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RADA-16: A Tough Peptide – Strategies for Synthesis and Purification

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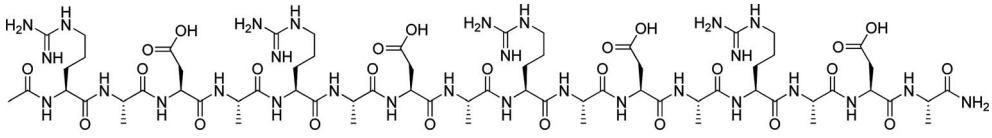
The self-assembling capacity of certain molecules can be exploited for a diverse range of biomedical applications. The ionic complementary peptide RADA-16 is well-known for its propensity to self-assemble, which derives from its architectural arrangement. Herein, we describe rational synthetic strategies of synthesis of RADA-16 based on fragment condensation, and its subsequent purification through optimized methods. Our methodology should prove suitable for the preparation of other peptides prone to self-assembly.

Introduction

Over the past few years nanobiotechnology and biomaterials based on peptide and protein self-assembly systems have garnered great attention.[1,2] The interest lies chiefly in the possibility of manufacturing new nanostructured scaffolds with customizable mechanical properties and biological functions.[3,4] Moreover, peptides and proteins are excellent model systems for studying biological self-assembly because they are highly biocompatible and their sequences can be modified to obtain defined molecular properties. Several types of self-assembling peptides have been systematically studied.[5] This class of biological materials has countless biomedical applications, including tissue regeneration,[6] drug delivery,[7] protein crystallization,[8] and cellular internalization.[9]

In this study, we chose the well-known self-assembling peptide Ac-$(RADA)_4$-NH_2 (RADA-16, Figure 1), a 16 amino acid peptide belonging to the family of ionic complementary peptides. It comprises repeated segments of hydrophobic (Ala) and hydrophilic (Arg and Asp) groups with alternating positive and negative charged amino acid residues. This structure enables RADA-16 to undergo ordered self-assembly in solution to form nanofibers.

RADA-16 is highly prone to self-assemble into very stable and highly organized β-sheet structures that tend to form hydrogels. It has been extensively used for three-dimensional cell culture scaffolds as well as for drug delivery and regenerative medicine.[10–12] Recently, several functional motifs have been used to generate RADA-16 analogues that promote cell adhesion, migration, neurite outgrowth, and cell differentiation.[13]



Ac-Arg^1-Ala^2-Asp^3-Ala^4-Arg^5-Ala^6-Asp^7-Ala^8-Arg^9-Ala^{10}-Asp^{11}-Ala^{12}-Arg^{13}-Ala^{14}-Asp^{15}-Ala^{16}-NH_2

Figure 1. Structure of RADA-16.

The self-assembling capacity of RADA-16, which results in a huge range of nanobiotechnology and biomedical engineering applications,[14–17] is not only its beauty, but also the cause of difficulty in both its synthesis in decent yield and its purification. Although RADA-16 is commercially available and has been synthesized by research groups, the absence of a detailed full chromatographic characterization[18] encouraged us to explore combined optimum synthetic and purification strategies as well as a convenient HPLC method for its characterization.[19]

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Prompted by the demand for RADA-16 in sufficiently high purity for clinical use, we sought to explore synthetic and chromatographic (HPLC) strategies to obtain this product in high purity. We rationalized that the peculiarity of repeated segments in the RADA-16 sequence would make this peptide an excellent model for developing different peptide synthetic strategies to obtain the desired target with maximum possible purity.

In this work, rationalized synthetic strategies to obtain RADA-16 have been proposed and carried out to establish the most suitable approach to the synthesis of this peptide with acceptable levels of purity. Stepwise solid-phase synthesis, in manually and automatic modes, were first attempted, since these are the most common and straightforward ways to synthesize peptides. Two more sophisticated strategies based on fragment condensation approaches ("solid-phase fragment condensation" and "fragment condensation in solution") were performed to evaluate which was the most suitable strategy to reach the peptide target.

Approaches outlined herein should prove valuable for other peptide targets that contain repetitive sequences and that undergo self-assembly into other structures.

Results and Discussion

Prior to the development of synthetic strategies to obtain RADA-16, an HPLC study of this peptide [in this case, Ac-$(RADA)_4$-NH_2 provided by different companies] was required to optimize the best analytical procedures with which to characterize the self-assembling peptide. Several HPLC factors were analyzed and optimized,[19] as an example, one of the analytical parameters that seriously affects the chromatographic analysis of RADA-16 was the concentration of peptide injected onto the HPLC column. This factor, which is not typically significant when other peptides are analyzed, is crucial when RADA-16 or other self-assembled peptides are studied. The appreciable differences in chromatographic profiles of RADA-16 dissolved at concentrations of 0.5, 1 and 2 mg/mL show the importance of this parameter (Figure 2). This study allowed us to conclude that the optimum concentration of HPLC injection for Ac-$(RADA)_4$-NH_2 was ca. 0.5 mg/mL, which reduces aggregation during application on the HPLC column while still allowing peptide purity evaluation.

