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Multigram-Scale Synthesis of Short Peptides via a Simplified Repetitive Solution-Phase Procedure

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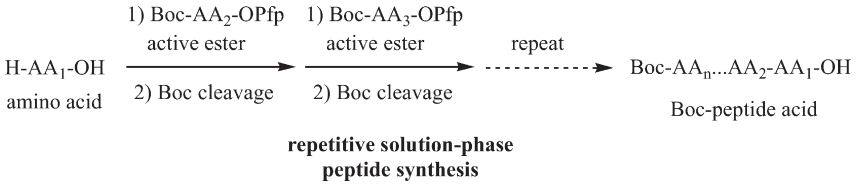

A rapid repetitive solution-phase synthesis of peptides is described. The procedure involves coupling of amino acids and peptide acids, instead of the usual amino esters and peptide esters, to slight excesses of pentafluorophenyl active esters in a THF/water solvent mixture. Due to their poor solubility, peptide acid intermediates are easily isolated in high purity by acidification under controlled conditions and removal of excess active esters by selective extraction. Contrary to modern repetitive solution-phase peptide synthesis procedures, our approach does not require time-consuming neutralization reactions and reduces significantly the number of operation units that are necessary to obtain peptide intermediates. Efficiency of the method was demonstrated by the rapid synthesis of short hydrophobic and hydrophilic peptides, the antimalarial cycloheptapeptide mahafacyclin B, and a protected form of the hydrophilic pentapeptide GRGDS.

Introduction

The development of rapid and efficient synthesis protocols has been a key factor in allowing the pharmaceutical potential of peptides to be realized. Development has proceeded such that in 2004 there were 40 peptide based drugs on the market with a further 200 in clinical phases.[1]

Peptide drugs possess a significant advantage over other small synthetic molecules as they are usually highly potent and decompose into safe metabolites in vivo. Recent advances in synthetic and drug delivery technologies have proved critical to lowering their production cost and counteracting their poor oral bioavailability.[2] Today, there are three distinct chemical methods used for the manufacture of peptides. The "classical solution-phase peptide synthesis" (CSPS) involves the use of optimized coupling reactions and purification techniques for each step. Consequently, development of synthetic routes for new peptides and the subsequent scale-up can be a very time-consuming process. For instance, the industrial production of the nine-residue peptide Atosiban, an antagonist of the oxytocin receptor, utilizes five different coupling methods.[3] The second synthetic method, Merrifield "solid-phase peptide synthesis" (SPPS), is the fastest way of producing peptides.[4] However, when applied to the production of multiton quantities the method is costly and generates large amounts of chemical waste.[5] Recently, SPPS was used in combination with CSPS for the large-scale production of Fuzeon, a 36-amino acid long peptide that inhibits cell infection by the HIV virus. The peptide fragments were produced on solid phase and subsequently assembled in solution.[6] The third synthetic method known as "repetitive (or continuous) solution-phase peptide

