Peptide Research

Evaluation of Cotton as a Carrier for Solid-Phase Peptide Synthesis

Jutta Eichler, Michael Bienert, Alena Stierandova[1] and Michal Lebl[1]
Institute of Drug Research, Berlin, and [1]Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague

ABSTRACT

Cotton as a new support for the solid-phase synthesis of peptides was evaluated under the conditions of both Boc/Bzl and Fmoc/But strategy. Syntheses of peptides containing only bifunctional amino acids gave comparable results, but peptides containing trifunctional amino acids are clearly advantageously synthesized using the Fmoc approach. The comparison of cotton with other planar carriers (paper or polypropylene membranes) and with classical pellicular ones has shown practical applicability of cotton as the replacement of more sophisticated carriers. N-Methylimidazole was shown to be the optimal acylation catalyst for the modification of the cellulose carrier. Stability of the ester bond to the cotton was studied and shown to be adequate for the syntheses of small to medium size peptides. A relatively low level of substitution of cotton carrier in connection with its ability to soak the solvent was used for the complete elimination of a synthetic vessel. Couplings can be performed by wetting the carrier with the coupling solution, and all solvents can be removed either by squeezing it together with a porous material (paper) or by centrifugation. This leads to a significant decrease of solvent consumption. The efficiency of solvent elimination by centrifugation was demonstrated by the synthesis of model peptide ACP 65-74 in which only one wash between coupling and deprotection and between deprotection and coupling was used and which afforded the product of the same quality as the product synthesized using the standard protocol. Bromophenol blue monitoring has been proven as the optimal way of monitoring the acylation reaction progress. Peptides directly bound to cotton were shown to be useful in enzyme-linked immunosorbent assay tests.

INTRODUCTION

The increase in efficiency by solid-phase synthesis of peptides (32) (for review see, e.g., Reference 4) over synthesis performed in solution was valued by biochemists, cell biologists, immunologists and others who needed a relatively rapid supply of defined peptide sequences. More recently, however, their demands are not being fulfilled as new biochemical techniques are becoming available, and the need for flexible and quickly responding sources of peptide sequences remains. The only solutions which would satisfy this demand would be either to perform the multiple peptide synthesis on compartmentalized supports or to synthesize several peptide sequences in one reaction vessel using the resolving power of HPLC after their cleavage from the carrier (14,38). Apparently the latter approach has its limitations, given the resolving power of HPLC which is complicated by the necessity of very careful analytical characterization of all products of the synthesis.

The first approach, simultaneous multiple peptide synthesis, depends on compartmentalizing the carrier used for the synthesis. Then on a given number of n carrier segments, n different peptides can be synthesized simultaneously, or one sequence can be synthesized under n conditions, allowing for very fast process optimization.

The segmentability of the carrier is required for selectively combining all carriers for identical processes (deprotection and wash) and also following unambiguous separation and resorting to individual acylation reactions without cross-contamination. The carrier segmentation can be achieved either by the inclusion of conventional beaded carrier resins into polypropylene bags (21), syringes (24) or microtiter plates (35), or by use of a compact carrier material that can be divided into distinct pieces such as polyethylene rods (18), membranes (6,11), cellulose paper (12,16) or cotton (26,28).

Planar carriers are well suited for segmentation because of their shape. Their size can be chosen individually according to the desired amount of peptides to be synthesized. Cellulose carriers are inexpensively available as paper and cotton in various shapes. Their hydroxyl groups can be easily modified for peptide synthesis.

MATERIALS AND METHODS

General

A cotton strip (width 3 cm, VEB Bandtex Pulsnitz) was used as the carrier for all investigations and syntheses. For comparative studies, Whatman 540 paper, polypropylene membrane coated with hydroxypropyl acrylate (11) and benzhydrylamine resin (Fluka, 0.08 mmol/g, 1% divinylbenzene (DVB)) were used. Boc-amino acids and bifunctional Fmoc-amino acids were prepared in our laboratory. *Tert*-butyl-type side-chain-protected Fmoc-amino acids were purchased from Serva or Bachem, Fmoc-His(Trt) and Fmoc-Arg(Mtr) from Fluka and Fmoc-Arg(Pmc) from Nova Biochem. Further chemicals were obtained from

