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SIMULTANEOUS MULTIPLE PEPTIDE SYNTHESIS UNDER CONTINUOUS FLOW CONDITIONS ON CELLULOSE PAPER DISCS AS SEGMENTAL SOLID SUPPORTS

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Abstract - A new approach to the simultaneous chemical synthesis of large numbers of different peptide sequences is described.[1] The particular features of the method presented[2] are: - cellulose paper discs serve as solid supports for individual peptide sequences, - the peptide chains are linked to the cellulose via a p-alkoxybenzyl ester anchor cleavable by mild treatment with trifluoroacetic acid in dichloromethane, - the syntheses of peptides are accomplished using N-α-Fmoc protected amino acids, - all reactions are carried out in a simple multi-column continuous flow device with the cellulose discs tightly stacked in column reactors, - as many as 100 discs can be reacted simultaneously with the same amino acid derivative in one reaction column.

Short synthetic peptides have become extremely useful tools for the localization and deciphering of structural determinants by which proteins interact specifically with other ligands such as posttranslationally modifying enzymes, receptors, and antibodies. Furthermore, peptides can be used to elicit antibodies with submolecular binding specificities to pre-selected sites on target proteins. In particular, the systematic screening with series of overlapping fragments or substitution analogues needs short peptides in large numbers, although small quantities (much less than a μmol) of these are sufficient.[3,4] A rational way to adapt synthesis capacities of the solid phase methodology to such drastically increasing demand from molecular biology and immunology is the simultaneous multiple synthesis following the 'segmental support' approach.[5] The term 'segmental support' refers to a mechanically and chemically stable, non-interchangeable entity of support material. This type of support allows one to combine many different growing oligomers, which require the coupling of the same monomer unit, into one common reaction cycle and to separate them thereafter without contamination. In this way, the number of reaction cycles necessary to assemble a set of different sequences can be reduced considerably compared to the number of individual synthesis steps, when performed separately. The advantage of this approach has been first demonstrated with the simultaneous multiple synthesis of oligodeoxyribonucleotides[5] using cellulose paper discs as solid supports. This 'filter method' is applied successfully following both the phosphotriester[6,7] and phosphoramidite[8,9] routes. Recently, R.A. Houghten[3] described a method to render conventional polystyrene resin non-interchangeable by sealing it into polypropylene bags and using these as supports in a simultaneous synthesis of 247 undecapeptides (tea-bag method).

This paper describes the straightforward adaptation of the filter-method to simultaneous multiple peptide synthesis. Cellulose paper has an excellent resistance towards most organic solvents but rapidly desintegrates upon the action of strong acids. This acid sensitivity

prohibits the use of an acid cleavable protection of the α-amino function such as tert-butoxycarbonyl (tBoc) in peptide synthesis on this support. The 9-fluorenylmethoxycarbonyl (Fmoc) group, however, permits a base catalyzed repetitive αN-deprotection and, hence, allows a mild-acid labile peptide-resin linkage.[10] This is perfectly compatible with the cellulose support. Cellulose discs are mechanically quite stable to even harsh manipulations and can be easily marked by writing numbers or letters with a pencil. The marking is sufficiently stable to follow unambiguously individual supports through an extended series of reaction cycles.[5-9] The rigid structure and low swelling properties of the cellulose paper allows the multiple coupling reactions to be carried out under low pressure continuous flow conditions with the discs tightly fitted and stacked in column reactors.

RESULTS AND DISCUSSION

The peptide-cellulose linkage

A benzyl ester type linkage is most commonly used for anchoring the first amino acid to a solid support. A large variety of simple bifunctional linkage reagents (handles) are described in the literature by which peptide-resin bonds can be formed of varying stability and cleavage by a wide range of reagent types (for a review see Ref. 11). On this basis, the p-alkoxybenzyl alcohol derivative (reagent **2**, Scheme 1) was conceived to release the benzyl ester bound peptide quantitatively by treatment with 50% or less trifluoroacetic acid in CH_2Cl_2 (see Figure 2). **2** provides a C_5-spacer arm and a carboxylic acid function for direct esterification of the cellulose hydroxyl groups. This reaction requires the intermediate protection of the benzyl alcohol function. The 4-methoxytrityl ether (MeOTr-O-) proved suitable as it can be cleaved readily under very mild acidic conditions with 3% dichloroacetic acid (DCA) in CH_2Cl_2 at room temperature. In addition, the yields of all reactions involving MeOTr-protected derivatives can be determined simply by photometric measurement of the orange $MeOTr^+$ cation formed upon acid treatment.

