activator, activator base and cleavage bottles. There is a separate tray for holding the deprotection bottle.

Computer setup—The Liberty peptide synthesizer is controlled by a laptop computer of the following configuration:

Base unit	Pentium M 1.6 GHz
Memory	1 GB
Hard drive	60 GB

Operating system Windows XP

The Liberty operating software program, which enables the control of the instrument and synthesis of peptides is called the PepDriver.

HPLC—The purification was carried out using RP HPLC diphenyl semi-prep column. A linear gradient of 0–70% acetonitrile (vol/vol) (solvent B) was maintained at a flow rate of 8 ml min⁻¹ for 60 min. Solvent A was water. Both solvents contained 0.1% TFA (vol/vol). The flow rate was maintained at 8 ml min⁻¹ for 60 mins. The UV detection wavelength was set at 235 nm. Injection volume was 2 ml. A blank run was carried out using exactly the same conditions as the actual run before injecting the sample. The analytical RP HPLC was carried out for pure product using diphenyl analytical column. A linear gradient of 0–70% acetonitrile (vol/vol) (solvent B) was maintained at a flow rate of 1.5 ml min⁻¹ for 25 min. Solvent A was water. Both solvents contained 0.1% TFA (vol/vol). The UV detection wavelength was kept at 214 nm. Injection volume was 20 µl. Blank run was carried out before actual injection.

Circular dichroism—Circular dichroism spectra were recorded in a 1 mm path length quartz cuvette supplied by Jasco with the CD instrument. The concentration of lipopeptide was 1 mg ml⁻¹ in aqueous solution. The solution was stored at 4 °C for 24 h before recording the actual spectra.

Circular dichroism parameters: Temperature, 25 °C; number of scans, 3; scan speed, 100 nm min⁻¹; response time, 0.25 s; and data pitch, 0.2 nm. The CD spectra were measured in a continuous scanning mode between 180–250 nm.

PROCEDURE

Creating the sequence in the PepDriver

- 1| Open PepDriver and select the sequence tab on the top to open the sequence editor window. In the sequence editor create a new folder. To create the new folder, highlight yellow folder marked CEM (Fig. 3) on the left side of the sequence editor, which on right click opens up the option for new sequence. Alternatively, create a completely new folder. Type a name for the new sequence, e.g., NAT PROT (Fig. 3). Start building the sequence by clicking on the amino-acid buttons (Fig. 3) placed in the middle of the sequence editor. For Hyp and stearic acid, click any external buttons on the right side (Fig. 3). Enter the correct molecular weight by subtracting the molecular weight of one water molecule and Fmoc or other protecting groups, which will not be present in the final peptide sequence. In this case choose EX-3 for Hyp and EX-4 for the fatty acid. Note the sequence total MW as calculated by the software. When it is over, click the save button and then close the window.

▲ **CRITICAL STEP** Recheck the sequence just created.

Creating a method in the PepDriver

- 2| Open method editor from the top of the PepDriver menu. Create a new folder by highlighting CEM folder in the extreme left (Fig. 4) and then right click to give a name for the new method. From the adjacent sequence window locate the desired sequence and click enter. This step will display the sequence created in the sequence editor. On the right hand side, check C-terminus as acid when using an acid resin as in this example. If N-terminus of the final peptide chain contains Fmoc group, then check 'Yes' on Final Deprotection. In this example the final peptide chain will contain stearic acid in the N-terminal so check 'No' on Final Deprotection (Fig. 4). Select the scale for synthesis and type the resin substitution as written on the resin bottle. Select the default cycles based on the protocol as shown in this example (Fig. 5). Additional comments can be typed in the method comment box. Enter the coupling information for each set of amino acids by highlighting each amino acid and selecting a coupling method by scrolling down the menu just above the sequence. In this case Arg is highlighted and special double coupling method is selected as shown in (Fig. 4). Finally, save the method and close the window. As soon as the method is saved it gets loaded into the method folder, which can be accessed from the main screen of the PepDriver.