The initial strategy described herein to obtain RADA-16 was based on stepwise solid-phase syntheses of RADA-16 in manual and automatic modes, using solid-phase peptide synthesis according to Fmoc/*t*Bu chemistry on ChemMatrix resin.[20–23] This resin, which is considered the best suited for this class of peptides, enabled us to overcome the challenge associated with strongly hydrophobic synthetic targets, including problems attributed to peptide aggregation or to poor resin solvation. Although after several recouplings, our manual synthesis ultimately afforded the desired peptide, however, final characterization by HPLC and MALDI-TOF MS revealed a rather poor final purity of the crude product. Most importantly, impurities associated with this strategy eluted close to the desired product, thus complicating the purification, which was already problematic due to the poor solubility of the peptide (Figure 3, A). In fact, one of the principal impurities was derived from deletion of the three amino acids Arg, Ala, and Asp, which are side-products that cannot be easily removed, especially when the stepwise approach is hampered by β-sheet formation. A similar HPLC profile was observed when automated synthesis was performed (Figure 3, B).

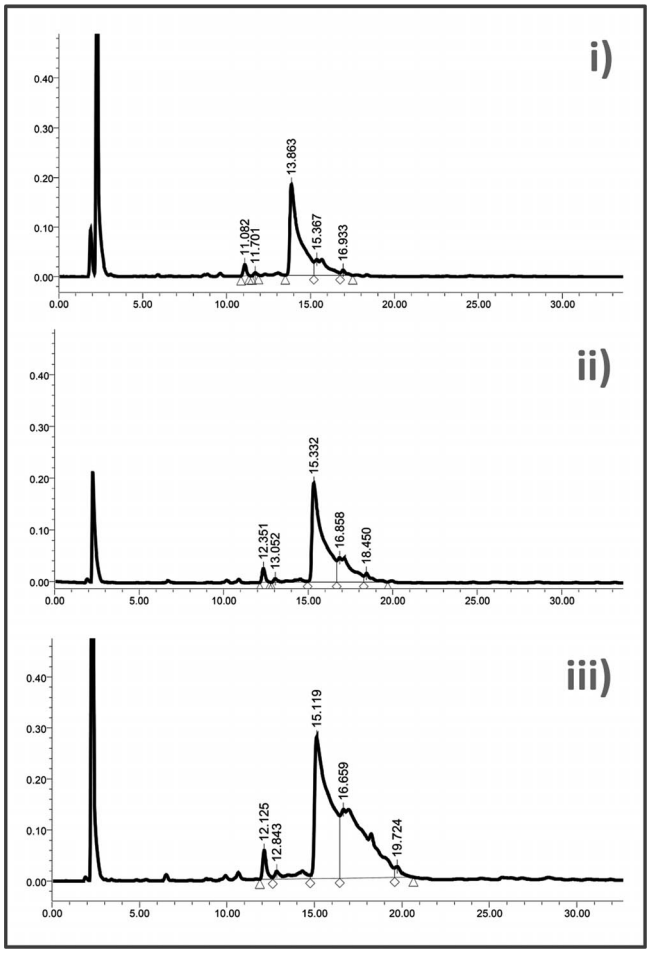

Figure 2. Comparison of HPLC profiles [conditions: column I heated at 50 °C, eluent based on TFA system, gradient (%B): 0–30] of Ac-$(RADA)_4$-NH_2 material provided by one chemical supplier dissolved with H_2O/0.1% TFA and analyzed at different concentrations: (i) 0.5, (ii) 1, and (iii) 2 mg/mL.

Since neither manual nor automated stepwise synthesis gave sufficiently pure peptide, we investigated the solid-phase fragment condensation strategy, whereby presynthesized, protected fragments were sequentially coupled onto the resin, enabling the peptide sequence to be completed on solid phase. By following this strategy, side products formed from incomplete couplings are more easily removed because they differ from the peptide by the length of one fragment, rather than by only one amino acid.[24–26] Taking into account that RADA-16 contains the tetrapeptide sequence RADA (Arg-Ala-Asp-Ala) in quadruplicate, we decided to