2 is prepared by selective tritylation of 4-hydroxymethylphenol with 4-methoxytrityl chloride and subsequent reaction of the phenolic hydroxyl with 6-bromohexanoic acid (Scheme 1). Overall yield is about 50%. The compound is obtained as an oil and stored dissolved in dry dioxane at -20°C.

$$\text{MeOTr-Cl} + \text{HO-CH}_2\text{-C}_6\text{H}_4\text{-OH} \xrightarrow{\text{I}} \text{MeOTr-O-CH}_2\text{-C}_6\text{H}_4\text{-OH} \quad (\mathbf{1})$$

$$\mathbf{1} + \text{Br-(CH}_2)_5\text{-COOH} \xrightarrow{\text{II}} \text{MeOTr-O-CH}_2\text{-C}_6\text{H}_4\text{-O-(CH}_2)_5\text{-COO}^-\ \text{Et}_3\text{NH}^+ \quad (\mathbf{2})$$

$$\mathbf{2} + \text{HO-(Cellulose)-OH} \xrightarrow{\text{III,IV,V}} \text{HO-CH}_2\text{-C}_6\text{H}_4\text{-O-(CH}_2)_5\text{-C(=O)-O-(Cellulose)-O-Ac}$$

Scheme 1: Introduction of benzyl alcohol functions onto cellulose. I) pyridine, RT; II) NaOH in ethanol, 80°C; III) esterification of **2**; IV) acetylation; V) detritylation.

The synthesis device

A schematic drawing of the manually operated device used to carry out all chemical reactions on the paper discs is shown in Figure 1. It is similar to that described by Dryland and Sheppard[12] and is built from inexpensive chromatography equipment. Flow of solvents and reagents (2 ml/min) is driven by a multi-channel peristaltic pump fitted with resistant Kalrez hose. Solvents from stock bottles are selected by valve I and distributed to the various reactor loops. Currently, a four-column device is used. Reactions other than amino acid couplings are carried out by connecting the appropriate solvent bottles to valve I. Stock bottles are pressurized to 0.01 MPa with dry nitrogen to avoid formation of bubbles

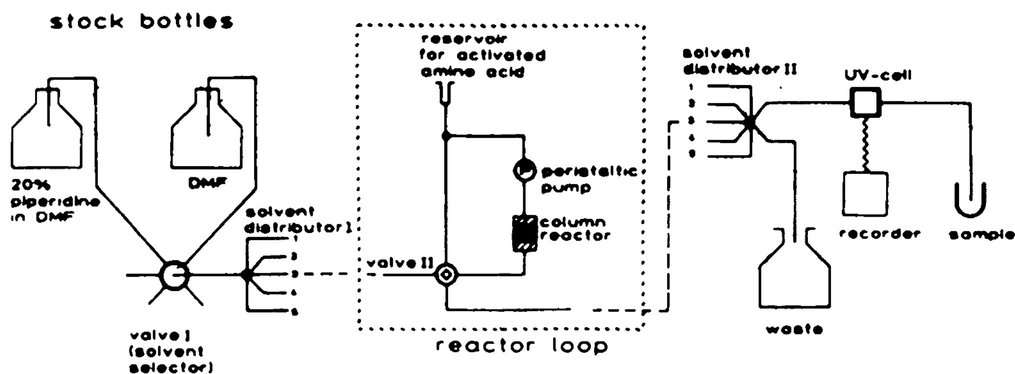


Figure 1: Schematic drawing of a manually operated multi-column continuous flow synthesizer.

caused by suction of the pump. The columns (1.5 cm ϕ x 10 cm glass tubes) have fixed upper and adjustable lower plungers to accomodate any number of discs up to 200. The volume of the reactor loop including the column without support is only 1.3 ml. Activated amino acid derivatives or any other reagents are introduced via a reservoir, a disposable plastic filtration column with a 20 µm polyethylene frit, connected to the reactor loop by a 3way valve. The maximum volume (void volume) which can be applied to displace the liquid between the reservoir and valve II by reagents is given in Table 1 as a function of the number of discs in the column. By switching valves II, the reactor loops can be closed individually and reagents are recirculated. The efflux of the columns is then collected by solvent distributor II and passes either into the waste or through a UV-cell for monitoring (280 nm) and sampling. This option allows the efficiency and completeness of cleavage reactions (Fmoc, MeOTr) and washing steps to be followed.

Table 1: Void volumes of the reactor loop as a function of the number of discs per column.

number of discs	void volume[a)]
0	0.8 ml
10	1.3 ml
20	1.8 ml
30	2.3 ml
40	2.8 ml
50	3.3 ml
100	5.8 ml

a) These volumes hold only for those discs specified below.