Table 1. Synthetic Protocols Used in the Syntheses on Planar Carriers

Step	Reagent	Time (min)
a) Boc-AA		
1. coupling	Box-AA/DCC or DIC/ HOBt in DMF + BB[a]	until decolorization of the carrier
2. wash	DMF	2 × 3
3. wash[b]	ethanol	2 × 3
4. wash	DCM	2 × 3
5. deprotection	25% TFA/DCM	20
6. wash	DCM	2 × 3
7. neutralization	10% DIPEA/DCM	2 × 3
8. wash	DCM	2 × 3
b) Fmoc-AA		
1. coupling	Fmoc-AA/DCC or DIC/ HOBt in DMF + BB[a]	until decolorization of the carrier
2. wash	DMF	2 × 3[d]
3. wash[b]	ethanol	2 × 3
4. wash[c]	DCM	2 × 3
5. deprotection	20% piperidine/DMF	12
6. wash	DMF	2 × 3[d]
7. wash[c]	DCM	2 × 3

[a]BB = bromophenol blue
[b]Only when DCC is used
[c]May be omitted
[d]Number of washes may be decreased

the following companies and used without pretreatment: TFA, DIPEA, BTFA, bromophenol blue, Merck; DCC, DIC, NMI, Fluka; HOBt, Berlin-Chemie; piperidine, Reachim (USSR); CAE, Chemisches Kombinat Bitterfeld.

Gel filtrations were carried out on a Sephadex® G-10 (column: 30 × 1 cm) using 5% acetic acid as eluant or on a Bio-Gel P-4 (100 × 1 cm) using 1 or 3 M acetic acid as eluant. Desalting and pre-purifications on CAE were carried out on a 10 × 1-cm column, which was first washed with 50 ml MeOH and afterwards equilibrated with 0.1% TFA. After injection of the peptide solution, the column was washed with 30 ml 0.1% TFA and then eluted with 50% MeOH/0.1% TFA. The fractions containing peptide were identified by chlorine/benzidine reaction on silica gel plates and combined and evaporated to 1 ml at 30°C. The pre-purification on Sep-Pak™ C_{18} cartridges was carried out analogously. The yields expressed as percentages are related to the determined substitutions of the carrier after attachment of the first amino acid.

Photometric measurements were carried out on a Specord M 40 (Carl Zeiss, Jena, FRG) and HPLC analyses on a Spectra Physics SP 8700 instrument. For prediction of HPLC retention times, the software ELEM/PREDICT (CSPS, Praha, Czechoslovakia) was used. Amino acid analyses were carried out after acidic hydrolysis (6 M HCl, 110°C) on a Durrum 500 device and on an Amino Acid Analyzer T 339 (Mikrotechna, Praha, Czechoslovakia), respectively. Fast atom bombardment (FAB) mass spectra were measured on a ZAB EQ spectrometer (VG Analytical, Manchester, UK) with xenon at 8 kV as bombarding gas. Electron microscopic investigations were carried out on a Phillips EM 400 ST device at an acceleration-voltage of 80 kV. Melting points were measured on a heating table microscope according to Boetius (PHMK, VEB Analytik, Dresden, FRG).

Modification of the Carrier

DMAP-catalyzed carbodiimide/HOBt acylation

a) The cotton or paper carrier (10 cm^2) was shaken successively in 3 ml 25% TFA/DCM (20 min), 2 × 3 ml DCM (3 min each), 2 × 3 ml 10% DIPEA/DCM (3 min each) and 2 × 3 ml DCM (3 min each) and dried between filter paper. Subsequently, the carrier was shaken overnight in 1 ml 0.1 M Fmoc-AA/DCC/HOBt/0.03 M DMAP in DMF. The carrier was washed successively with 2 × 3 ml DMF (3 min each), 2 × 3 ml ethanol (3 min each) and 2 × 3 ml DCM (3 min each) and dried between filter papers. A sample of the carrier was used for the determination of substitution. After shaking in 5 ml 20% piperidine in DMF and washing by the same solution, the absorption of the solution was read at 301 nm. The substitution was calculated according to the formula:

$$\text{Substitution} = \frac{A \times V\,(\text{ml}) \times 1000}{8100 \times F\,(\text{cm}^2)}\ (\mu\text{mol/cm}^2)$$

where A = absorption; V = volume; F = area of the carrier. The absorption coefficient (8100) was determined by a calibration curve using 2×10^{-5} M (A = 0.16), 4×10^{-5} M (A = 0.33) and 8×10^{-5} M (A = 0.65) solutions of Fmoc-Ala in 20% piperidine/DMF. It deviates slightly from the value of 7800, determined by Meienhofer et al. (30), in more diluted solution (7% piperidine in DCM/DMF/isopropanol 3:2:2).

b) The reaction was performed in the same way, with the exception that the solution was placed 4 times for 15 min in the ultrasonic bath (UC 405- BJ, Tesla Lanškroun). In this way, larger quantities of cotton were modified (3 × 300 cm).