■ **PAUSE POINT** Check 'Skip Resin Load' under Sequence (Fig. 4) only if the resin is loaded manually, otherwise leave it blank.

▲ **CRITICAL STEP** If coupling method is not specified then by default it takes 0.1 mmol single coupling for all the amino acids.

Getting reagent weights

- 3| Access the main screen of the PepDriver and locate the method just saved by selecting the method folder from the lower left side (Fig. 5). Check whether all the information has been incorporated correctly. Then load the method into one of the gray circles marked '1'–'12' on top by dragging and dropping. The color of the circle will change from gray to green (Fig. 5, circle 6). Find the total volume and weight of the reagents required by clicking the calculator tab on top of the screen and then select 'usage'. The usage calculator will calculate the minimum volume and weights corresponding to minimum volumes of each reagent. However, to ensure that no reagent bottle empties out during the synthesis, alternatively add 4–5 ml of excess solvent to the minimum volume. This will be the altered volume. Once the total volume to be used has been decided, click on the calculator tab and select 'reagent'. Under 'reagent' six tabs are listed for each type of reagents e.g., resin, amino acids, activator, bases, deprotection and cleaving. By clicking on the reagent of choice and typing in the altered volume the corresponding weights can be calculated. For external reagents, type the actual molecular weight, including all the protective groups, on the reagent calculator or use the following formula to calculate the number of grams to be weighed out. $\text{Weight in grams} = (\text{Molecular weight (g/mol)} \times 0.2$

(M) × volume needed (ml))/(1,000). This formula is valid for 0.1-mmol scale synthesis.

■ **PAUSE POINT** The method loaded can be cleared by right clicking on the green circle and opting for a clear method.

Reagent preparation

- 4| Weigh and transfer the desired amounts of natural amino acids into 125 ml reagent bottles. Weigh the resin and external amino acids and transfer them in the 50 ml centrifuge tubes. Use the 250 ml amber color bottle for the activator solution and prepare activator base and cleavage solution in the 250 ml clear bottles. Use the 1 liter bottle for preparing the deprotection reagents and dissolve the solid by using shaker for 30 min. See Table 1 and Table 2 for exact weights and volume used for preparing the reagents in this synthesis.

■ **PAUSE POINT** The amino acid solutions are stable for 2 weeks while the deprotection, activator and activator bases are stable for 1 week. If not used immediately, refrigerate all the solutions (except deprotection) at 4 °C. Store the deprotection mixture at room temperature (25–30 °C) to ensure all the piperazine remains in the solution.

▲ **CRITICAL STEP** Always use freshly prepared cleavage solution.

! **CAUTION** Wear lab coat, gloves and safety goggles while preparing the reagents. Work in a fume hood for preparing activator base and cleavage solutions.

Starting the synthesis

- 5| Replace old-line filters and screw filters with new filters. Empty the waste solvent carboy. Use two equally filled 4 liter DMF bottles and a 4 liter DCM bottle. Check the desired sequence and then click start. On the left hand side the PepDriver will display a table indicating the resin position and status. Status should read 'running'. Place an empty centrifuge tube (50 ml) on the product manifold for collecting the final product. Match the position number of the product tube with the position number of the resin tube in their respective manifold. If the product tube is placed in a different numbered slot in the product manifold then the product will not come out after automatic cleavage.

■ **PAUSE POINT** If cleavage is carried out manually then the synthesized peptide attached to the solid support will collect in the resin tube.

? TROUBLESHOOTING

Resin cycle

- 6| Follow the standard-resin cycle (Table 3) to transfer and wash the resin.

Fmoc deprotection

- 7| Carry out two deprotection treatments using the microwave irradiation with piperazine dissolved in DMF for complete removal of Fmoc groups. Follow the standard microwave method with the method information as shown below (Table 4):

Initial deprotection: Microwave power 35 W, temperature 75 °C and time 30 s.

Final deprotection: Power 35 W, temperature 75 °C and time 180 s.

To make changes, select 'Microwave Editor' by selecting 'Setup' from the main screen and then type in the necessary changes.

