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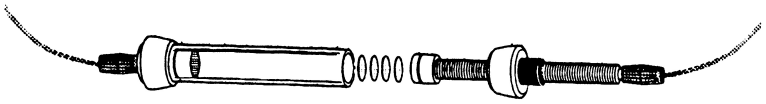
J. Comb. Chem., **2003**, 5 (1), 1-7• DOI: 10.1021/cc0200078 • Publication Date (Web): 19 October 2002



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JOURNAL OF combinatorial CHEMISTRY

Volume 5, Number 1 January/February 2003

Articles

A Simple and Effective Method for Producing Nonrandom Peptide Libraries Using Cotton as a Carrier in Continuous Flow Peptide Synthesizers

Felikss Mutulis, Marcus Tysk, Ilze Mutule, and Jarl E. S. Wikberg*

Department of Pharmaceutical Biosciences, Division of Pharmacology, Uppsala University, Box 591, Biomedicum, SE-751 24 Uppsala, Sweden

Received February 12, 2002

A method has been developed for generating nonrandom peptide libraries on cotton. Disks of cotton fabric were chemically modified to enable peptide synthesis. Incorporation of a 6-aminocaproic acid residue handle on the cellulose turned out to be advantageous. Disks were labeled with silver ink, stacked one on top of another in a continuous flow peptide synthesizer column, and simultaneously subjected to automated synthesis procedures. Depending on the sequences to be synthesized, the automatic synthesis procedure was stopped, and the disks were removed from the column, sorted, and reapplied to subsequent synthesis steps. In this way, individual peptides could be easily prepared in milligram quantities on each of the cotton disks.

Introduction

Since Merrifield's pioneering work on solid-phase peptide synthesis,[1] numerous methods have been developed to accelerate the synthesis of peptide libraries. Different carrier materials and methodologies have been introduced to create high-yield peptide libraries while minimizing the number of coupling steps and increasing the speed of synthesis. Among them, peptide synthesis on cellulose (paper or cotton) supports has been used (for review, see e.g., Terrett, Hudson,[2] and Lebl[3]). Paper support was actually first introduced in the preparation of multiple oligonucleotides.[4,5] Soon after, Frank and Döring reported simultaneous multiple peptide synthesis on paper disks using a homemade semiautomatic continuous flow peptide synthesizer.[6] A similar approach was used by Krchòák et al. for the synthesis of paper-bound peptides for ELISA screening.[7] An alternative cellulose material, namely cotton, received significant attention for solid-phase peptide synthesis about 10 years ago.[8−12] A few articles were also published more recently on the matter.[13] Cotton shows a number of unique features that make it suitable for combinatorial peptide synthesis, such as easy handling, low cost, relative high substitution degree, and good mechanical stability.

Because of our needs of access to multiple peptide synthesis, we decided to develop a modified Frank and Döring method using a commercial peptide synthesizer and cotton as the carrier, and we here report our experience with the approach.

Results and Discussion

Our approach is illustrated by the flowchart shown in Figure 1. To test it, we used a synthesis object consisting of a small library of 16 hexapeptides (structures shown in Table

* Corresponding author. Phone: +46-18-471 42 38. Fax: +46-18-55 97 18. E-mail: Jarl.Wikberg@farmbio.uu.se.