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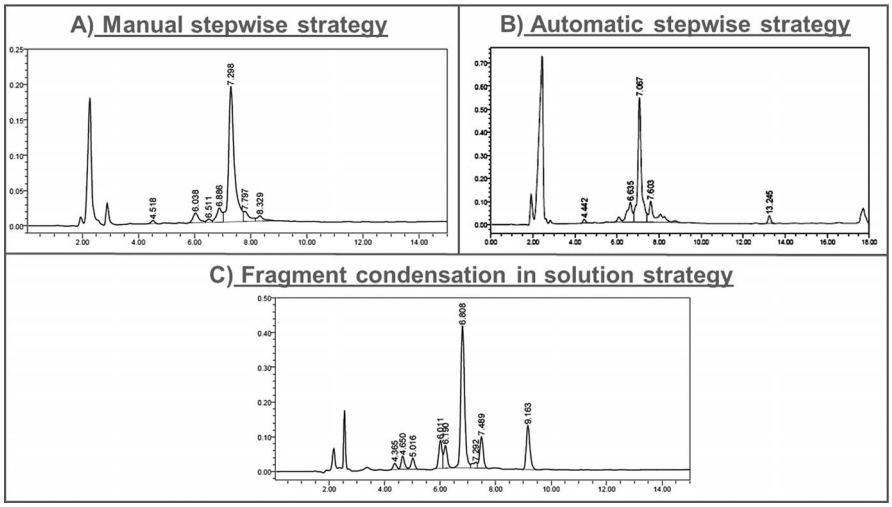
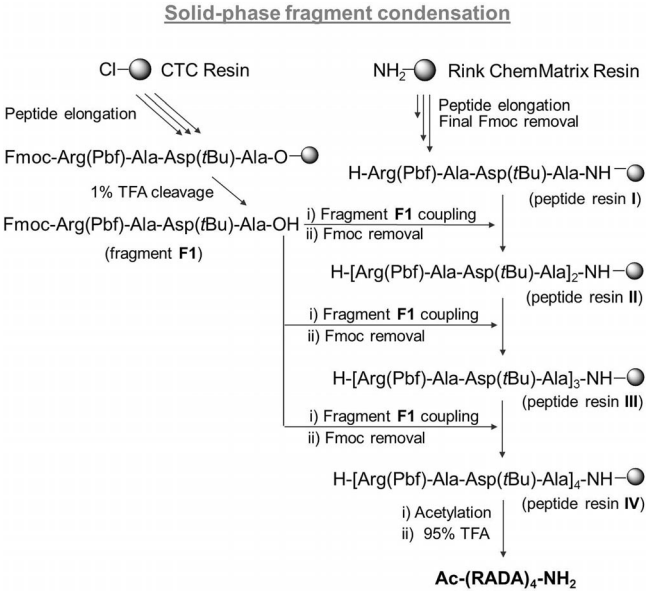


Figure 3. HPLC profiles of the crude peptide obtained in three different syntheses of RADA-16: (A) manual stepwise solid-phase, (B) automatic stepwise solid-phase, and (C) fragment condensation in solution.

prepare the Fmoc-protected tetrapeptide Fmoc-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH (Fragment **F1**) on solid-phase, and then link, consecutively, **F1** until the desired sequence of RADA-16 was complete (Scheme 1).



Scheme 1. Synthesis of Ac-$(RADA)_4$-NH_2 (RADA-16) by solid-phase fragment condensation.

To synthesize the protected fragment **F1**, with a C-terminal carboxyl moiety, we accomplished peptide elongation on CTC resin.[27] Cleavage of the peptide from the resin under mild acidic conditions provided **F1** in high purity (99%), thus obviating the need for further purification. Although the solubility of the obtained product – a requisite for successful couplings on resin – was unpredictable,[28] fortunately, the target protected tetrapeptide was completely soluble in *N*,*N*-dimethylformamide (DMF), which is an appropriate solvent for solid-phase coupling. To incorporate the synthesized protected fragment **F1** onto the resin, careful choice of resin,[29] peptide loading,[30] coupling reagents,[31,32] and reaction conditions[33] is essential. Again, the ChemMatrix support was chosen for this strategy. At this point, we first tried to incorporate fragment **F1** directly onto the polymer support by using a (4 + 4 + 4 + 4 + resin) strategy. However, this approach was unsuccessful and was abandoned. We then turned to the solid-phase fragment condensation (4 + 4 + 4 + RADA-resin) strategy (Scheme 1); thus, first four amino acids (RADA) were coupled stepwise conferring "peptide resin **I**". Subsequently, the first fragment **F1** was incorporated onto peptide resin **I** through a double-coupling protocol to afford the resin-bound octapeptide (peptide resin **II**).

The best way to monitor each coupling of **F1** onto the resin was to cleave an aliquot of peptide–resin and analyze it by reverse-phase HPLC, which, although more tedious, was essential because standard tests used in stepwise synthesis, such as the Kaiser test and the TNBS-test only give a first indication. After Fmoc-removal, the **F1** fragment was incorporated (three times) using relatively harsh coupling reagents to afford the dodecapeptide on resin (peptide resin **III**). HPLC analysis revealed a mixture of products, although the desired peptide was obtained in 46% purity. After the last fragment **F1** incorporation, the Fmoc group was removed and the resulting deprotected peptide was acetylated and then cleaved from the resin. The main advantage of this strategy was that it minimized formation of side products that differ from the desired product by only one or two residues, as indicated in narrower peaks observed by reverse-phase HPLC (Figure 3, C). Although for peptides that are not prone to self-assembly, purification of the crude material obtained from the fragment coupling strategy was more convenient than from crude material obtained from a stepwise strategy, in our case, the poor solubility of the crude material precluded that.