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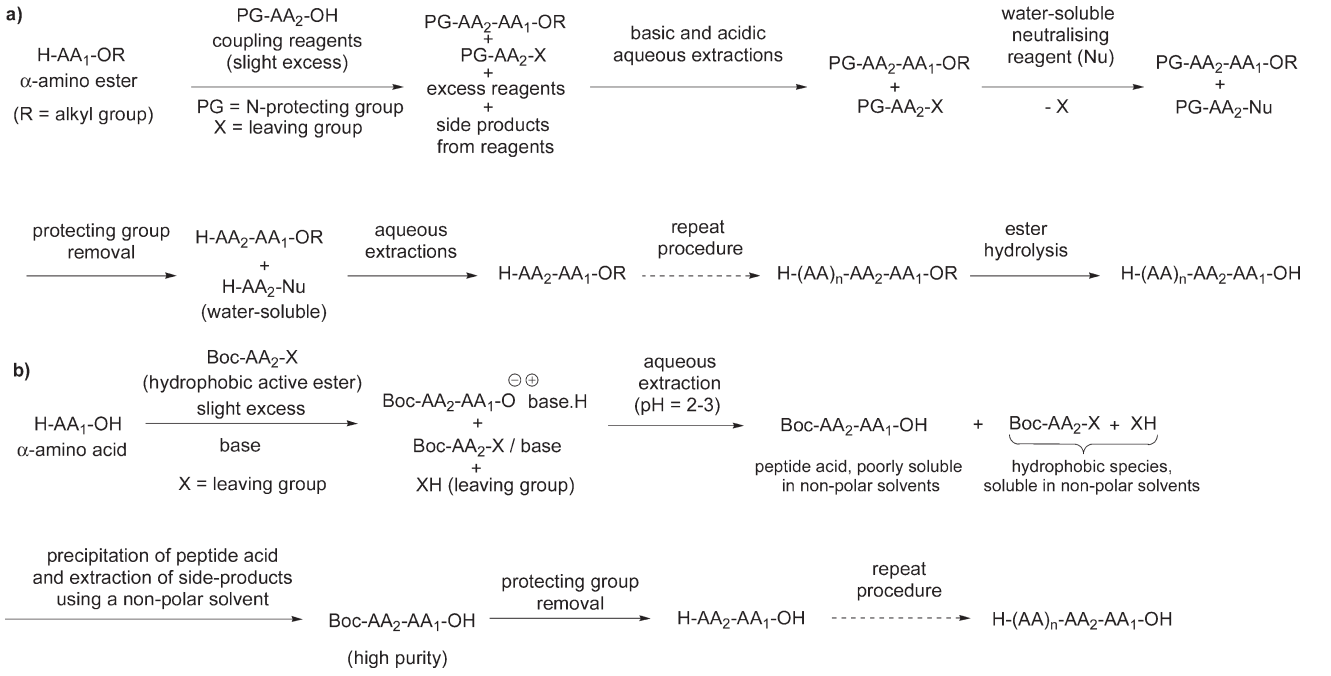
SCHEME 1. Synthesis of Peptides by (a) Typical Repetitive Solution-Phase Peptide Synthesis (RSPS); (b) Proposed Simplified Procedure



synthesis" (RSPS) is a methodology that aims to produce peptides via a repetitive procedure without the need for purification of intermediates.[7] The advantage of this approach is that the same coupling and isolation procedures are repetitively used, thus simplifying peptide production (Scheme 1a). One of the key features of this method is the utilization of superstoichiometric amounts of activated amino acids to drive reactions to completion, an approach similar to that utilized in SPPS. However, unlike SPPS, excesses cannot be removed by simple washing and filtration procedures. Instead, water-soluble nucleophilic reagents are added to the reaction mixture to neutralize the excess of activated species, after which the byproducts are removed by acidic and basic aqueous extractions. The *N*-protecting group is subsequently cleaved, and the pure deprotected peptide obtained after additional aqueous washings. The coupling cycles are repeated until the desired peptide sequence has been reached. Recently, some of these techniques have been automated, most notably the DioRaSSP procedure as reported by Eggen et al.[8] In comparison to SPPS, RSPS procedures use significantly lower quantities of expensive coupling reagents and produce less waste, which is particularly advantageous for synthesis on a larger scale. Additionally, RSPS techniques are less likely to lead to truncated peptides when synthesizing challenging sequences, but insertion sequences may arise if removal of activated amino acids after the coupling step is incomplete.

In this paper, we describe a simplified repetitive solution-phase synthesis of peptides (RSPS) using Boc-protected pentafluorophenyl esters of amino acids as the activated species for coupling reactions. The new method is illustrated by the synthesis of small hydrophobic and hydrophilic peptides and mahafacyclin B, a natural cycloheptapeptide with antimalarial properties.[9]

Methodology

The main weaknesses of RSPS are 2-fold: (1) additional reactions are necessary to neutralize the excess of coupling reagent employed and (2) several acidic and basic aqueous extractions are required to isolate the amino peptide esters in high purity (Scheme 1a). These postsynthetic treatments are time-consuming and undesirable. Our new procedure aims to decrease significantly the number of unit operations that are necessary for each coupling cycle, providing a rapid and facile method of peptide synthesis.