Functionalization of cellulose discs

Cellulose paper is readily available in a large variety of quality, thickness and shape. Throughout the described experiments, 1.55 cm ϕ discs cut out from Whatman 3MM paper sheets (average weight 33 mg) were used. Mild acid treatment affords a limited and irreversible swelling of the cellulose matrix. By using 10% or less TFA in CH_2Cl_2, the swelling process is slow enough to become controllable. In this way, paper can be pre-treated to achieve a maximal accessible surface area while maintaining the necessary mechanical stability.

Table 2 summarizes the results from several experiments to evaluate proper reaction conditions for a high degree of benzyl alcohol functionalization of the cellulose. Paper discs were pre-swollen for various time intervals in 10% TFA in CH_2Cl_2 and then reacted with reagent **2**. Activation of the carboxyl group of **2** was carried out either with mesitylene sulfonyl nitrotriazolide (MSNT) in pyridine[5], dicyclohexyl carbodiimide (DCC)/dimethylamino-pyridine (DMAP) in CH_2Cl_2, or diisopropyl carbodiimide (DIC)/DMAP in dimethylformamide. Consistently, the highest yields were obtained by the MSNT activation. No further increase of yields is obtained by prolonged pre-swelling of the paper, where upon the mechanical

Table 2: Functionalization of cellulose discs with reagent 2[a)]

pre-swelling [min]	A(MSNT/pyridine) [µmol/disc]	[µmol/g]	B(DCC/DMAP/CH_2Cl_2) [µmol/disc]	[µmol/g]	C(DIC/DMAP/DMF) [µmol/disc]	[µmol/g]
0	1.0	30	0.1	3	0.9	27
10	2.9	87	0.4	12	1.3	39
20	3.0	90	0.4	12	1.5	45
30	2.9	87	0.4	12	1.5	45
45	2.7	81	0.4	12	1.6	48
60	2.9	87	0.4	12	1.5	45

a) reaction conditions are given in the Experimental section

stability gradually decreases. 20 min in TFA/CH_2Cl_2 (1/9) is sufficient and stability remains almost unchanged. Optimized conditions for large scale preparation of functionalized discs include a double coupling of 2 (0.5 mmol/100 discs) with 2.5 mmol MSNT and 2.5 mmol N-methylimidazole[6,7] each lasting 2 h. This procedure consistently yields 2-3 µmol/disc in the first coupling and up to 6 µmol/disc in the second. Thus, a functionalization of almost 0.2 mmol/g is practically feasible with this type of paper material and reaction conditions. Residual accessible hydroxyl groups on the cellulose are blocked by acetylation and the benzyl alcohol function is liberated by a 10 min wash with 3% DCA in CH_2Cl_2.

Loading of functionalized cellulose discs

Attachment of the first Fmoc-amino acids is carried out as described for other benzyl alcohol substituted supports using a 5-fold excess over benzyl-OH of preactivated amino acid derivatives catalyzed by DMAP[13] and following the steps listed in Table 3 (loading cycle). Residual hydroxyl functions are again blocked by acetylation and the Fmoc-protection is cleaved with 20% piperidine in DMF. Loaded discs may be prepared in large quantities and stored over a long period.

Table 3: Amino acid coupling cycle

step	time	reagent
1 wash	10 min	DMF (until all air bubbles between discs have disappeared)
2 recycle	60 min	5 eq. symm. anhydride in DMF[a] (normal cycle)
	or 120 min	5 eq. HOBt ester in DMF[a] (normal cycle)
2a (as 2)		+ 5 eq. DMAP (loading cycle)
3 wash	10 min	DMF
4 recycle	10 min	10 eq. Ac_2O, 5 eq. DIPEA in DMF[a,b]
5 wash	10 min	DMF
6 wash	10 min	20% piperidine in DMF
7 wash	10 min	DMF

a) volumes are taken from Table 1
b) always freshly prepared
After step 7, the colums are disassembled; the discs are pressed out into a beaker, washed with CH_2Cl_2, and dried in vacuo.

The TFA promoted cleavage of the benzyl ester bond formed between peptide and linkage reagent 2 was examined using amino acid loaded discs. As shown in Figure 2 even less than 10% TFA is sufficient to release the amino acid quantitatively within a reasonably short time.

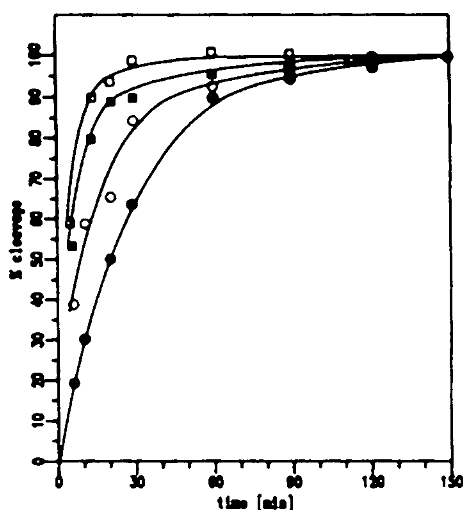


Figure 2: Kinetics of the release of amino acid from the cellulose support by TFA in dichloromethane containing 5% anisole. ● 5% TFA; ○ 10% TFA; ■ 20% TFA; □ 50% TFA. The discs have been loaded with valine. Experimental conditions are described in the Experimental section.