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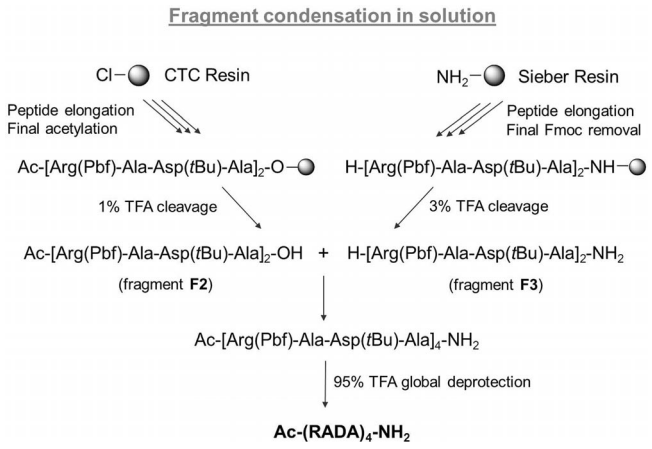
Finally, seeking to establish an optimized strategy for RADA-16 synthesis that would ultimately enable synthesis on a larger scale, we explored fragment condensation in solution.[34] Numerous examples of this type of synthesis, which combines the respective advantages of solid-phase and solution phase synthesis, have been reported. We chose the 8 + 8 strategy (Scheme 2), which required two protected fragments: the N-terminal fragment (Ac-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH; Fragment **F2**) and the C-terminal fragment (H-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-NH_2; Fragment **F3**). The success of fragment condensation depends on various factors: (1) the solubility and final purity of the protected fragments, (2) the scale of synthesis, (3) the coupling reagents and conditions used, and most importantly (4) the final isolation of the product with concomitant removal of any remaining reagents. This strategy has proved to be the strategy of choice for industrial synthesis of several difficult peptide targets[35,36] because it can produce sufficiently pure final product for clinical use.



Scheme 2. Synthesis of Ac-$(RADA)_4$-NH_2 (RADA-16) by fragment condensation in solution.

In this work, we synthesized the two protected fragments by solid-phase, manually, on medium scale (0.8 mmol) with two different resins. In each case, the peptide was cleaved from the resin without affecting the protecting groups. Because the two fragments were obtained in high purity, no further purification was required. The key point of this strategy was that both fragments were completely soluble in DMF, because, as previously mentioned, although the solubility of fragments in DMF was mandatory for coupling, the solubility of any designed protected fragment is unpredictable. The choice of coupling reagents PyBOP/DIEA with HOAt as additive, and the use of equimolar amounts of protected fragments, was found to be suitable for this strategy. Phosphonium salts are better than aminium salts as activating agents for reactions in which the carboxylic component was not in excess (e.g., fragment condensation or cyclization), because they do not react irreversibly with the amino function, as aminium salts tend to do.[37,38] A clear advantage of fragment condensation in solution over fragment condensation on solid-phase is the quality of the crude peptide obtained.

Chromatographic methods are generally unsuitable for purification of peptides such as RADA-16, which are typically insoluble in standard chromatography solvents. Thus, the relatively pure crude material with impurities that are chemically differentiated from the expected product ob-