A major problem in repetitive methods is the separation of the peptide products from the excess of activated amino acids and byproduct. We envisioned that this problem could be easily solved if we used hydrophobic active esters and produced hydrophilic peptide products (Scheme 1b). This way, excess active esters would remain soluble in nonpolar solvents but the peptide products would easily precipitate. Boc-protected pentafluorophenyl esters seemed the ideal candidate for use as the activated amino acid component, because they are hydrophobic, easily accessible in pure form,

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SCHEME 2

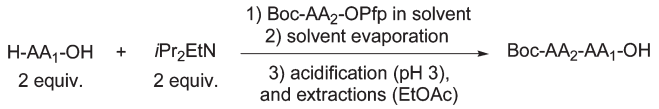


TABLE 1. Reaction Times and Yields of Peptides Synthesized under Varying Solvent Conditions

entry	product	solvent	reaction time (h)	yield[a] (%)
1	Boc-L-Phe-L-Phe-OH, **1**	DCM	5	92
2	Boc-L-Phe-Gly-OH, **2**	DCM	10	89
3	Boc-L-Val-L-Thr-OH, **3**	DCM	24	traces
4	Boc-L-Phe-L-Phe-OH, **1**	THF/H_2O	2	97
5	Boc-L-Phe-Gly-OH, **2**	THF/H_2O	1.5	98
6	Boc-L-Phe-L-Thr-OH, **4**	THF/H_2O	2	92
7	Boc-L-Ser(Bzl)-Gly-OH, **5**	THF/H_2O	2	94
8	Boc-L-Cys(Bzl)-Gly-OH, **6**	THF/H_2O	2	93
9	Boc-L-Val-L-Thr-OH, **3**	THF/H_2O	6	84

[a]Epimerization level was $<99.9\%$ as demonstrated by chiral HPLC.

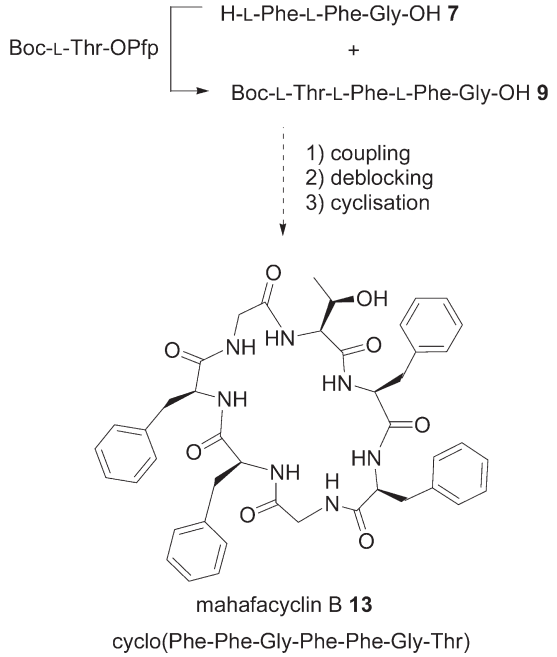
stable to storage and known to rapidly undergo aminolyzes with amino acids to yield epimerization-free peptide products.[10,11] Reacting pentafluorophenyl esters with free amino acids or side-chain protected peptide acids would produce Boc-protected peptide acid products, which should be hydrophilic enough to be poorly soluble in nonpolar organic solvents like petroleum ether or diethyl ether. Excess active esters and the hydrophobic byproduct could be then easily removed from the precipitated peptide acid product. The coupling cycle would be repeated after removal of the Boc-protecting group until the desired peptide sequence has been obtained.