All curves reach the same level which corresponds to 100% of the amount of benzyl-OH introduced with reagent 2. This proves that all linker functions are accessible for the loading and cleavage reactions and that both reactions are quantitative. However, even after prolonged acid treatment up to 10% of total amino acid loading may remain bound to the cellulose and can be cleaved off only by alkaline hydrolysis with aqueous triethylamine. Obviously, this represents amino acid coupled directly to the cellulose by an acid stable alkyl ester bond. Despite the careful acetylation of the cellulose after functionalization with linker reagent 2, some new reactive sites must have become accessible, probably due to further swelling during the acid detritylation. This reaction is enhanced by the DMAP catalyst and was not observed under usual coupling conditions (less than 10 nmol/disc in the normal cycle). As the alkyl ester is not cleaved with TFA, any subsequent minor coupling to the cellulose matrix will not lead to contamination of the product with failure sequences.

Peptide assembly

The method is exemplified by the synthesis of 13 heptapeptides listed in Figure 3. These peptides are variants of the sequence H_2N-Thr-Lys-Ile-Tyr-Asn-Pro-Val-COOH and represent the active sites of a corresponding series of genetically engineered protease inhibitors.[14] The computer printout is a protocol form to support the multiple peptide synthesis. It is generated by the program SYNPEP which is part of the menu-driven package GENMON, designed to work with protein and nucleic acid sequences.[15] SYNPEP accepts sequences from a GENMON amino acid sequence file, from an ASCI file, or directly from the keyboard. Optionally, the loaded sequence can be transformed into series of overlapping fragments with any offset. SYNPEP then calculates an optimized scheme for the distribution of discs resulting in a minimum number of successive coupling cycles required for a given set of peptide sequences. It includes the two options of choosing any number of parallel operated column reactors and of setting a threshhold for the maximal number of discs to be included in one column. In this particular example using a one-column mode, only 16 cycles are required, compared to 78 (13x6) for their individual syntheses. This is almost an 80% saving of coupling reactions.

Four discs corresponding to 10 µmol of valine loading per peptide sequence were unambiguously marked with a pencil using the numbers of Figure 3. Single amino acid additions were carried out as described in Table 3 (normal cycle) using the symmetrical anhydrides essentially as described by Dryland and Sheppard.[12] The capping steps (steps 4 and 5 in Table 3) were omitted. Only Fmoc-Asn was coupled as HOBt ester including the capping steps. After

a)

b)

c)

d)

Figure 3: The SYNPEP protocol form for the simultaneous multiple peptide synthesis. a) alphabetical listing of project names under which peptide sequences have been defined and are to be included; b) peptide numbers and sequences together with some useful data; c) sum of all individual coupling reactions; d) scheme for the sorting of individually numbered supports into the multiple coupling reactions (one column mode), cycle 0 = loading cycle, in brackets = number of supports per reaction / number of underlined supports for which the respective cycle is the last one, - = numbers of discs which are not included in the respective cycle.

completion of the 16 cycles listed in Figure 3, the peptides were cleaved from the cellulose discs separately with TFA/anisole/dichloromethane (55/5/40 by volume - 1ml/disc) for 2.5h at RT. This treatment also removes all tert-butyl side chain protecting groups. To cleave the Mtr-group, arginine containing peptides were further treated with TFA/5% phenol at RT

overnight. After ether extraction and lyophilization, 8 to 10 mg of crude products were obtained. Some HPLC profiles of these are shown in Figure 4. The identities of the peptides from the major HPLC peaks were confirmed by amino acid analysis and FAB-MS (Figure 5).