10.1021/cc0200078 CCC: $25.00
Published on Web 10/19/2002

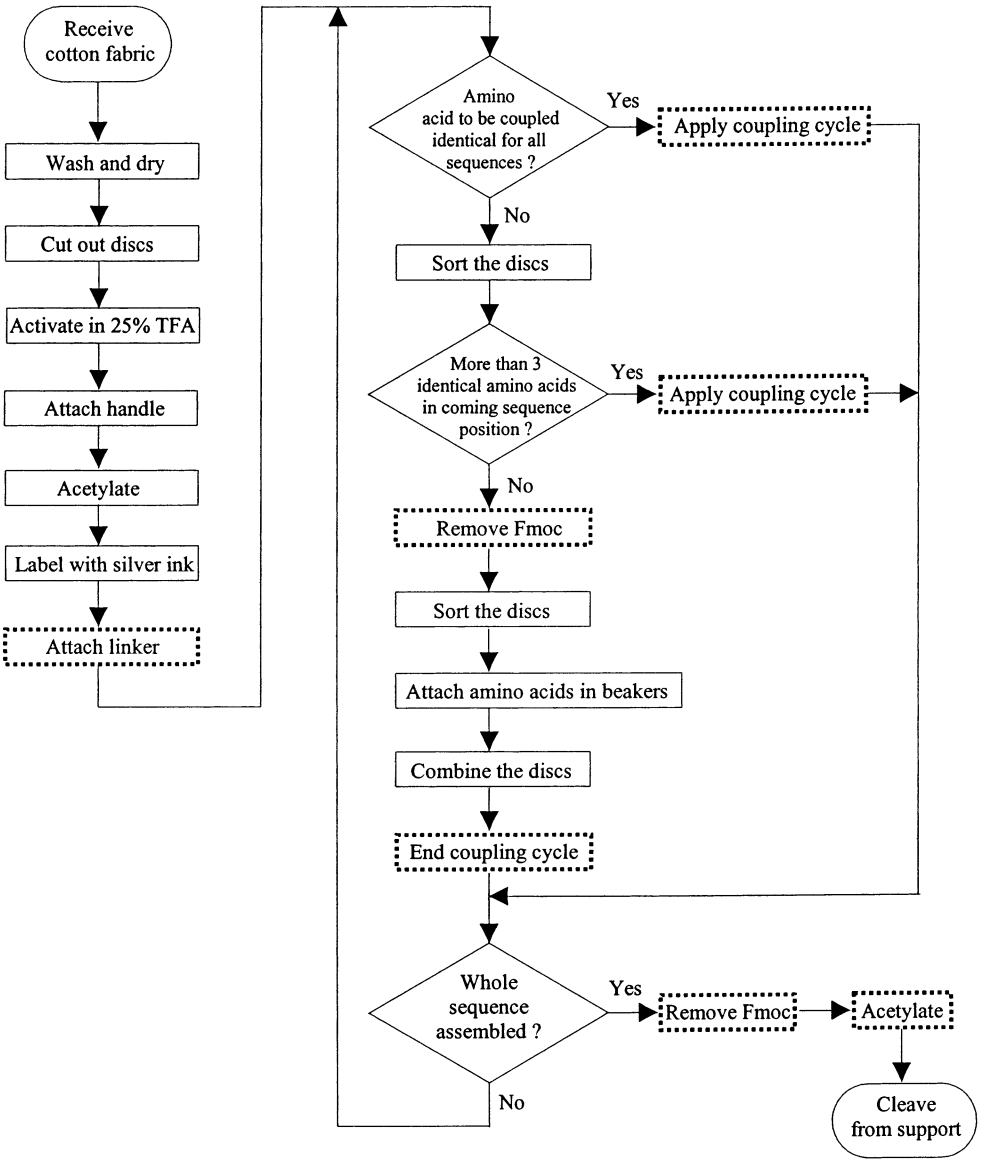

Figure 1. Sequence of actions for preparation of a peptide library on cotton. Synthesizer operations are indicated by dashed boxes.

1). Synthesis of it started by preactivation of the cotton with 25% trifluoroacetic acid (TFA) in CH_2Cl_2. To enable peptide synthesis, a handle and a linker had to be attached to the cotton (Figure 2). In previous investigations, Fmoc−glycine was used to introduce a handle on the cotton.[8−13] Here, we investigated the use of the corresponding derivative of 6-aminocaproic acid (Fmoc-ϵAhx-OH), expecting that the increased flexibility and presumed larger distance between the nitrogen atom and the carbonyl group in the handle could lead to better access for reagent molecules and higher reaction yields. Indeed, we found that a higher degree of substitution could be obtained by the use of derivatives of 6-aminocaproic acid (140 μmol/g cotton compared to 98 μmol/g for glycine; see Experimental Section for details). The residual hydroxyl groups of the cellulose were subsequently blocked by acetylation [i.e., disks were soaked in a solution of acetic anhydride (Ac_2O) and *N*-methylimidazole in DMF].

The disks were then labeled with silver ink for easy identification, and all the 16 disks were placed into the continuous flow peptide synthesizer column, stacking them one on the top of the other (Figure 3). The subsequent automated synthesis procedures were based on Fmoc chemistry, including amino acid derivative activation by *O*-(7-azabenzotriazol-1-yl)-1.1.3.3-tetramethyluronium hexafluorophosphate (HATU) (except for attachment of cysteine, where pentafluorophenyl ester and HOAt were used).