Results and Discussion

Synthesis of Boc-Protected Dipeptide Acids/Optimization of the Method. The coupling conditions were optimized for the synthesis of a series of dipeptides (Scheme 2, Table 1). The Boc-protected active esters were readily obtained by coupling the corresponding Boc-amino acids with pentafluorophenol (PfpOH) in the presence of 1-[3[(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC·HCl).[12] We found that pure active esters could be readily purified in excellent yields by filtration of the crude mixture containing the urea byproduct through a short pad of silica gel. The pentafluorophenyl esters were then reacted with a variety of amino acids, and the reactions were monitored by thin layer chromatography. In these experiments, substoichiometric amounts of active esters were used as excesses of amino acids would be easily removed by aqueous extraction.

Amino acids are poorly soluble in organic solvents, so one would expect coupling reactions to be very slow or non existent. To our surprise, Boc-L-Phe-OPfp was found to react with phenylalanine (suspension) in dichloromethane (DCM) in the presence of Hünig's base, and the reaction reached completion after 5 h stirring at room temperature. Excess amino acid and protonated base were conveniently removed by washing the organic layer with a 10% aqueous solution of citric acid (pH 3). The organic solvent was dried and evaporated to give the crude peptide product, contaminated only by pentafluorophenol. While the dipeptide product is insoluble in petroleum ether, pentafluorophenol is soluble, thus allowing its facile removal after three sonication extractions with this solvent. Ultrasonic-assisted extraction (USE) has been shown to be an effective method for analytical extractions; hence, it is used here to facilitate sample purification.[13] Samples were sonicated by indirect sonication using a sonic bath. The product was isolated in 92% yield in high purity (entry 1). Importantly, NMR and HPLC analyses indicated that no epimerization had taken place during coupling. The encouraging results of this initial experiment were short-lived: reaction of the same active ester with glycine required 10 h reaction to reach completion (entry 2), and almost no reaction was observed when the more hydrophilic (and therefore less soluble) amino acid threonine was used (entry 3). Attempts to increase the amino acid solubility and reaction rates in various polar solvents did not give satisfactory results. In a final attempt, the reaction was carried out in a THF−water (~2:1) solvent mixture.[14] In this homogeneous system, the amino acid and active ester are highly soluble, even in the presence of the Hünig's base. To our satisfaction, coupling reactions are much faster than hydrolysis of active esters; hence, a series of dipeptide products could be prepared in high yields

SCHEME 3. Approach for the Synthesis of Mahafacyclin B



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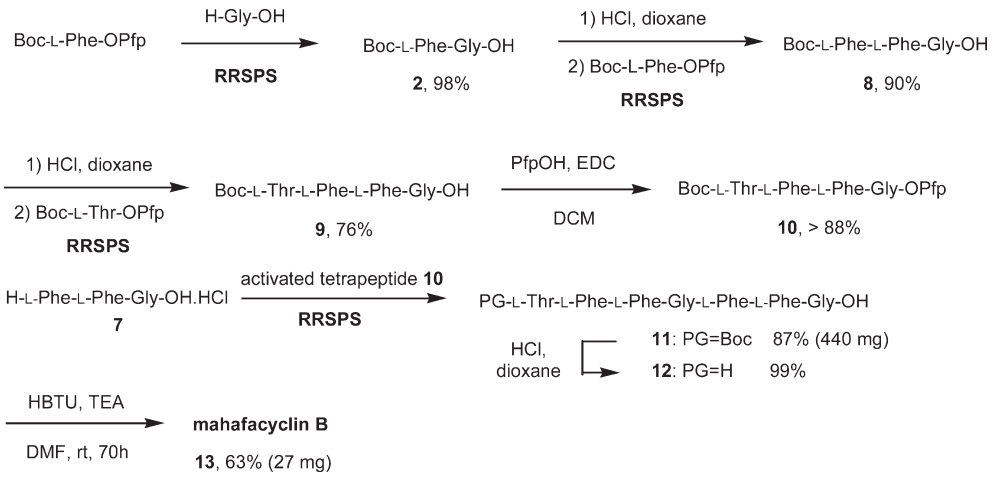
SCHEME 4. Synthesis of Mahafacyclin B through Rapid Repetitive Solution-Phase Peptide Synthesis



and excellent purity in significantly shorter reaction times (Table 1, entries 4−9). Reactions of active esters of Boc-amino acids with high susceptibility to racemization[15] afforded the corresponding dipeptides in good yields without stereomutation (entries 7 and 8). In contrast to earlier reactions in DCM, both hydrophilic and hydrophobic amino acids reacted equally well in this solvent system (entries 4 and 9).