N-Methylimidazole (NMI)-Catalyzed Carbodiimide/HOBt-Acylation

The cotton or paper carrier (10 cm^2 was pretreated with TFA/DCM, as described above, and shaken overnight in 1 ml 0.1 M Fmoc-AA/DIC/HOBt/ 0.2 M NMI in DMF or soaked with 0.2 ml 0.5 M Fmoc-AA/DIC/HOBt/1 M NMI and left overnight. After that, the carrier was shaken successively in 2 × 3 ml DMF (3 min each) and 2 × 3 ml DCM (3 min each) and dried between filter papers. The substitution was determined as above.

Attachment of the Acid Labile Handle and the Starting Amino Acid

The handle HO-CH_2-C_6H_4-O-(CH_2)$_2$-COOTcp (HPP-OTcp) was synthesized according to the original procedure described by Albericio and Barany (1). The AA-carrier (10 cm^2) was shaken in 1 ml 0.1 M HPP-OTcp/HOBt in DMF after the addition of 20 µl of a 0.01 M bromophenol blue/DMA solution until the disappearance of the blue color from the carrier (30–60 min), or the carrier was soaked with 0.2 ml 0.3 M HPP-OTcp/HOBt in DMF and left after the addition of bromophenol blue until decolorization. After that, the carrier was shaken successively in 2 × 3 ml DMF (3 min each) and 2 × 3 ml DCM (3 min each) and dried between filter paper. The HPP-AA-carrier formed by this reaction was then acylated with the starting amino acid as described above.

The attachment of the handle already containing the first amino acid of the intended sequence was performed in the same way.

Synthetic Protocol

The protocols given in Table 1 were applied with appropriate modifications to all syntheses described. The carriers were dried between filter papers before and after each coupling. The volumes of the solutions for coupling, deprotection and wash were chosen depending on the carrier size according to the following modes:

Mode S (shaker)—Coupling: 0.1 ml 0.1 M solution per cm^2 carrier; deprotection wash: 0.3 ml solution per cm^2 carrier.

Mode G (glass)—Coupling: 0.02 ml 0.3 M solution per cm^2 carrier; deprotection wash: like in Mode S.

To each coupling, 10–20 µl of a 0.01 M bromophenol blue/DMA solution were added, or this solution was applied first (spiked with HOBt) and the solvent was removed (by centrifugation or filter paper). Couplings according to mode S were carried out under gentle shaking. Using mode G, the carrier was soaked with the acylation reagent and left between two layers of glass until the disappearance of the blue color from the carrier. Syntheses in a shaker vessel using resin carrier were performed in a standard way.

Table 2. Amino Acid Analysis of Peptides I–VIII Synthesized on Acid Labile Handle

Amino Acid[a]	I	II	III	IV	V	VI	VII	VIII
Ala	--	0.93	0.87	0.87	0.92	0.88	0.95	0.92
Tyr	0.72	--	0.73	0.75	0.77	0.78	0.72	0.76
His	1.0	1.0	--	1.0	1.0	1.0	1.0	1.0
Asp	0.9	0.94	0.92	--	0.91	0.86	0.90	0.93
Lys	0.88	0.90	0.87	0.92	--	0.90	0.88	0.92
Thr	0.97	0.93	0.81	0.98	0.85	--	0.93	0.97
Arg	1.02	1.11	1.29	1.02	1.07	1.16	1.02	--

[a]Tryptophan was destroyed under the acidic hydrolysis conditions.

Table 3. Predicted and Found Values of FAB-MS and HPLC Retention Times

Compound	I	II	III	IV	V	VI	VII	VIII
Predicted R_T[a]	15.3	14.2	15.7	15.6	16.3	15.1	12.6	15.9
Found R_T	15.3	11.8	15.9	15.8	16.3	14.3	9.8	16.0
Predicted M+H$^+$	1005	913.5	939.5	961.5	948.4	975.5	890.4	920.4
Found M+H$^+$	1006	914	940	962	949	976	891	921

Experimental conditions: Vydac C_{18} (25 × 0.4 cm), 0%–80% MeOH in 0.05% TFA in 40 min.
[a]Predicted values adjusted to the retention time of peptide I (15.3 min).