Synthesis started by addition of Fmoc Rink linker, followed by acetylation of the residual amino groups of the handle. Thereby, structure **2** (see Figure 2) was generated. For monitoring the completeness of the couplings, we tried to use the bromophenol blue test, as suggested previously,[8,10,13] but in our hands, this approach failed as even untreated cotton turned blue. Obviously, the fabrics that we used differed from those used in previous studies. Anyhow, 4 h of coupling time seemed to be reasonable for the attachment of the linker, followed by 60 min for coupling of the first amino acid. For the subsequent steps, the coupling time was set to 30 min. The automated process for all 16 disks was then continued to attach the C-terminal dipeptide,

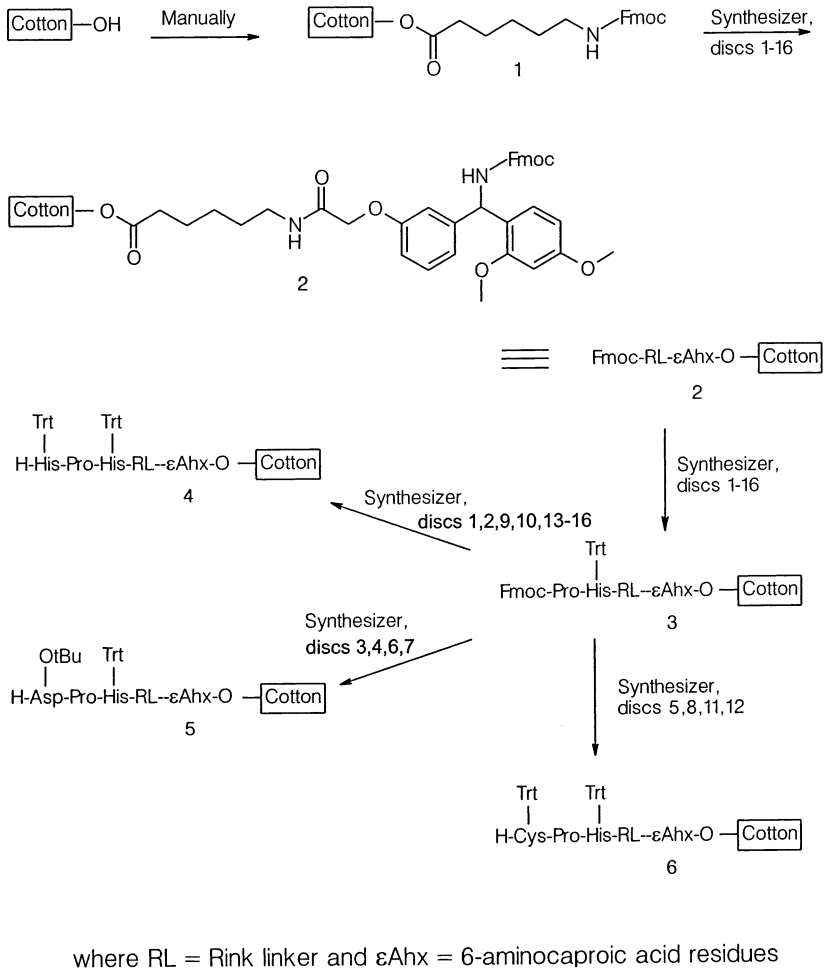

Figure 2. Addition of 6-aminocaproic acid, Rink linker, and the first three amino acids.

Table 1. The 16 Peptides of the Hexapeptide Library Synthesized in This Study

peptide no.	sequence	mass spectrometry ($M + H^+$, m/z)	HPLC (retention time, 45-min run, min)
1	Ac-TYMHPH-NH_2	826.4	37.7
2	Ac-THAHPH-NH_2	740.4	17.6
3	Ac-RYCDPH-NH_2	831.4	32.8
4	Ac-RHSDPH-NH_2	789.4	16.1
5	Ac-PYVCPH-NH_2	756.4	17.9[a]
6	Ac-PYCDPH-NH_2	772.4	38.7
7	Ac-PHPDPH-NH_2	740.4	25.5
8	Ac-PHLCPH-NH_2	744.4	18.2[a]
9	Ac-MYTHPH-NH_2	826.4	36.1
10	Ac-MHAHPH-NH_2	770.4	26.7
11	Ac-EYTCPH-NH_2	790.4	34.4
12	Ac-EHTCPH-NH_2	764.4	22.5
13	Ac-CYCHPH-NH_2	800.4	35.5
14	Ac-CHAHPH-NH_2	742.4	20.7
15	Ac-AYMHPH-NH_2	796.4	38.5
16	Ac-AHPHPH-NH_2	736.4	20.2

[a] 30-min run.

which in our case was identical for all peptides (see Table 1).