Coupling conditions

- 8| Carry out the standard 0.1-mmol single coupling for each amino acid with the exception of N-terminal stearic acid and amino acid Fmoc-Arg(Pbf)-OH. Adopt the standard protocol for 0.1-mmol single coupling by using the following microwave method: microwave power 25 W, temperature 50 °C and time 300 s (Table 4).
- 9| Use multi-step temperature control microwave method for Arg double coupling. See (Table 5) for method details.
- 10| Adopt the method and operations for amino-acid cycle 0.10 mmol double for stearic acid (EX-4) coupling to the N-terminal Gly (Table 6). Standard condition: microwave power 25 W, temperature 50 °C and time 300 s. This condition is repeated twice.

Microwave-assisted cleavage from the solid support

- 11| Perform cleavage from the solid support under microwave condition by applying microwave power of 35 W for 2,100 s at 36 °C (Table 7).

? TROUBLESHOOTING

Precipitation and isolation of crude peptide ● TIMING 3 h

- 12| Add diethyl ether (15–20 ml) to the solution collected in the centrifuge tube after automated cleavage. Keep the solution in a freezer at a temperature of –20°C for 2–3 h to ensure complete precipitation of the crude peptide as a white solid.

Isolation and drying of the crude product ● TIMING ~5 h

- 13| Centrifuge for 25 min at 4 °C at 2,000g. Decant the supernatant liquid. Add fresh ether and repeat this two more times. Finally, dry the crude product in a vacuum pump for 5 h.

! CAUTION Do not discard the ether washings. If the yield of the lipopeptide is low, evaporate the decanted liquid in a rotary evaporator, add ether and repeat Step 12.

Purification and analysis of crude lipopeptide ● TIMING ~2 h

- 14| Purify the crude lipopeptide in semi-preparatory RP HPLC using the conditions stated in EQUIPMENT SETUP. Inject 20 mg in the column. Collect the pure product with the help of a fraction collector in small disposable centrifuge tubes. In this example, collect the peak corresponding to retention time 25.2 min (Fig. 6).

? TROUBLESHOOTING**Solvent removal from the purified product ● TIMING ~2 h**

- 15| Transfer the collected liquids to a 250 ml round bottom flask. Evaporate the organic solvents in the rotary evaporator at a temperature of 40 °C, at 10-mbar pressure for 15 min. Then remove the round bottom flask from the rotary evaporator and transfer the aqueous solution to 4-dram vial or 25 ml polypropylene centrifuge tube. Add < 1 ml of deionized water for rinsing. Rinse with small volume of water thrice.

Freeze drying ● TIMING ~2–24 h

- 16| Freeze the solution under liquid nitrogen and then lyophilize.

Analysis of the pure product by HPLC ● TIMING 1 h

- 17| Analyze the lyophilized solid in analytical HPLC (Fig. 7) under the same conditions as mentioned before (see HPLC under EQUIPMENT SETUP).

Mass spectral analysis ● TIMING 30 min

- 18| Measure the mass of the pure lipopeptide using in MALDI-ToF mass spectrometer (Fig. 8).

? TROUBLESHOOTING**Determination of triple helical conformation using CD spectroscopy ● TIMING 24 h for storing, 15 min for recording the spectra**

- 19| Use a CD spectrometer and scan from wavelength 180–250 nm to observe any collagen-like triple helical structure (Fig. 9). Prepare the solution by weighing 1 mg of the pure peptide in a small centrifuge tube (1.5 ml) and then dissolve in 1 ml of deionized water as described in reference 21. Store the solution for 24 h at 4 °C before recording the CD spectra to ensure that the lipopeptides have reached the monomer to trimer equilibrium³⁵.

Calculation of Rpn value ● TIMING 5 min

- 20| Calculate the Rpn value (ratio of positive peak intensity at 225 nm to negative peak intensity at 200 nm) and compare it with Rpn value for collagen. The Rpn value calculated in this example is 0.1. Rpn value for natural collagen is 0.13 (ref. 14).