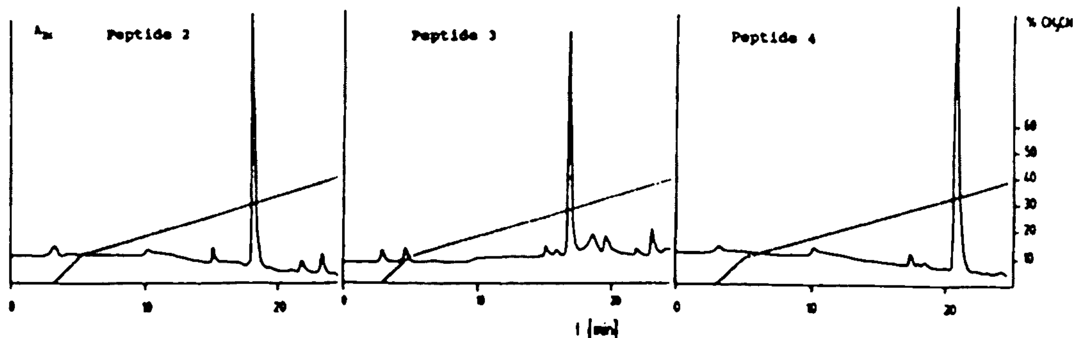
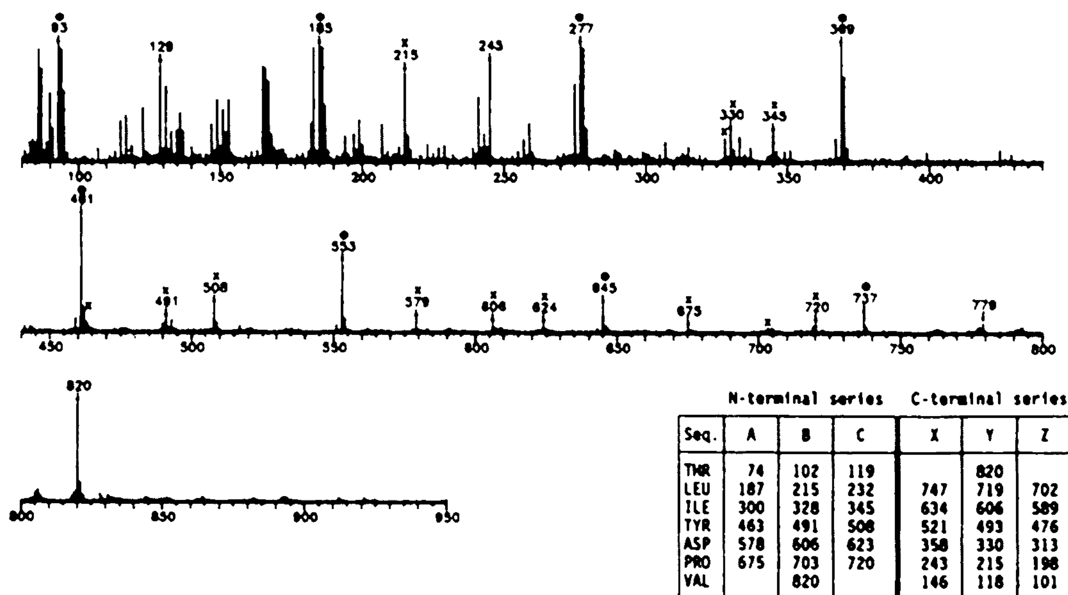


Figure 4: HPLC profiles of some crude peptides. Peptide numbers correspond to Figure 3.



	N-terminal series			C-terminal series		
Seq.	A	B	C	X	Y	Z
THR	74	102	119		820	
LEU	187	215	232	747	719	702
ILE	300	328	345	634	606	589
TYR	463	491	508	521	493	476
ASP	578	606	623	358	330	313
PRO	675	703	720	243	215	198
VAL		820		146	118	101

Figure 5: Positive ion FAB mass spectrum of peptide 2 with a nominal mass of 819 ($M \cdot H^+$ = 820). Peaks marked with a dot are from the glycerol matrix, those marked with a cross are sequence specific fragment ions (see table)[16].

The crude peptides were pure enough for direct use as antigens in the production of monoclonal antibodies. Several stable lines of hybridoma clones were obtained that produce antibodies which specifically bind to the corresponding variant of the protease inhibitor.[17] To date several hundred such peptides up to 12 residues in length have been synthesized on cellulose discs with equal success. Hence, the method described represents a rational, economic and flexible approach for the preparation of large numbers of different peptide sequences: cellulose paper is an inexpensive and easy to handle material; paper discs are suitable segmental supports to carry out the simultaneous multiple peptide synthesis under very efficient continuous flow conditions; the synthesis scale for individual peptides can be easily adapted to specific demands by variation of disc size, number and loading; without technical problems syntheses can be carried out even on a very small scale (e.g. <1μmol). Investigations directed towards the synthesis of longer peptides by stepwise- or fragment-coupling strategies are in progress.

EXPERIMENTAL

Materials and general methods

Reagents and solvents were of highest available purity and purchased from various commercial suppliers. Triple-distilled pyridine and MeOTr-Cl were from Cruachem, Scotland, DMF (p.a.) from Fluka. Piperidine and dioxane were distilled from KOH under N_2. Petroleum ether of boiling range 60-80°C was used throughout. MSNT was prepared as described[18] and routinely checked by melting point (136-137°C) prior to use as it decomposes slowly even if stored at-20°C. 6-Bromohexanoic acid was recrystallized from petroleum ether (mp 35°C). Paper discs (1.55 cm ϕ, average weight 33 mg) were cut out individually from Whatman 3MM paper sheets with a punch. Polypropylene filtration columns with 20 µm polyethylene frits were from J.T. Baker Chemicals B.V. (Holland), and 2.2 ml polypropylene tubes from Eppendorf (Hamburg,FRG).