In the following synthesis cycle, three different amino acids—histidine, cysteine and aspartic acid—had to be added. This was achieved by removing the disks from the column, sorting them, and then placing them back into the column for three short consecutive runs. In each case, addition of

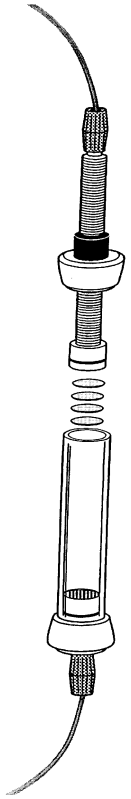

Figure 3. Exploded view of the cotton disks stacked in the synthesizer column.

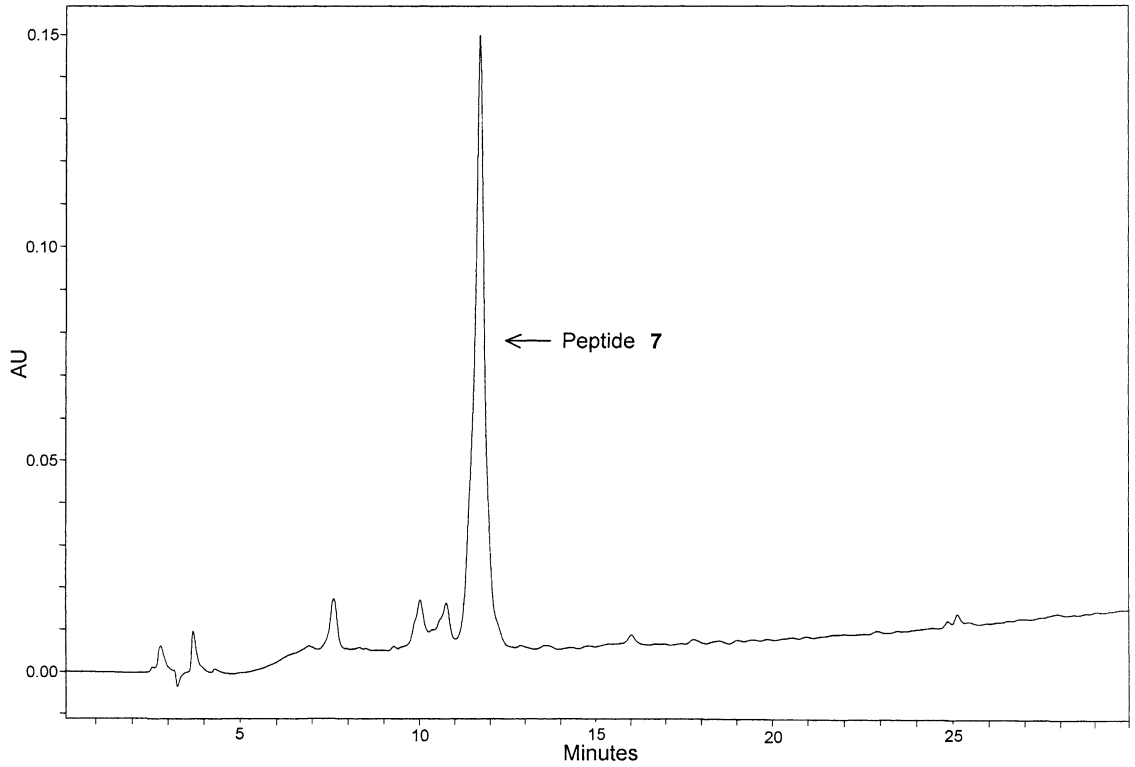

Figure 4. RP-HPLC analysis of peptide **7** synthesis product. Conditions set as described in the text.

the corresponding amino acid, capping, and Fmoc removal were performed in an automated fashion. This resulted in products **4**, **5**, and **6** (Figure 2).