● TIMING

Steps 1–3: Creating the sequence, method and loading a method in the PepDriver takes 20 min. This can be carried out any time before the actual synthesis

Step 4, Reagent preparation: 2 h (it can vary depending on the sequence of the peptide)

Step 5, Starting the synthesis: 1 min

Steps 6–11: 14 h 25 min. This is the total synthesis time. The synthesis time can vary depending on the peptide sequence, coupling and cleavage conditions

Step 12: Precipitation of the crude product, 3 h

Step 13: Isolation and drying of the crude product, about 5 h

Step 14: Purification of the crude product by HPLC, 2 h

Step 15: Solvent removal from the purified product, 15 min

Step 16: Freeze drying, 24 h

Step 17: Analysis of the pure product by HPLC, 1 h

Step 18: Mass spectral analysis, 30 min

Step 19: Sample preparation and CD spectral analysis, 24 min for storing, 15 min for recording the spectra

Step 20: Calculation of Rpn value, 5 min

? TROUBLESHOOTING

If the system is unable to pressurize DMF or DCM, check whether the 'O' ring has fallen off from the caps or if there is enough nitrogen in the nitrogen tank (Step 5). In the case of valve problems, seek technical assistance from CEM. If the product transfer is not detected after automatic cleavage (Step 11) try changing the F4 line filter. Make sure that the product tube is placed in the right position and fitted properly. Check for leaks on any other manifold. In case a leak is detected from a different position in the product or resin manifold, put some empty centrifuge tubes in those positions. In case of deleted sequence or impurity detected by MALDI-ToF (Step 18), apply double coupling for those amino acids. Alternatively, increase the microwave coupling times and temperature. In case of difficulty purifying by the semi-preparatory HPLC, use a different column, solvent system or higher temperature to get better peak resolution (Step 14).

ANTICIPATED RESULTS

The coupling temperature has been optimized for the amino acids including Arg by employing the automatic, microwave-assisted peptide synthesizer. At first, we attempted the coupling and the stearic acid conjugation reactions at 25 °C; the crude lipopeptide was difficult to purify by HPLC because of peaks with very close retention times (Figs. 10 and 11). Conducting the coupling and the conjugation reactions at 40 °C improved the quality of

the crude lipopeptide to some degree (Figs. 12 and 13). Increasing the coupling and conjugation temperature to 50 °C solved the problem (Figs. 7 and 8). Further increase in reaction temperature (70 °C) did not provide any additional improvement for the quality of the crude lipopeptides. Using the described optimized conditions, the average yield of the crude lipopeptide isolated is 116 mg (50%). Purity determined from HPLC chromatogram for the purified lipopeptide is >94% (Fig. 8). The peptide mass was verified using MALDI-ToF (Fig. 9) and the propensity of the lipopeptide to acquire a triple helical structure in aqueous solution was confirmed by CD spectrometry (Fig. 10).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The steps to be followed in microwave-assisted, solid-phase synthesis of peptides.

Figure 2.
CEM Liberty automatic, microwave-assisted peptide synthesizer.

Figure 3. Sequence Editor window in the software PepDriver for creating a new sequence.

Figure 4. Method Editor window for creating a method for the desired peptide sequence in the PepDriver software.

Figure 5.
Part of the window displaying the method loaded at position '6' on main screen of the software PepDriver.

Figure 6. High-performance liquid chromatography (HPLC) chromatogram for the synthesized crude lipopeptide.

Figure 7. High-performance liquid chromatography (HPLC) chromatogram of the purified lipopeptide.

Figure 8.
Mass spectrum of the purified lipopeptide.

Figure 9. Circular dichroism (CD) spectrum of the purified lipopeptide recorder at 25 °C. The positive peak at 225 nm and the negative peak at 200 nm is indicative of collagen triple helix formation.

Figure 10. High performance liquid chromatography (HPLC) chromatogram for the synthesized crude lipopeptide with 25 °C coupling temperature for the amino acids.

Figure 11.
Mass spectrum of the purified lipopeptide with 25 °C coupling temperature.

Figure 12.
High performance liquid chromatography (HPLC) chromatogram for the synthesized crude lipopeptide with 40 °C coupling temperature.