Fmoc-protected amino acid derivatives were from Novabiochem, Switzerland. Side chain protection was tBut and tBoc, for arginine Mtr. Activated intermediates of these were always freshly prepared and used directly. - Symmetrical anhydrides: Fmoc-amino acid was dissolved in the minimum volume of CH_2Cl_2 with the aid of a drop of DMF and 0.5 eq. DCC (0.33 M solution in CH_2Cl_2) was added; after 10 min the solution was evaporated and the residue dissolved in the appropriate volume (Table 1) of DMF and transferred to the reservoir of the synthesizer; precipitated dicyclohexylurea was filtered off by the polyethylene frit in the reservoir. - HOBt esters: Fmoc-amino acid and 1.5 eq HOBt were dissolved in the appropriate volume (Table 1) of DMF and 1.2 eq. DIC was added; after 1 h at RT the solution was transferred to the reservoir of the synthesizer. DMAP catalyst (loading cycle), 1 M solution in DMF, was added only to the activated amino acid in the reservoir just prior to transport onto the reaction column.

Short column chromatography was carried out as described[19] on silica gel 60H (E. Merck) and TLC on Si-60 F_{254} 0.2 mm coated glass plates (E. Merck). Products were visualized by UV absorption and by spraying+heating with anisaldehyde/sulfuric acid reagent.[20] - All parts to build the manual synthesis device were from Omnifit, Cambridge UK, except for the 4-channel peristaltic pump (Meredos, Bovenden FRG) fitted with special, solvent resistant Kalrez hoses (Pharmacia, Sweden) and the UV-monitor (Isco, USA).The PTFE frits in the plungers of the original Omnifit columns were replaced by perforated PTFE plates. Solvent lines were made of 0.4 mm i.d. PTFE tubing. Gas-tight glass syringes were from Hamilton. - HPLC-separations were carried out on an analytical reversed-phase silica gel column (Nucleosil 300-7C18,4x250 mm, Macherey-Nagel, Düren, FRG) with gradients of acetonitril/0.1% TFA in water/0.1% TFA; flow was 0.5 ml/min. - Proton-NMR spectra were recorded at room temperature on a Bruker WM-400 NMR spectrometer locked at the deuterium resonance of the solvent. Chemical shifts are reported relative to internal tetramethylsilane (TMS) and coupling constants in Hz. - Mass spectra were recorded on a Kratos MS 50RF with a high field magnet (mass range ca. 10.000 at 8 kV) and a Kratos FAB source using a beam of neutral xenon atoms at 8-9 kV and glycerol as matrix. Peak fractions from HPLC separations containing 5 to 10 µg peptide were evaporated to dryness, dissolved in 10 µl water and aliquots thereof were injected into the glycerol matrix. - Amino acid analyses were carried out on a Biotronik LC200. - Computer programs run as VAX/VMS- or IBM-PC/DOS versions. - UV/VIS extinction coefficients were measured with a Zeiss PMQII spectrophotometer. $MeOTr^+$ was determined in 3% DCA in dichloromethane (ϵ_{470}=57.000). Solutions containing the dibenzofulvene-piperidine adduct in 20% piperidine in DMF were evaporated to dryness and the residue dissolved in dichloromethane (ϵ_{267}=18.980; ϵ_{301}=8.550).

The quantitative ninhydrin assay was carried out in 2.2 ml plastic tubes essentially as described.[21] The assay does not give satisfactory results with amino acids linked to the cellulose support. Therefore, discs or part of them were treated either with acid (A) or base (B) and amino acid determined in solution.
(A) TFA/anisol/dichloromethane (55/5/40 by volume -1 ml/disc) at RT for 2.5 h.
(B) 1 M aqueous triethylamine (1ml/disc) at 50°C overnight.
For A as well as B, the liquid was then removed and filtered through a plastic filtration column. The disc and the column were washed twice with 1 ml 2-propanol and the combined eluates evaporated to dryness. The residue was dissolved in 0.1 to 1 ml ethanol/water (1/1 by volume) and aliquots thereof subjected to the ninhydrin reaction.