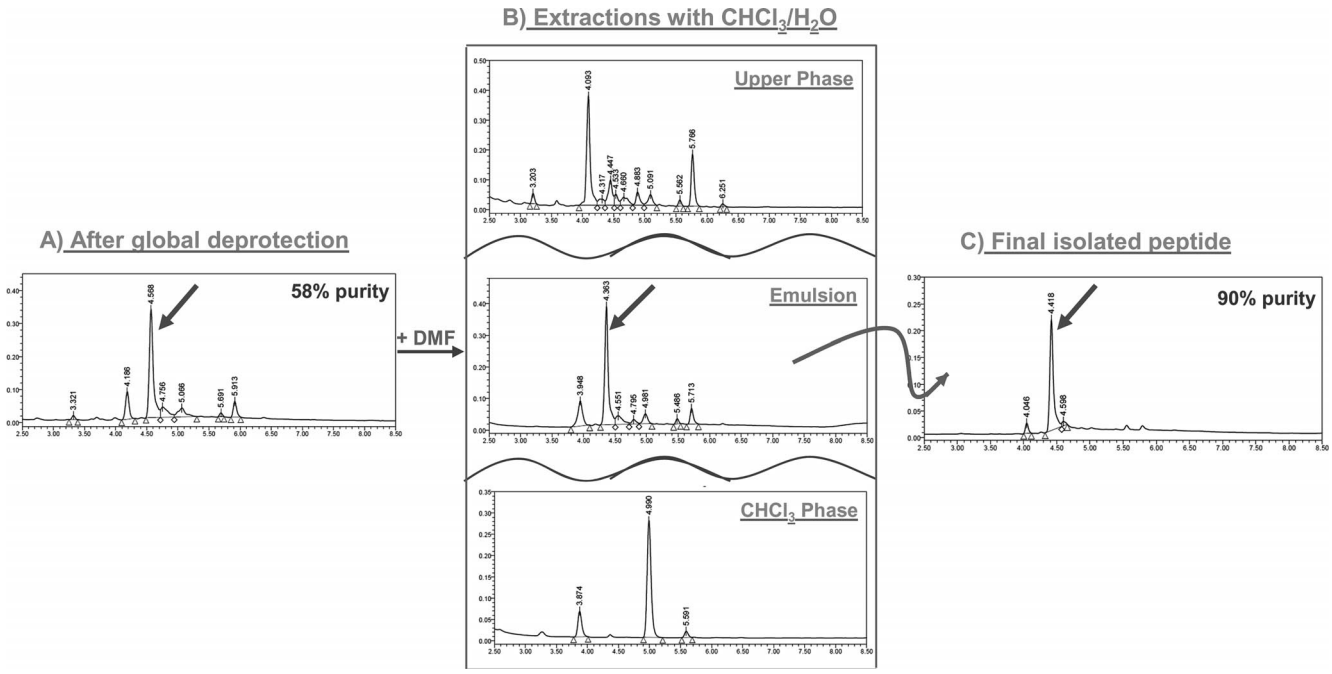


Figure 4. HPLC profiles obtained (A) before, (B) during, and (C) after $CHCl_3/H_2O$ extractions performed in the synthesis of RADA-16 by fragment condensation in solution.

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tained from fragment condensation in solution should be purified by washing and precipitation. Washing with *tert*-butyl methyl ether helped to remove HOAt, which very often remains on the peptide. However, although this step can be very efficient in small-scale syntheses, as was the case in our attempts, for a larger scale a deeper work-up was needed (Figure 4). During this procedure, several impurities were removed (Figure 4, B); one of the principal impurities that entered the organic phase had the UV profile of HOAt (as indicated by photodiode array detector from HPLC analysis). The expected product was mainly detected in the emulsion phase and was isolated as described in detail in the experimental part and presented in (Figure 4). This purification procedure gave RADA-16 in higher purity (90%; see Figure 4, C) than did either of the stepwise syntheses or the solid-phase fragment condensation synthesis.

Conclusions

It is clear that Ac-$(RADA)_4$-NH_2 is a tough peptide to synthesize and purify. The strategy outlined herein, which combines the advantages of solid-phase methodology and solution chemistry, has allowed the preparation of the target peptide with sufficient purity for biological purposes. The strategy described herein is based on the solid-phase preparation of two protected fragments with sufficient purity that they do not require purification. Their coupling in solution gives the target product in a purity that can be resolved further by standard laboratory operations. In addition, a wide range of chromatographic parameters to establish the optimum HPLC conditions to characterize the RADA-16 were analyzed.

This work has shown that, despite the sophistication of recently developed synthetic strategies for the preparation of peptides (solid supports, handles, coupling reagents, etc.), the synthesis of self-assembled peptides remains a major challenge. The methodology developed herein should be of broad applicability to similar peptide targets.

Experimental Section

General: Analytical HPLC was carried out with an instrument comprising two solvent delivery pumps, an automatic injector, and a variable wavelength detector (photodiode array). UV detection was performed at 215 and 220 nm. Two HPLC columns were used: column I [C18 column (XTerra, Waters: 4.6 × 150 mm, 5 μm)] or column II (SunFire, Waters: C18, 3.5 μm 4.6 × 100 mm). All peptide fragments analyzed from different synthetic strategies of RADA-16 were run using linear gradients of two eluents at room temperature (25 °C) and a flow rate of 1.0 mL/min: eluent A (H_2O + 0.045% TFA) and eluent B (MeCN + 0.036% TFA). HPLC/ESMS was performed on a reverse-phase C18 column (3.9 × 150 mm, 5 μm) using aqueous (+ 0.1% formic acid) and MeCN (+ 0.07% formic acid) as eluents. MALDI-TOF MS was carried out with a Voyager-DETMRP Biosystem, using α-cyano-4-hydroxycinnamic acid (ACH) as matrix.

RADA-16 Syntheses

Stepwise strategies: We explored manual and automatic stepwise solid-phase syntheses of RADA-16, with the double objective of optimizing the preparation and also to obtain sufficient amount of peptide for use as a reference material.