Total Synthesis of Mahafacyclin B. The results above allowed us to design a new rapid repetitive solution-phase synthesis of peptides (RRSPS). In this new methodology, Boc-protected dipeptide acids are synthesized as before using a superstoichiometric amount of free amino acid to drive the coupling reactions to completion. Extension of the peptide sequence then proceeds by reaction of the deblocked dipeptide with an excess of activated Boc-amino acid in a THF−water mixture. After evaporation of the organic solvent and acidification of the aqueous solution, the tripeptide acid product is precipitated during ultrasound-assisted extraction of the remaining active ester. The procedure is repeated until the desired peptide sequence is obtained.

The versatility and simplicity of the method was demonstrated by the synthesis of the bioactive cycloheptapeptide mahafacyclin B (Scheme 3). The natural cyclopeptide contains two consecutive Phe-Phe-Gly fragments followed by a threonine residue. We decided to synthesize the heptapeptide precursor **12** (H-Thr-Phe-Phe-Gly-Phe-Phe-Gly-OH) via fragment condensation of tripeptide H-Phe-Phe-Gly **7**, with Boc-protected tetrapeptide **9**, which was to be obtained by coupling of a threonine residue to **7**. The peptide fragments were synthesized using the new RRSPS (Scheme 4).

Dipeptide acid **2** was obtained in 98% yield by reaction of glycine with Boc-Phe-OPfp. Deblocking of the Boc group using HCl 4 M in dioxane followed by reaction with excess of the same active ester afforded Boc-protected tripeptide **8** in 90% yield after ultrasound-assisted extraction with petroleum ether. The Boc group was then cleaved and the resulting peptide reacted with activated Boc-threonine to produce **9** in 76% yield. The lower yield obtained in this coupling is explained by the fact that a more polar petroleum ether/ethyl acetate solvent system was required for the ultrasound extractions of Boc-Thr-OPfp, because we chose to use it without protecting the alcohol side chain. This should not be necessary if the side chains of polar amino acids were protected with hydrophobic groups. This peptide was subsequently activated using EDC·HCl and PfpOH and coupled to the deblocked tripeptide **7** in THF−water to produce 87% of Boc-protected heptapeptide **11**. Reversed-phase HPLC and NMR analysis of the deblocked heptapeptide **12** (Figure 1) showed that even after four RRSPS cycles no purification was required. Finally, mahafacyclin B was obtained in 63% yield (27 mg, 24% overall yield) after purification by preparative HPLC by cyclization using HBTU in the presence of triethylamine in DMF. NMR spectra of the cyclopeptide were identical to those described for the natural product (see Supporting Information).[16]

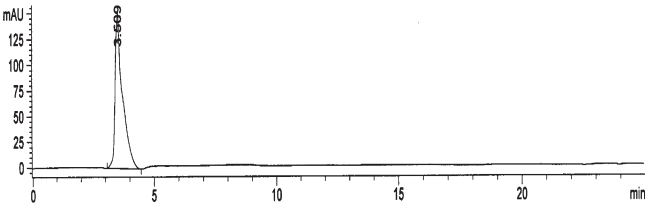


FIGURE 1. HPLC analysis (reversed-phase column) of crude heptapeptide **12**, synthesized by RRSPS.