Preparation of 4-methoxytrityl-6[4'-(oxymethyl)phenoxy]hexanoic acid (linker reagent 2)

4-Hydroxymethyl phenol (50 mmol, 6.21 g) was dried by coevaporation twice with 2 ml pyridine and the residue was dissolved in 10 ml pyridine. MeOTr-Cl (55 mmol, 21.8 g) was added and the solution stirred at room temperature. After 10 min, TLC ($CHCl_3$/MeOH = 95/5 by volume) indicated completion of the reaction and water (2 ml) was added. The mixture was diluted with 0.4 l CH_2Cl_2 and extracted three times with 0.15 l water. The combined aqueous layers were reextracted with 0.1 l CH_2Cl_2. The combined organic phases were dried with sodium sulfate and evaporated. The product was purified in two parts by short column chromatography on 300 g silica gel using CH_2Cl_2/petroleum ether (8/2) as starting solvent. The product was eluted with CH_2Cl_2 containing increasing concentrations of methanol to 5%. Product containing fractions, identified by TLC ($CHCl_3$/MeOH = 95/5 by volume), were pooled, a few drops of triethylamine were added, and evaporated. The oily residue was dissolved in 50 ml dioxane and stored at -20°C. The yield was determined by photometric measurement of the MeOTr+ absorption of a 10 µl aliquot and was 35 to 40 mmol, 70 to 80%.

A solution of 4-MeOTr-(4'-oxymethyl)phenol (40 mmol) in dioxane was evaporated and the residue dissolved in 120 ml ethanol containing NaOH (128 mmol, 5.12 g). 6-Bromohexanoic acid (60 mmol, 11.8 g) and KJ (40 mg) were added and the mixture was refluxed overnight. After cooling, the solution was added to water (0.5 l) and CH_2Cl_2 (0.2 l) in a separation funnel

and neutralized with citric acid. The organic layer was separated and the aqueous layer extracted again with 0.2 l CH_2Cl_2. The combined organic phases were reextracted twice with 2M TEAB buffer (0.1 l), dried with sodium sulfate, and evaporated. The product was purified by short column chromatography on 300 g silica gel using 0.5% triethylamine in CH_2Cl_2 as starting solvent and containing increasing concentrations of methanol to 5%. Product containing fractions, identified by TLC ($CHCl_3$/MeOH/Et_3N = 95/5/0.5 by volume), were pooled and evaporated. Attempts to crystallize, lyophilize, or precipitate the product failed. Therefore, a stock solution in dioxane was stored at -20°C. The yield was determined as described above and was 50 to 60%.

4-Methoxytrityl-(4'-oxymethyl)phenol: ^1H NMR (DMSO-d_6) δ = 9.371 [bs, 4-OH], 7.44-7.22 [m, Ph x 2, H-2', H-6'], 7.126 ["d", H-2, H-6, J(2-3)+J(2-5) 8.4], 6.924 ["d", H-3', H-5', J(3'-2')+J(3'-6') 8.9], 6.741 ["d", H-3, H-5], 3.926 [s, CH_2], 3.743 [s, OCH_3].

4-Methoxytrityl-6[4'-(oxymethyl)phenoxy]hexanoic acid: ^1H NMR ($CDCl_3$) δ = 7.510 ["d", H_o-Ph], 7.390 ["d", H-2", H-6", J(2"-3")+J(2"-5") 8.8], 7.31-7.20 [m, H_m-Ph, H_p-Ph, H-2', H-6'], 6.862 ["d", H-3', H-5', J(3'-2')+J(3'-6') 8.6], 6.841 ["d", H-3", H-5"], 4.079 [s, OCH_2Ar], 3.958 [t, H-6, J(6-5) 6.4], 3.791 [s, OCH_3], 2.386 [t, H-2, J(2-3) 7.5], 1.808, 1.718, 1.529 [pentetx3, H-3, H-4, H-5].

Test reactions described in Table 2

Paper discs were treated with TFA/CH_2Cl_2 (1/9 by volume, about 1 ml/disc) for various time intervals as indicated. They were immediately washed with DMF and CH_2Cl_2 and dried in vacuo overnight. Two discs from each group were marked with a pencil and reacted together with 0.1 mmol reagent **2** overnight under the following conditions (reaction volume 2 ml):

A: 1 mmol MSNT in pyridine
B: 0.12 mmol DCC, 0.1 mmol DMAP in CH_2Cl_2
C: 0.12 mmol DIC, 0.1 mmol DMAP in DMF

Reactions were carried out in 3 ml flat bottom septum-stoppered glass vials. All solvents and reagents were transferred by gas tight syringes. After washing out excess reagents with the corresponding solvent, 2-propanol and CH_2Cl_2, the discs were dried in vacuo. The discs from all reactions were then reacted together for 6 h with an excess of acetic anhydride in pyridine (2/8 by volume) followed by washing with pyridine and CH_2Cl_2 and drying in vacuo. The yields of linker attachment were determined individually for each disc by placing it into 2 ml of 3% DCA in CH_2Cl_2 and measuring the trityl color after 5 min.