Comparative Syntheses Following Fmoc/Bu^t and Boc/Bzl Strategies

Syntheses with bifunctional amino acids. The peptide Ala-Pro-Phe-Ala-Val-Gly was synthesized on 4 × 2 cm^2 (A–D) Gly-cotton prepared according to the procedure described above (NMI catalysis, 2.8 µmol/cm^2) with Boc-Gly used instead of Fmoc-Gly for peptide D. Carrier B was treated with 0.5 ml Ac_2O/NMI/DMF 1:2:3 (v/v/v) for 1 h and afterwards was washed with 2 × 1 ml DMF and DCM (3 min each) before the Fmoc deprotection. Carrier A was acylated with HPP-OTcp and then again with Fmoc-Gly. Peptides A–C were synthesized simultaneously according to protocol b, mode S, and peptide D was synthesized separately according to protocol a, mode S. For the cleavage, carriers B, C and D were shaken separately in 0.5 ml 1 M NaOH and afterwards in 1 ml water. The combined cleavage and wash solutions were adjusted to pH 3 by 1 M HCl, evaporated to 1 ml at 30°C, desalted on CAE and frozen. Carrier A was shaken in 0.5 ml 75% TFA and afterwards in 1 ml TFA. The combined cleavage and wash solutions were evaporated at 30°C, taken up in 2 ml water, filtrated and further treated as described for B–D.

Syntheses with trifunctional amino acids. The alanine replacement set of Arg-Trp-Thr-Lys-Asp-His-Tyr was synthesized on 16 × 5-cm^2 cotton carriers, which were acylated with Fmoc-Gly (see above) for set A and with Boc-Tyr(Bzl) (B/I, B/III–VIII) and Boc-Ala (B/II) for set B, respectively. The determined substitutions amounted to 2.9 µmol/cm^2 for set A, and 0.8 µmol/cm^2 (B/I, B/III–VIII) and 1.9 µmol/cm^2 (B/II) for set B. The carriers of set A were acylated with HPP-OTcp, and, subsequently, the starting amino acids were coupled to it (resulting substitutions A: A/I, A/III–VIII: 1 µmol/ cm^2; A/II: 2.1 µmol/cm^2). The carriers of set B were acetylated using acetic anhydride and NMI in DMF (1 h, room temperature). Set A was synthesized according to coupling cycle b, mode S, and set B was synthesized according to coupling cycle a, mode S. The alanine couplings were carried out separately while all other carriers of each set were coupled simultaneously. Deprotection

Table 4. Substitutions of Carriers Used for the Synthesis of FMDV VP_1 Sequences

Amino acid coupled	Carrier substitution (μmol/cm²)[a]		
	Cotton	Paper	Polypropylene
Ala	2.6	1.9	0.4
Leu	2.7	1.7	0.35
Val	2.6	1.6	0.4
Gln	1.8	1.9	0.3
Leu	2.2	1.6	0.4
Asp(OBu^t)	2.3	1.75	0.35

[a] 1 μmol/cm² = 0.08 mmol/g (paper) or 0.05 mmol/g (cotton) or 0.25 mmol/g (polypropylene)

and washes were carried out together for all carriers of a set.

After the last coupling, the carriers of set A were shaken separately for 2 h in 1.5 ml TFA/EDT (8:2) containing 2.5% indole and washed by 2 ml TFA. The combined cleavage and wash solutions were evaporated at 30°C, taken up in 5 ml water, filtered, extracted with 5 ml ether, evaporated to 2 ml and lyophilized. The crude peptides were gel-filtered on Sephadex G-10 and lyophilized yielding 1.8–4.0 mg peptide (39%–50%). Their amino acid analysis is given in Table 2; HPLC retention times and results of FAB-MS are given in Table 3.

The carriers of set B were shaken in 1 M BTFA/TFA (10% EDT, 2.5% indole) for 2 h and washed with TFA, DCM, ethanol, DCM, 10% DIPEA/DCM and DCM (2 × 10 ml, 3 min each). The peptides were cleaved separately in 1.5 ml 1 M NaOH as described above and lyophilized yielding 1.3–3.1 mg of peptides (36%–51%).