Manual Synthesis: RADA-16 was synthesized manually by solid-phase peptide synthesis by using 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) chemistry on an aminomethyl-ChemMatrix resin[39] (Matrix Innovation, scale 0.1 mmol, loading: 0.62 mmol/g, 35–100 mesh). Peptide elongation was performed in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. The linker Rink-Amide (3 equiv., Iris Biotech) was anchored to the resin with *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBt) (3 equiv. each) in DMF for 12 h. At this point, a positive ninhydrin test indicated free amino groups, so the linker reaction was repeated, this time for 2 h. The Fmoc groups were removed by treatment with piperidine/DMF (1:4) in the presence of 0.1 M HOBt (1 × 2 min, 2 × 10 min), which was used to minimize aspartimide formation.[40] The following Fmoc-L-protected amino acid derivatives were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Asp(*t*Bu)-OH. Chain extension was carried out by using 5 equiv. of the amino acid, at each coupling step, in the presence of 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) (5 equiv.) and diisopropylethylamine (DIEA) (10 equiv.) for 1 h. In case of a positive ninhydrin test, a recoupling was performed, using a fivefold molar excess of the Fmoc-amino acid, activated essentially in situ by DIPCDI/7-aza-1-hydroxybenzotriazole (HOAt) in DMF for 3 h. Incorporation of the following residues required multiple recouplings: Ala^2, Asp^3, Ala^4, Arg^5, Ala^6, Arg^9, Ala^{10}, Asp^{11}, and Ala^{12}. After Fmoc removal of the last Arg residue, acetylation was performed by treatment with Ac_2O (10 equiv.) and DIEA (10 equiv.) for 30 min. All washings of the peptidyl-resin between Fmoc removal, amino acid coupling and the final acetylation were done with DMF. Once the fully protected peptide had been synthesized, it was subject to global deprotection (removal of the side-chain protecting groups and cleavage from the resin) by treatment with a mixture of TFA/triisopropylsilane (TIS)/H_2O (95:2.5:2.5) for 4 h. The peptide was isolated by precipitation with cold diethyl ether, centrifuged, dissolved in H_2O/MeCN/TFA (7:2.8:0.2, starting with neat TFA), and sequentially lyophilized. It was then characterized by HPLC [Column I; t_R = 7.29 min, 75% purity, gradient (%B): 5–25 in 15 min] and MALDI-TOF MS showed the mass of the desired peptide {*m*/*z* calcd. for Ac-$(RADA)_4$-NH_2 ($C_{66}H_{113}N_{29}O_{25}$) 1711.8; found 1712.8 $[M + H]^+$}. Other impurities were also detected from the HPLC analysis and characterized by MALDI-TOF MS: the peak that eluted at t_R = 6.88 min (peak area: 6.84% purity) corresponded to a byproduct that lacks the tripeptide RDA {*m*/*z* calcd. for Ac-$(RADA)_4$-NH_2 – RAD ($C_{53}H_{91}N_{23}O_{20}$) 1369.6; found 1370.7 $[M + H]^+$}.

Automated Synthesis: The automated synthesis was conducted on the same resin used for the manual synthesis (aminomethyl-ChemMatrix), with an ABI 433A synthesizer (Applied Biosystems, Foster City), on 0.1 mmol (peptide) scale, following standard Fmoc chemistry and using a 10-fold excess of Fmoc-protected L-amino acids and *N*-[(1*H*-benzotriazol-1-yl)-dimethylamino-methylene]-*N*-methylmethanaminium tetrafluoro-borate *N*-oxide (TBTU, 0.45 M) in the presence of HOBt as coupling reagents in DMF. Each deprotection step was carried out in 15 min and each coupling step in 35 min. Acetylation, cleavage and isolation of the completed peptide were done as described for the manual synthesis. The product

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was characterized by HPLC [Column I, t_R = 7.06 min, 60% purity, gradient (%B): 5–25 in 15 min] and by MALDI-TOF MS, which showed the mass of the desired peptide {*m*/*z* calcd. for Ac-(RADA)$_4$-NH$_2$ ($C_{66}H_{113}N_{29}O_{25}$) 1711.8; found 1712.6 [M + H]$^+$}.

Solid-Phase Fragment Condensation Strategy (4 + 4 + 4 + RADA-resin)

Fragment F1: Fmoc-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH was synthesized manually by using standard Fmoc protocols on 2-chlorotrityl chloride (CTC) resin (1.55 mmol/g, 10 g). The first Fmoc-Ala-OH (1.00 mmol/g resin, scale of synthesis 10 mmol) was incorporated in the presence of DIEA (10 equiv.), which was added in two portions: first, 1/3 of the volume and, after 10 min, the remaining 2/3. The mixture was allowed to react for 1 h. Next, a capping step with MeOH (0.4 mL/g) was done. In each deprotection step, removal of the Fmoc group was performed with piperidine/DMF (1:4) (2 × 2 min, 2 × 10 min). The remaining couplings were done using threefold molar excess of the corresponding Fmoc-amino acid activated essentially in situ by DIPCDI/HOBt in DMF for 3 h. Between coupling and subsequent deprotection steps, the resin was washed with DMF (3 × 3 min), CH_2Cl_2 (1 × 5 min), DMF (1 × 3 min), and CH_2Cl_2 (3 × 3 min), using 10 mL of solvent/g of resin per wash. The complete protected peptide was cleaved from the resin by using TFA/CH_2Cl_2 (1:99) (6 × 3 min), and then collected in H_2O to avoid side-chain deprotection. After evaporation of CH_2Cl_2 and lyophilization, the peptide was characterized by HPLC [Column I, t_R = 9.14 min, 99% purity, gradient (%B): 40–100 in 15 min] and by HPLC/ES-MS, which confirmed the desired product Fragment **F1** {*m*/*z* calcd. for Fmoc-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH ($C_{48}H_{63}N_7O_{12}S$) 961.4; found 962.6 [M + H]$^+$}. This product was sufficiently pure that it did not require any further purification.