Addition at the fourth position involved many different amino acids. This coupling step was therefore performed manually by placing 1−3 cotton disks in eight glass beakers. Disks to which the same amino acid had to be attached were placed together. Coupling was then achieved by shaking the disks in a DMF solution containing 3-fold excess of the corresponding amino acid derivative, 3-fold excess of HATU, and 6-fold excess of diisopropylethylamine (DIPEA). Disks were then sorted, and the fifth amino acid was attached in two synthesizer runs. Both of these automated synthesis cycles included cleavage of the Fmoc group, addition of the protected amino acid followed by acetylation of the residual amino groups, and repeated cleavage of the Fmoc group. For the attachment of the sixth amino acid, the couplings were again performed manually, each with two or three disks. Thereafter, all 16 disks were placed in the synthesizer column for the final Fmoc removal and acetylation of the cotton-attached peptide chains. The disks were also washed according to the standard synthesizer program and then removed and dried in a vacuum. During the entire above synthesis process, the silver ink labels could be clearly seen. After drying, treatment with trifluoroacetic acid and additives for cleavage from cotton and simultaneous side chain deprotection followed. The reaction mixture was filtered to remove the cotton, the filtrate was evaporated, and the residue was treated with anhydrous ether. Precipitates formed were filtered off and dried.

Isolated end products were of satisfactory quality for screening studies (Figure 4) and showed the expected *m*/*z* of protonated molecular ions and HPLC retention times (Table 1). Approximately 3−4 mg (yield ∼30% calculating from capacity of cotton with attached handle) of peptide could be obtained from each disk. No silver ions that could possibly have originated from the ink could be detected in the synthesized peptides (i.e., hydrogen sulfide applied to probes of products did not produce any black precipitate).

Our data thus show that simultaneous multiple peptide synthesis can be performed on cotton disks using continuous flow synthesizers, which were originally designed for synthesis of single peptides on resin. Until the cleavage step, each disk contained one unique peptide (one disc, one peptide). Such disks can be stored, small portions of peptidyl-cotton can be cut out by scissors and cleaved by trifluoroacetic acid and additives, and the product can be analyzed by LC/MS. Similar portions of several disks can be easily put together and cleaved together, yielding any desired sublibrary. To increase the amount of a particular peptide, the same peptide can be synthesized simultaneously on several disks. To improve the purity of particular library members, they can be purified separately, for example, by preparative HPLC.

The present method is particularly suited when the set of peptides to be synthesized contains a large number of identical amino acids that can be coupled at combined synthesis steps. Obviously, a larger time saving per peptide is obtained when one synthesizes a larger peptide library.

Compared to previously described methods for simultaneous peptide synthesis (see, e.g., Terrett[2] for a review), the present one does not result in any random mix of the incorporated amino acids. We like to term the present type of library “additive”, because amino acids are added to a

finite number of growing peptide chains. This distinguishes them from the libraries obtained by the split and mix approach, which can be said to be "multiplicative", because the number of peptides is the multiplication of the number of different amino acids to be coupled at each position. To obtain the present 16 hexapeptides, the popular split and mix approach[14] would not be an appropriate choice, because it would result in the generation of a multiplicative library consisting of $7 \times 2 \times 8 \times 3 \times 1 \times 1 = 336$ peptides. Hence, 95% of the peptides obtained by this approach would be of no immediate use, and it would not be reasonable to isolate the 16 peptides of interest from this mixture.

We believe that the need for additive libraries will increase in the future. This is due to the growing number of studies that apply statistical molecular design (SMD) to combinatorial chemistry.[15−17] The purpose of SMD is to reduce the number of synthesized peptides without losing chemical diversity. The multiplicative approach turns out here to be inappropriate, because the libraries created by SMD can invariably not be fitted into the growing tree structure required for synthesis of a multiplicative library. By contrast, the additive approach is well-suited because it can be used for the simultaneous synthesis of a peptide library of any design. We estimate that with a commercial continuous flow peptide synthesizer, it would be possible to accomplish at least six different amino acids couplings/day on at least 50 disks (i.e., different peptides) in each run (i.e., one channel), resulting in 300 amino acid couplings/day.