Multigram-Scale Synthesis of a Protected Form of the Hydrophilic Pentapeptide GRGDS. Having successfully prepared short hydrophobic peptides by RRSPS, we undertook the multigram-scale synthesis of the Boc-protected hydrophilic peptide model Boc-Gly-L-Arg(Cbz)$_2$-Gly-L-Asp(Bzl)-L-Ser(Bzl)-OH (BocGRGDS, **14**), for which the side chain of the arginine (R) residue is protected by Cbz groups, and the aspartic acid (D) and serine (S) residues are protected with benzyl groups (Scheme 2).[17]

The synthesis started with the hydrochloride salt of the benzyl protected serine **15** and the desired pentapeptide sequence BocGRGDS **14** was obtained in over 90% purity (Figure 2) after four cycles of RRSPS using only 1.1 equiv of appropriate pentafluorophenyl amino esters (Scheme 5). The

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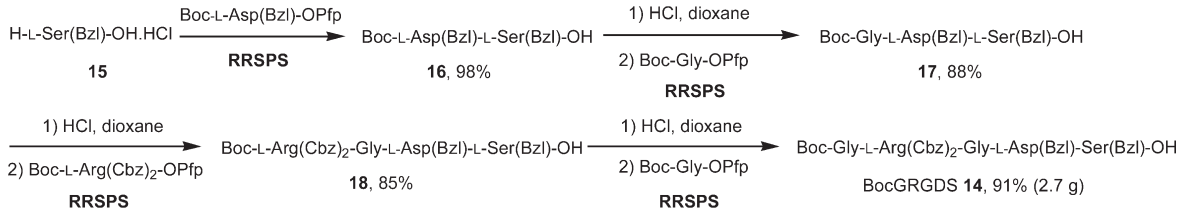
SCHEME 5. Synthesis of BocGRGDS 14 through Rapid Repetitive Solution-Phase Peptide Synthesis



yields for each coupling step were in excess of 85%, and the protected pentapeptide was obtained in 67% overall yield (nonoptimized) after 7 steps.

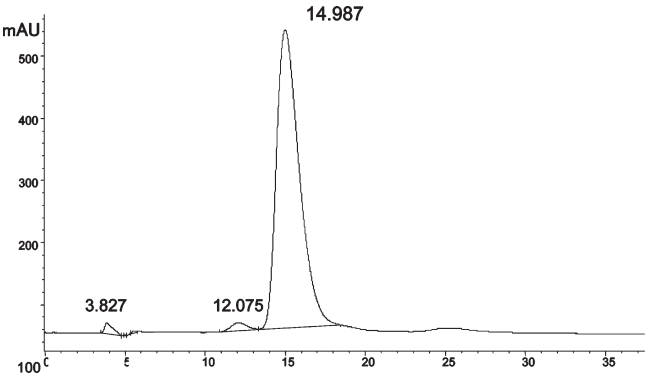


FIGURE 2. HPLC analysis (chiral column) of crude pentapeptide BocGRGDS **14**, synthesized by RRSPS.

Conclusions

We have developed an efficient and rapid method for solution-phase synthesis of short peptides. Compared to other existing solution-phase methods, our approach has the advantage of not requiring fastidious and time-consuming neutralization and washing steps. The THF−water solvent system utilized for coupling reactions seems advantageous over most nonpolar and polar solvents used in peptide synthesis as peptides appeared very well soluble, and THF is easily removed by rotary evaporation. In this study, peptides were produced in good to excellent yields on a multigram-scale. Vitally, the repetitive method was shown to be epimerization-free and provide peptide intermediates of high purity. The method proved applicable to the synthesis of both hydrophobic and hydrophilic peptides sequences, as demonstrated by the rapid synthesis of the cyclic heptapeptide mahafacyclin B and a protected form of the pentapeptide GRGDS.

Experimental Section

Chiral HPLC was performed using a cellulose tris-3,5-dimethylphenyl carbamate-coated column (4.6 mm ID, 250 mm) with a flow rate of 1 mL/min, using a 0.1% TFA + 10% isopropanol in hexane solvent system for Boc-protected dipeptides and a 0.1% TFA + 50% isopropanol in hexane solvent system for Boc-GRGDS pentapeptide **14**. Analytical reversed-phase HPLC was performed using a C18 column (4.6 mm ID, 150 mm) with a flow rate of 0.8 mL/min. Semipreparative reversed-phase HPLC was performed using a C18 column (10 mm ID, 250 mm) with a flow rate of 1.3 mL/min.