Peptide Resin I (RADA-CM Resin): Synthesized on aminomethyl-ChemMatrix resin (0.062 mmol scale) following the same procedure used for the manual synthesis of RADA-16. At the end, an aliquot was taken and treated with TFA/TIS/H_2O (95:2.5:2.5) for 2 h. HPLC/ES-MS confirmed the desired (H-Arg-Ala-Asp-Ala-NH$_2$) product (*m*/*z* calcd. for $C_{16}H_{30}N_8O_6$ 430.2; found 431 [M + H]$^+$).

Peptide Resin II ([RADA]$_2$-CM resin): Fragment **F1** (3 equiv.), which was completely soluble in DMF, was coupled onto peptide resin **I** in the presence of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU) (3 equiv.) and DIEA (6 equiv.) for 1 h. Due to a positive ninhydrin test, a recoupling was performed using fragment **F1** (3 equiv.) and equimolar amounts of DIPCDI/HOAt for 3 h. Complete coupling was confirmed by a negative ninhydrin test. An aliquot was taken and treated with TFA/TIS/H_2O (95:2.5:2.5) for 2 h. HPLC [Column I; t_R = 5.88 min, 89% purity, gradient (%B): 20–80 in 15 min] and HPLC/ES-MS confirmed the desired product (*m*/*z* calcd. for $C_{47}H_{67}N_{15}O_4$ 1065.5; found 1066.1 [M + H]$^+$).

Peptide Resin III ([RADA]$_3$-CM resin): The Fmoc group of peptide resin **II** was removed by treatment with piperidine/DMF (1:4) in the presence of 0.1 M HOBt. Three couplings were required until a negative ninhydrin test was obtained. Fragment **F1** (1.5 equiv.) was incorporated onto peptide resin **II** in three steps: first, using HCTU (1.5 equiv.)/DIEA (3 equiv.) for 1 h; second, with DIPCDI (1.5 equiv.)/HOAt (1.5 equiv.) for 3 h; and finally, with HCTU (1.5 equiv.)/DIEA (3 equiv.) for 1 h. An aliquot was taken and treated with TFA/TIS/H_2O (95:2.5:2.5) for 3 h. HPLC [Column II; t_R = 3.89 min, 98% purity, gradient (%B): 5–100 in 8 min] and MALDI-TOF MS indicated the desired peptide {*m*/*z* calcd. for Fmoc-(RADA)$_3$-NH$_2$ ($C_{63}H_{94}N_{22}O_{20}$) 1478.7; found 1479.8 [M + H]$^+$}.

Ac-(RADA)$_4$-NH$_2$: Fragment **F1** (1.5 equiv.) was coupled onto peptide resin **III** in two steps: first, with HCTU (1.5 equiv.)/DIEA (3 equiv.) for 1 h, which gave a positive ninhydrin test; and then, using DIPCDI/HOAt (1.5 equiv. of each) for 3 h. After Fmoc group removal, the acetylation step was performed by using Ac_2O (10 equiv.)/DIEA (10 equiv.) for 30 min. An aliquot of peptide resin was taken and then treated with TFA/TIS/H_2O (95:2.5:2.5) for 4 h. The HPLC revealed a mixture of products [Column II; main peak t_R = 4.24 min, 41% purity, gradient (%B): 5–25 in 8 min], including the desired peptide, RADA-16. MALDI-TOF MS showed the desired product [*m*/*z* calcd. for Ac-(RADA)$_4$-NH$_2$ ($C_{66}H_{113}N_{29}O_{25}$) 1711.8; found 1712.6 (M + H)$^+$], as well as two incorrect mass signals: one corresponding to Ac-(RADA)$_3$-NH$_2$ (*m*/*z* calcd. for $C_{50}H_{86}N_{22}O_{19}$ 1298.6; found 1299.8 [M + H]$^+$) and another impurity (*m*/*z* found 1671.9 [M + H]$^+$) that corresponds to the nonacetylated peptide [H-(RADA)$_4$-NH$_2$ (*m*/*z* calcd. 1669.8)]. Based on these results, the acetylation treatment was repeated. The peptide-resin was cleaved using TFA/TIS/H_2O (95:2.5:2.5) for 4 h. HPLC results still revealed a mixture of products, but the peak at t_R = 4.94 min had disappeared and the purity of the desired peptide was superior to that previously seen in the aliquot [Column II; main peak t_R = 4.26 min, 46% purity, gradient (%B): 5–25 in 8 min].

Fragment Condensation in Solution (8 + 8): Two protected octapeptides Ac-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH (Fragment **F2**) and H-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-NH$_2$ (Fragment **F3**) were synthesized separately on different resins, and then cleaved from the resin with full preservation of protecting groups.