Figure 13.
Mass spectrum of the purified lipopeptide with 40 °C coupling temperature.

TABLE 1

Amount, concentration and final volume of amino acids and external reagents prepared for 0.1-mmol scale synthesis.

Amino acids and external reagents	Amount (g)	Concentration (M)	Solvent	Final volume (ml)
Fmoc-Ala-OH	0.498	0.2	DMF	8
Fmoc-Arg(Pbf)-OH	1.298	0.2	DMF	10
Fmoc-Gln(Trt)-OH	1.221	0.2	DMF	10
Fmoc-Gly-OH	1.487	0.2	DMF	25
Fmoc-Ile-OH	0.636	0.2	DMF	9
Fmoc-Pro-OH	1.215	0.2	DMF	18
Fmoc-Hyp(tBu)-OH (Ext 3)	1.228	0.2	DMF	15
Stearic acid (Ext 4)	0.569	0.2	DMF	10

DMF, N,N-dimethylformamide.

TABLE 2

Amount, concentration and final volume of coupling reagents, deprotection, resin and cleavage cocktail prepared for 0.1-mmol scale synthesis.

Reagent type	Reagents	Amount	Concentration	Solvent	Final volume (ml)
Activator	HBTU	7.207 g	0.5 M	DMF	38
Activator	HOBt	2.567 g	0.5 M	DMF	38
Deprotection	Piperazine	5.0 g	5% (wt/vol)	DMF	450
Activator base	DIEA	7.0 ml	0.5 M	NMP	20
Resin ^a	Fmoc-Gly-Clear Acid Resin	227 mg			
Cleavage	TFA	19.0 ml	95% (wt/vol)		20
Cleavage	TIS	0.5 ml	2.5% (wt/vol)		20
Cleavage	Deionized water	0.5 ml	2.5% (wt/vol)		20

DMF, N,N-dimethylformamide; NMP, 1-methyl-2-pyrrolidone.

^aThe weight of the resin is calculated based on resin substitution and synthesis scale. About 1 ml of DMF is added to wet the resin.

TABLE 3

The standard resin cycle for the transfer of the resin to the reaction vessel and subsequent wash.

No.	Operation	Parameter	Volume (ml)	Drain	Cycles	Pause
1	Clean reaction vessel		10	Yes	1	No
2	Add DMF to resin		7	No	1	No
3	Add DCM to resin		7	No	1	No
4	Transfer resin to reaction vessel		10	Yes	1	No
5	Add DMF to resin		7	No	1	No
6	Add DCM to resin		7	No	1	No
7	Transfer resin to reaction vessel		10	Yes	1	No
8	Add DMF to resin		7	No	1	No
9	Add DCM to resin		7	No	1	No
10	Transfer resin to reaction vessel		10	Yes	1	No
11	Add reagent	DMF	5	No	1	No
12	Add reagent	DCM	5	No	1	No
13	Wait state	900 s		No	1	No
14	Drain-filtered			Yes	1	No

DCM, dichloromethane; DMF, N,N-dimethylformamide.

TABLE 4

The single coupling steps using microwave.

No.	Operation	Parameter	Volume (ml)	Drain	Cycles	Pause
1	Clean resin dip tube			Yes	1	No
2	Wash-top	DMF	7	Yes	1	No
3	Add deprotection		7	No	1	No
4	Microwave method	Initial deprotection		Yes	1	No
5	Wash-top	DMF	5	Yes	1	No
6	Add deprotection		7	No	1	No
7	Microwave method	Deprotection		Yes	1	No
8	Wash-top	DMF	7	Yes	1	No
9	Wash-bottom	DMF	7	Yes	1	No
10	Wash-top	DMF	7	Yes	1	No
11	Clean resin dip tube			Yes	1	No
12	Wash-top	DMF	7	Yes	1	No
13	Add amino acid		2.5	No	1	No
14	Add activator		1	No	1	No
15	Add activator base		0.5	No	1	No
16	Microwave method	Coupling		Yes	1	No
17	Wash-top	DMF	7	Yes	1	No
18	Wash-bottom	DMF	7	Yes	1	No
19	Wash-top	DMF	7	Yes	1	No

DMF, N,N-dimethylformamide.