Large scale preparation of functionalized cellulose paper discs

100 paper discs were added to 30 ml of TFA/CH_2Cl_2 (1/9 by volume) in a stoppered glass filter funnel. After 20 min the acid solution was filtered off and excess DMF was added. The discs were then washed twice with 2-propanol and CH_2Cl_2 and dried in vacuo. The discs were carefully pressed successively into the reaction column and connected to the synthesizer. The column was flushed with pyridine until all air bubbles had disappeared. MSNT (2.5 mmol, 0.74 g) was dissolved in 4 ml pyridine and transferred with a gas-tight glass syringe to a flask containing the linker reagent **2** (0.5 mmol, dried by twice coevaporation with 10 ml pyridine). N-methylimidazole (2.5 mmol, 0.2 ml) was added and the solution was transferred to the reaction column . The mixture was recirculated for 2 h followed by flushing the column with pyridine (10 min). If required, this treatment may be repeated. Then 10 ml acetylation mixture (10% acetic anhydride, 15% collidine, 5% DMAP in acetonitrile, by volume) were injected and recirculated for 30 min. After washing the column with pyridine (10 min) and CH_2Cl_2 (10 min) 3%, DCA in CH_2Cl_2 was passed through until the orange colour was washed out (10 min) followed by flushing for 15 min with CH_2Cl_2. The $MeOTr^+$ containing efflux was collected and used to determine the yield of linker attachment as described under general methods. The column was then disconnected from the synthesizer. The discs were dried in the column by passing a stream of N_2 and then stored at -20°C.

Loading of functionalized discs with Fmoc-amino acids

See below under 'Peptide Synthesis'.

Cleavage reactions from Figure 2

Per reaction, one disc (functionalized to 4.06 µmol with linker reagent **2** and loaded with 4.5 µmol valine as determined by Fmoc-cleavage) was placed into a 10 ml flat bottom septum-stoppered glass vial. 2.5 ml CH_2Cl_2 containing 5% anisole and the indicated concentration of TFA was added and the vial was gently shaken. 0.1 ml aliquots were removed after the time intervals indicated and subjected to the ninhydrin assays described under general methods. Finally, the disc was removed from the solution, washed with 2-propanol (10 ml) and CH_2Cl_2 (10 ml) and dried in vacuo. Residual amino acid on the disc was then cleaved with 1 ml 1M aqueous triethylamine and again determined with the ninhydrin assay. This yielded 0.4 µmol.

Peptide synthesis

52 paper discs (functionalized with about 2 µmol benzyl-OH) were subjected to a complete amino acid loading cyle (Table 3) with Fmoc-valine which yielded an average loading of 2.5 µmol/disc. This was determined from the eluate of the Fmoc-cleavage step as described under general methods. 4 discs per heptapeptide sequence were marked with a soft pencil using the corresponding numbers of Figure 4 and then subjected to the series of coupling cycles (single amino acid additions) listed in the protocol form. The appropriate amount of Fmoc-amono acids was calculated from 5-times the average loading and the number of discs/reaction (twice this amount is needed to prepare the symmetrical anhydrides). It was then activatd as described under general methods. All amino acids were coupled as symm. anhydrides for 1 h except Fmoc-Asn as HOBt-ester for 3 h. Only the Asn coupling cycle included a capping step (acetylation steps 4 and 5, Table 3).

After the final coupling cycle the discs were distributed for each individual sequence into 10 ml flat-bottom stoppered glass vials and TFA/anisole/CH_2Cl_2 (55/5/40 by volume - 1 ml/disc) was added. The vials were gently shaken for 2.5 h at room temperature. The liquids were then removed and filtered through plastic filtration columns into pear shaped flasks, followed by washing the discs three times alternately with each 2 ml 10% aqueous TFA and 2-propanol. The combined filtrates were evaporated to dryness. For cleavage of the Mtr-protecting groups, arginine containing peptides were further treated with TFA/phenol (95/5 by volume, 5 ml/reaction) at room temperature overnight and again evaporated. The residues were each dissolved in 5 ml 0.1% aqueous TFA, extracted three times with 5 ml diethyl ether, and lyophilized. 8 to 10 mg of crude peptides were obtained.

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2. Abbreviations: Ac, acetyl; DCA, dichloroacetic acid; DCC, dicyclohexylcarbodiimide; DIC, diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, N-hydroxybenzotriazole; HPLC, high pressure liquid chromatography; MeOTr-, 4-methoxytrityl; MSNT, 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole; RT, room temperature; TEAB, triethylammonium bicarbonate (pH 7.5); TFA, trifluoroacetic acid; TLC, thin layer chromatography.
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