Fragment F2 [Ac-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH]: Synthesized manually by using standard Fmoc protocols on CTC resin (1.55 mmol/g, 10 g). The first Fmoc-Ala-OH (0.8 mmol/g resin, scale of synthesis 8 mmol) was incorporated using DIEA (10 equiv.) added in two portions: first, 1/3 of the volume and, after 10 min, the remaining 2/3. The mixture was allowed to react for a total of 1 h. Next, a capping step with MeOH (0.4 mL/g) was performed. In all deprotection steps, removal of the Fmoc group was carried out with piperidine/DMF (1:4) in the presence of HOBt 0.1 M (1 × 2 min, 2 × 10 min). The remaining couplings were accomplished by using threefold molar excess of each Fmoc-amino acid activated in situ by DIPCDI/HOBt in DMF for 2 h. Washings between coupling and subsequent deprotection steps were performed with DMF (3 × 3 min), CH_2Cl_2 (1 × 3 min), DMF (1 × 3 min) and CH_2Cl_2 (3 × 3 min), using 10 mL of solvent/g of resin per wash. No positive ninhydrin tests were observed at any point during the synthesis; therefore, no recouplings were performed. Acetylation was accomplished with Ac_2O (10 equiv.)/DIEA (10 equiv.) for 30 min. The protected peptide was cleaved from the resin by using TFA/CH_2Cl_2 (1:99) (8 × 3 min), and then collected in H_2O to avoid side-chain deprotection. After evaporation of CH_2Cl_2, MeCN was added to increase solubility. The product was then lyophilized and characterized by HPLC [Column II; t_R = 5.50 min, 98% purity, gradient (%B): 40–100 in 8 min] and by HPLC/ES-MS, which confirmed the desired product, fragment **F2** (*m*/*z* calcd. for $C_{68}H_{106}N_{14}O_{20}S_2$ 1502.7; found 1504.0 [M + H]$^+$). Owing to the high purity (98%) of the final product, no further purification was performed.

Fragment F3 [H-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-NH$_2$]: Synthesized on Sieber amide resin[41] (loading: 0.53 mmol/g, 15 g), which allows cleavage of protected peptides from the resin using 3% TFA. In each deprotection step, removal

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of the Fmoc group was accomplished with piperidine/DMF (1:4) in the presence of HOBt 0.1 M (1 × 2 min, 2 × 10 min). The couplings were done using a threefold molar excess of each Fmoc-amino acid activated by DIPCDI/HOBt (3 equiv. each) in DMF for 2 h. Only one recoupling was required: Arg[1] (recoupled using the same coupling reagents in half-molar quantity for 2 h). The protected peptide was cleaved from the resin by using TFA/CH_2Cl_2 (3:97) (3 × 3 min), and then H_2O was added. After evaporation of CH_2Cl_2, MeCN was added to increase the solubility of the protected peptide. The product was lyophilized and then characterized by HPLC [Column II; t_R = 3.21 min, 97% purity, gradient (%B): 40–100 in 8 min] and by HPLC/ES-MS, which confirmed the desired structure fragment **F3** (*m/z* calcd. for $C_{66}H_{105}N_{15}O_{18}S_2$ 1459.7; found 1462 $[M + H]^+$, 731 $[M + 2H]^+/2$). Due to the excellent purity of the product, no further purification was required.

Fragment Condensation in Solution: The coupling was carried out on 0.2 mmol scale. Thus, Fragment **F2** (0.2 mmol) was dissolved in DMF (30 mL). To the solution were added DIEA (2 mmol), then, added dropwise over 30 min, a solution of 1-benzotriazole-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)/HOAt (0.2 mmol each) in DMF (43 mL). Fragment **F3** (0.2 mmol) was dissolved in DMF (30 mL) and added slowly to the solution. The reaction mixture was left at room temperature for 16 h and monitored by HPLC. The DMF was then evaporated in the presence of toluene and the product was precipitated by adding cold *tert*-butyl methyl ether (3 times). The precipitate was dissolved in MeCN/H_2O (1:1), with sonication (3 min), and then lyophilized. This process (from the addition of cold *tert*-butyl methyl ether up to the lyophilization) was then repeated twice. The side-chain protecting groups were removed by treatment with TFA/TIS/H_2O (95:2.5:2.5) for 4 h. The peptide was precipitated by adding cold diethyl ether, centrifuged, washed with diethyl ether, dissolved in MeCN/H_2O (1:1), and then lyophilized. The product was characterized by HPLC, which exhibited one main peak (Column II; t_R = 4.56 min, 58% purity, gradient (%B): 5–25 in 8 min), and by MALDI-TOF MS, which confirmed the desired product, RADA-16.

To isolate the desired product and remove any remaining coupling reagents or by-products, the following work-up was performed. Water (0.75 mL) was added to a part of the reaction mixture (200 mg) in DMF (1.5 mL), and a precipitate appeared. The upper phase was extracted three times with $CHCl_3$, and the combined upper phases together with the pellet/emulsion were centrifuged (4000 rpm for 8 min at 4 °C) to isolate the solid and to remove the upper phase. The solid material was washed with cold diethyl ether, and then centrifuged twice. The precipitate was then dissolved in MeCN/H_2O (1:1) and lyophilized. It was then characterized by HPLC, which exhibited a main peak (Column II; t_R = 4.41 min, 90% purity, gradient (%B): 5–25 in 8 min). Aliquots from different phases during the work-up were taken ($CHCl_3$ phase, emulsion, upper phase), and then characterized by HPLC.

Supporting Information (see footnote on the first page of this article): An accurate HPLC analysis of six RADA-16 materials provided by different chemical suppliers has been performed.

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