TABLE 5

Two-step microwave method for coupling of amino acid Fmoc-Arg(Pbf)-OH.

No.	Operation	Parameter	Volume (ml)	Drain	Cycles	Pause
1	Clean resin dip tube			Yes	1	No
2	Wash-top	DMF	7	Yes	1	No
3	Add deprotection		7	No	1	No
4	Microwave method	Initial deprotection		Yes	1	No
5	Wash-top	DMF	5	Yes	1	No
6	Add deprotection		7	No	1	No
7	Microwave method	Deprotection		Yes	1	No
8	Wash-top	DMF	7	Yes	1	No
9	Wash-bottom	DMF	7	Yes	1	No
10	Wash-top	DMF	7	Yes	1	No
11	Clean resin dip tube			Yes	1	No
12	Wash-top	DMF	7	Yes	1	No
13	Add amino acid Arg		2.5	No	1	No
14	Add activator		1	No	1	No
15	Add activator base		0.5	No	1	No
16	Microwave method	Coupling		Yes	1	No
17	Clean resin dip tube			Yes	1	No
18	Add amino acid Arg		2.5	No	1	No
19	Add activator		1	No	1	No
20	Add activator base		0.5	No	1	No
21	Microwave method	Coupling		Yes	1	No
22	Wash-top	DMF	7	Yes	1	No
23	Wash-bottom	DMF	7	Yes	1	No
24	Wash-top	DMF	7	Yes	1	No

DMF, N,N-dimethylformamide.

TABLE 6

The double coupling steps for conjugating stearic acid (EX-4) to the peptide on the resin.

No.	Operation	Parameter	Volume (ml)	Drain	Cycles	Pause
1	Clean resin dip tube			Yes	1	No
2	Wash-top	DMF	7	Yes	1	No
3	Add deprotection		7	No	1	No
4	Microwave method	Initial deprotection		Yes	1	No
5	Wash-top	DMF	5	Yes	1	No
6	Add deprotection		7	No	1	No
7	Microwave method	Deprotection		Yes	1	No
8	Wash-top	DMF	7	Yes	1	No
9	Wash-bottom	DMF	7	Yes	1	No
10	Wash-top	DMF	7	Yes	1	No
11	Clean resin dip tube			Yes	1	No
12	Wash-top	DMF	7	Yes	1	No
13	Add amino acid		2.5	No	1	No
14	Add activator		1	No	1	No
15	Add activator base		0.5	No	1	No
16	Microwave method	Coupling		Yes	1	No
17	Clean resin dip tube			Yes	1	No
18	Add amino acid		2.5	No	1	No
19	Add activator		1	No	1	No
20	Add activator base		0.5	No	1	No
21	Microwave method	Coupling		Yes	1	No
22	Wash-top	DMF	7	Yes	1	No
23	Wash-bottom	DMF	7	Yes	1	No
24	Wash-top	DMF	7	Yes	1	No

DMF, N,N-dimethylformamide.

TABLE 7

The steps for the cleavage of the lipopeptide from the resin.

No.	Operation	Parameter	Volume (ml)	Drain	Cycles	Pause
1	Wash-top	DCM	7	Yes	4	No
2	Wash-bottom	DCM	7	Yes	1	No
3	Clean resin dip tube			Yes	1	No
4	Wash-top	DCM	7	Yes	1	No
5	Add cleavage mixture		8	No	1	No
6	Microwave method	Cleaving (3.5 min)		No	1	No
7	Transfer product			No	1	No
8	Add reagent	DCM	5	No	1	No
9	Transfer product			No	1	No
10	Add reagent	DCM		No	1	No
11	Transfer product			No	1	No
12	Clean reaction vessel		10	No	3	No
13	Reaction vessel neutralization		10	No	3	No
14	Wash-top	DCM	7	Yes	2	No

DCM, dichloromethane.