In summary, we have here shown how one can combine the advantages of cotton with the automation, efficiency, and easy handling of common peptide synthesizers for the synthesis of designed peptide libraries.

Experimental Section

Reagents. All amino acid derivatives except those of glycine and 6-aminocaproic acid were of L-configuration. Fmoc-protected amino acids were from Neosystem, except Fmoc-Thr(tBu)-OH, which was obtained from Nova-Biochem. The side-chain protective groups used in the synthesis were tertiary butyl (tBu) for Tyr, Thr, Ser, and Glu; trityl (Trt) for Cys and His; and 2,2,4,6,7-pentamethyldihydrogenbenzofurane-5-sulfonyl (Pbf) for Arg. The Rink linker, *p*-(*R*,*S*)-α-[1-(9H-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid was from NovaBiochem. Bromophenol blue was from Biorad, and silver nitrate was from Sigma. DIPEA, HATU, and 1-hydroxy-7-azabenzotriazole (HOAt) were from Perseptive Biosystems. Reagent grade TFA, triisopropylsilane, *N*-methylimidazole, hydrochloric acid, sodium hydrogencarbonate, and ammonium hydroxide were from Aldrich, and gum arabic was from Fluka. Reagent grade Ac_2O and dichloromethane were obtained from Merck. DMF and 20% piperidine in DMF (Applied Biosystems) were of peptide synthesis grade. Ethanol was a product of Primalco.

Pretreatment of the Cotton Carrier. 100% factory-bleached cotton fabric was used for the synthesis. It was washed at 60 °C in a common washing machine before use. Five disks were cut out (diameter 6.0 cm, weight 850 mg) and soaked in 50 mL of 25% TFA in CH_2Cl_2 for 60 min. The disks were then stacked one on top of the other in a Büchner funnel with the same inner diameter as the diameter of the disks. After removal of the excess TFA with vacuum filtration, the disks were washed successively with CH_2Cl_2 (3×30 mL), 5% DIPEA/CH_2Cl_2 (3×30 mL), and CH_2Cl_2 (3×30 mL). The washings were carried out by soaking the disks in the washing solution for 3 min, followed by its removal by vacuum filtration. After the final wash, the disks were dried under vacuum.

Attachment of the Handle. Attachment of the handle was carried out by soaking the disks for 16 h in a solution of Fmoc-ϵAhx-OH, 1-hydroxybenzotriazole, 1,3-diisopropylcarbodiimide (0.6 M in relation to each of the components), and *N*-methylimidazole (1.2 M) in a volume of DMF equal to the absorption capacity of cotton (1.1 mL/g).[8] For the first hour the wet disks were placed in an ultrasonic bath (Sonorex RK100, Bandelin). The disks were then washed successively with DMF (3×30 mL), ethanol (3×30 mL), and CH_2Cl_2 (4×30 mL) in a Büchner funnel. After completion of the washing, the disks were dried under vacuum. The degree of substitution was then determined as described[8] using a spectrophotometer (Ultraspec III, Pharmacia LKB) and found to be 140 μmol/g cotton. (The corresponding experiment using Fmoc-Gly-OH instead of Fmoc-ϵAhx-OH gave a capacity of 98 μmol/g.) The disks were subsequently soaked in 9 mL Ac_2O/*N*-methylimidazole/DMF 1:2:3 (v/v/v) for 1 h. After washing with DMF (3×30 mL) and CH_2Cl_2 (3×30 mL), the disks were dried under vacuum. The five disks were then cut into 16 smaller disks, all with a diameter of 2.5 cm (weight, 140 mg), corresponding to the inner diameter of the peptide synthesizer column.

Labeling. Disks were labeled with silver ink to aid their identification. The ink was prepared as follows: $NaHCO_3$ (0.2 g) was dissolved in a minimum volume of distilled water. The solution was placed in a mortar, and gum arabic (0.2 g) added and triturated until dissolution. Separately, $AgNO_3$ (0.1 g) was dissolved in 0.5 mL of 28% ammonium hydroxide, the previous solution containing gum arabic was added, and the mixture was heated gently to the boiling point. Numbers were then written on the disks using a calligraphy pen. The disks were developed under a short-wave UV lamp (Spectroline, model ENF-240C/FE, Spectronics Corporation) for 20 min, after which they could be used in the peptide synthesis.