NMR spectra were recorded on 250 and 400 MHz spectrometers. Melting points are uncorrected and reported in degrees Celsius. Ultrasound-assisted extraction was performed in an ultrasound cleaning bath by indirect sonication at the frequency of 40 kHz. Reactions were monitored by thin-layer chromatography using a mixture of 1% acetic acid in ethyl acetate.

Abbreviations: PfpOH, pentafluorophenol; EDC·HCl, 1-[3-[(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; DIEA, *N*,*N*-diisopropylethylamine; HBTU, (*O*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′- tetramethyluronium hexafluorophosphate; Bzl, benzyl; Cbz, carboxybenzyl; USE, ultrasonic-assisted extraction; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; RRSPS, rapid repetitive solution-phase peptide synthesis.

Synthesis of Pentafluorophenyl Esters (Procedure A). PfpOH (1 equiv) was added to a stirred solution of Boc-amino acid/peptide in dichloromethane (0.2 M) followed by EDC·HCl (1.2 equiv). After 2 h, silica (10 times the mass of EDC·HCl used) was added to the solution, and the resulting suspension was filtered over a bed of silica and Celite. The solvent was removed under reduced pressure and the crude pentafluorophenyl ester used directly in the next step.

Synthesis of Boc-AA_2-AA_1-OH (Procedure B). Boc-AA_2-OPfp (1 equiv) in THF (0.2 M) was added to an aqueous solution (1 M) of AA_1 (2 equiv) with constant stirring. DIEA (2 equiv) was then added, and the reaction left to proceed. Once the reaction had reached completion (TLC) the THF was removed under reduced pressure, the aqueous mixture acidified to pH = 3 using a 10% solution of citric acid and extracted with ethyl acetate. The combined extracts were dried ($MgSO_4$) and the solvent evaporated under reduce pressure. The crude was subjected to USE with petroleum ether or diethyl ether to give the desired dipeptide.

Synthesis of Mahafacyclin B 13 and Boc-Gly-L-Arg(Cbz)$_2$-Gly-L-Asp(Bzl)-Gly-L-Ser(Bzl)-OH 14

Boc Deprotection (Procedure C). Typically, Boc-peptide (1.2 mmol) was added to an ice-cold solution of 4 M HCl in dioxane (10 mL). The reaction mixture was allowed to warm to room temperature and stirred for 2 h. The solvent was removed in vacuo and the resulting ammonium chloride salt was washed with dry diethyl ether (3 × 15 mL) and collected by filtration. The deblocked peptide was used in the coupling step without further purification.

Coupling Protocol (Procedure D). A solution (0.1 M) of the Boc-protected amino acid (or peptide fragment) pentafluorophenyl ester (1.1−1.5 equiv) in THF was added to an aqueous solution (or suspension; 0.2 M) of deblocked peptide (1 equiv) containing 2−2.1 equiv of DIEA (pH aqueous solution = 8.5−9), resulting in a homogeneous mixture. The progress of the reaction was monitored by TLC (silica gel). Once the reaction had reached completion (ca. 3 h), the THF was removed in vacuo, and the aqueous mixture acidified to pH = 3 using a 10% citric acid solution resulting in the precipitation of the Boc-protected peptide. The product was extracted three times with ethyl acetate, the combined extracts were dried over $MgSO_4$, and the solvent was evaporated under reduce pressure. The product, contaminated by PfpOH and the excess active ester, was subjected to USE with petroleum ether or diethyl ether (3×) to give the purified Boc-protected peptide acid, which was collected by filtration.

Protocols C and D were repeated until the desired peptide sequence had been reached.

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Supporting Information Available: Experimental details and analyses for all peptides. This material is available free of charge via the Internet at http://pubs.acs.org.