Automated Synthesis. Automated synthesis was performed on a Pioneer Peptide Synthesis System (PerSeptive Biosystems, Framingham). The modified cellulose disks were stacked one on the top of the other in the synthesizer column and wetted with DMF, and the height of the bed was adjusted by screwing the column end-piece down until it was fitted just above the disks. The synthesis was based on the "capping cycle", which is incorporated in the synthesizer's software package. The slightly modified cycle included the following operations on the cellulose-derivatized disks, in sequence: (1) pumping of 20% piperidine (flow rate, 5 mL/min for 5 min); (2) washing with DMF (flow rate, 30 mL/min, for 100 sec); (3) dissolution of Fmoc-amino acid (3-fold molar excess), HATU (3-fold molar excess), and DIPEA (6-fold molar excess) in 4 mL of DMF; (4) recycling of the above-

mentioned solution through the support (flow rate, 30 mL/min, for 30 min); (5) washing with DMF (flow rate, 30 mL/min, for 30 s), (6) soaking in 0.3 M Ac_2O/DMF for 5 min, and (7) washing with DMF (flow rate, 30 mL/min, for 40 s). To attach cysteine, solely Fmoc-Cys(Trt)-OPfp (Pfp = pentafluorophenyl) and HOAt (both in 3-fold molar excess) were introduced at step 3.

Assembling of the Peptide Library. The automated process started by cleavage of the Fmoc group from the 6-aminocaproic acid residue. Then it continued by consecutive attachments of Rink linker, N^{im}-tritylhistidine, and proline. Coupling times for attachment of the linker and the first amino acid were set to 4 h and 60 min, respectively. After attachment of proline, the synthesizer was set to "hold", whereupon the disks were washed five times with methanol and then dried with a nitrogen stream. The column was disassembled and the disks were removed and sorted. Disks 1, 2, 9, 10, and 13−16 were first put back into the column. A "capping cycle" was applied to attach N^{im}-tritylhistidine. Thereafter, additionally the following automated procedures were applied: (1) treatment with 20% piperidine, (2) washing with DMF, (3) washing with methanol, (4) washing with CH_2Cl_2, and (5) drying with nitrogen stream. Disks were removed from the column and dried in a vacuum. In the second run, disks 3, 4, 6, and 7 were treated the same way to attach *β-tert*-butyl aspartic acid. In the third run, (*S*)-Trt-cysteine was attached similarly to the peptidyl cellulose on disks 5, 8, 11, 12.

For the addition of the fourth amino acid, the disks were divided among eight glass beakers. In the following list, beaker numbers together with corresponding disk numbers in parentheses are shown: 1 (7, 16), 2 (1, 15), 3 (2, 10, 14), 4 (9, 11, 12), 5 (3, 6, 13), 6 (4), 7(5), and 8 (8). Coupling was attained by shaking the disks for 60 min in a DMF solution containing a 3-fold excess of the corresponding amino acid derivative, a 3-fold excess of HATU, and a 6-fold excess of DIPEA, except for the fifth beaker, in which Fmoc-Cys(Trt)-OPfp and HOAt (both in a 3-fold molar excess) were introduced. The amount of solvent (DMF) was increased as more disks were to be treated. Two milliliters of DMF was used for soaking of one or two disks, whereas 3 mL DMF was used for soaking of three disks. The following amino acid derivatives were used: Fmoc-Pro-OH (beaker 1), Fmoc-Met-OH (beaker 2), Fmoc-Ala-OH (beaker 3), Fmoc-Thr(tBu)-OH (beaker 4), Fmoc-Cys(Trt)-OPfp (beaker 5), Fmoc-Ser(Trt)-OH (beaker 6), Fmoc-Val-OH (beaker 7), and Fmoc-Leu-OH (beaker 8). After coupling, the excess solution was removed, and all of the disks were transferred to a Büchner funnel and washed together with DMF (3 × 30 mL) and ethanol (3 × 30 mL). The disks were then dried under vacuum.

For addition of the fifth amino acid, disks 1, 3, 5, 6, 9, 11, 13, and 15 were first placed in the synthesizer column. An automated "capping cycle" was applied, as described above using Fmoc-Tyr(tBu)-OH as amino acid derivative and a coupling time of 60 min. Thereafter, additionally the following automatic procedures were applied: (1) treatment with 20% piperidine, (2) washing with DMF, (3) washing with methanol, (4) washing with CH_2Cl_2, and (5) drying with a nitrogen stream. In the same way, Fmoc-His(Trt)-OH was attached to peptidyl cellulose on disks 2, 4, 7, 8, 10, 12, 14, and 16 (coupling time, 30 min). Additional steps were as described above in this paragraph. All disks were then dried under vacuum.

For addition of the sixth amino acid, the disks were distributed among seven glass beakers. Beaker numbers together with corresponding disk numbers in parentheses were as follows: 1 (1, 2), 2 (3, 4), 3 (5−8), 4 (9, 10), 5 (11, 12), 6 (13, 14), and 7 (15, 16). The following amino acid derivatives were used: Fmoc-Thr(tBu)-OH (beaker 1), Fmoc-Arg(Pbf)-OH (beaker 2), Fmoc-Pro-OH (beaker 3), Fmoc-Met-OH (beaker 4), Fmoc-Glu(OtBu)-OH (beaker 5), Fmoc-Cys(Trt)-OPfp (beaker 6), and Fmoc-Ala-OH (beaker 7). The procedure was identical to that described for addition of the fourth amino acid. All disks were then placed together in the synthesizer column, and the following automatic procedures were applied: (1) pumping of 20% piperidine (flow rate, 5 mL/min for 5 min), (2) washing with DMF (flow rate, 30 mL/min, for 100 sec), (3) soaking in 0.3 M Ac_2O/DMF for 5 min, (4) washing with DMF (flow rate, 30 mL/min, for 40 s), (5) washing with methanol (flow rate, 30 mL/min, for 40 s), (6) washing with CH_2Cl_2(flow rate, 30 mL/min, for 40 s), and (7) drying with a nitrogen stream. Then the disks were removed from the column and dried in a vacuum.

Cleavage and Isolation of Peptides. A segment (20% of area) of each disk was cut out by scissors. The strips were then transferred to 16 small glass flasks. To each of them, a mixture TFA/1,2-ethanedithiol/triisopropylsilane/water (95:2.5:2.5:2.5, 1.2 mL) was added. After shaking for 1.5 h (during this treatment, fabrics fell apart into a lump of fibers), the mixtures were filtered through porous glass filters. Fibers on filters were washed with TFA (1 mL). Filtrates obtained from the reaction mixture and washing were pooled and evaporated under vacuum at room temperature, and the residue was triturated with anhydrous ether. Precipitates formed were filtered off and dried in a vacuum in the presence of KOH and P_2O_5. The substances obtained were dissolved in a mixture of acetonitrile/water (3:7, 0.7 mL) and freeze-dried. Yields of products were ∼30% (calculated from capacity of Fmoc-6-aminocaproyl-cotton), and the purity (HPLC) reached 80%. Substances showed the expected *m*/*z* values according to mass spectrometry.

LC-MS and HPLC Conditions. LC/MS analyses were performed using a PE Sciex API 150EX system equipped with a Perkin-Elmer HPLC system and TurboIonspray as the ion source. A 10-μL portion of the peptide solution were applied onto a Reprosil-Pur C_{18}-column (150 × 3 mm) (Dr. Maisch) and eluted with acetonitrile/water (5mM) CH_3COONH_4 using a linear gradient (10−30% acetonitrile in 15 min) and a flow rate of 300 μL/min. Before injection, the column was equilibrated for 5 min with 10% acetonitrile/water (5mM) CH_3COONH_4. Full-scan mass spectra were acquired in the positive ion mode over the mass range *m*/*z* 100−1000.

Analytical HPLC was performed on a Waters system equipped with a Millenium 32 workstation, 2690 separation module, and 996 photodiode array detector. A Vydac RP

C_{18} column (4.6 × 250 mm, 300 Å, 218TP54) was used, and the eluent was applied in a linear concentration gradient increasing MeCN content from 0 to 40% in water + 0.1% TFA in 30 min or from 0 to 15% MeCN in water + 0.1% TFA at a flow rate of 1 mL/min. Detection was at 220 nm.

Acknowledgment. This research was supported by the Swedish MRC (04X-05957) and TFR (230-2000-291).

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CC0200078