Comparative Syntheses on Polystyrene Resin and Planar Carriers

Ala-Asp-Phe-Ser-Pro-Lys-Leu-Val (IX). The title sequence was synthesized together on 200 mg benzhydrylamine resin cross-linked with 2% divinylbenzene (Chemisches Kombinat Bitterfeld, 1 mmol/g) and 6 cm² (128 mg) of an Ala-paper carrier prepared as described above (NMI catalysis) (2.2 μmol/cm²). Both carriers were first acylated separately with HPP-OTcp and then with Fmoc-Val (substitutions—resin: 0.55 mmol/g; paper: 1.1 μmol/cm² or 0.05 mmol/g). The paper carrier was cut into small pieces and mixed with the resin. A glass column (10 × 2 cm) sealed on the bottom with sintered glass served as a reaction vessel. The amino acids were coupled under stirring according to cycle b using 1.5 ml 0.2 M Fmoc-AA/DIC/HOBt in DMF for coupling and 4 ml of deprotection and wash solutions. After the last coupling, the carriers were separated and shaken in 2 ml 50% TFA/DCM (5% anisole) for 2 h and washed with 2 ml 50% TFA/DCM. The combined cleavage and wash solutions were evaporated at 30°C, taken up in 5 ml water, extracted with 5 ml ether and filtered. The peptide cleaved from the resin was pre-purified on CAE and lyophilized, yielding 41 mg (43%) product. The peptide cleaved from the paper was pre-purified on a Sep-Pak C_{18} cartridge and lyophilized, yielding 4.4 mg (76%) product.

Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly (X, ACP_{65-74}). a) The sequence of ACP_{65-74} was synthesized simultaneously on 1 g of benzhydrylamine resin (0.3 mmol NH_2/g) and cotton strip (18 × 3 cm, modified by Fmoc-Gly, substitution 0.1 mmol/g). Carriers were coupled with Fmoc-Gly-$OCH_2C_6H_4OCH_2CH_2$ $CH_2COOTcp$ in a classical shaker vessel using a semi-automatic home-made solid-phase synthesizer. The synthesis using Fmoc-Asn-OH, Fmoc-Ile-OH, Fmoc-Tyr(Bu^t)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Ala-OH, Fmoc-Gln-OH and Fmoc-Val-OH was performed by DIC/HOBt-coupling, 20% piperidine/DMF (10 min) deprotection and bromophenol blue monitoring (25). Peptide was cleaved from the separated carriers by a TFA/anisole/dichloromethane (50:2:48) mixture (1 h, room temperature), the solution was evaporated, and the residue dissolved in 3 M acetic acid and lyophilized. Products were gel-filtered on Bio-Gel P-4; the resulting yields were 216 mg on resin (72%) and 49 mg on cotton (46%). Amino acid analysis: Asp 2.05, Glu 1.04, Gly 1.14, Ala 2.03, Val 0.91, Ile 1.97, Tyr 0.85. FAB-MS $M+H^+$ = 1064; theory 1063.5.

b) The synthesis was performed on a strip of cotton (18 × 3 cm) in mode G. The same cleavage as in a) afforded 62 mg (58%) of peptide X. Comparison of the product quality is given in Figure 5.

c) Synthesis was performed as in case b). The washing solution (DMF) was added only once after the coupling and after the deprotection and removed by centrifugation. The same cleavage as in a) was applied, and the product was analyzed by HPLC (Figure 5).

Synthesis of FMDV VP_1 Sequences

These peptides were synthesized on 18 × 7-cm² cotton, paper and HPA polypropylene carrier (HO-$CH(CH_3)$-CH_2-O-CO-PP), respectively, which were acylated with Fmoc-Gly according to the procedure mentioned above using NMI as the acylation catalyst (substitution—C: 2.8 μmol/cm²; P: 1.8 μmol/cm²; PP: 0.4 μmol/cm²). HPP-OTcp and the C-terminal amino acids were coupled as above. The substitution determined for all carriers is given in Table 4.

The amino acids were coupled according to cycle a, mode G, except for Boc-Arg(HCl) (cycle a, mode S) with the addition of another carrier of each type to every coupling. After the last coupling, the carriers were shaken separately for 2 h in 1 ml 5% anisole/TFA and washed with 2 ml TFA. The combined cleavage and wash solutions were evaporated at 30°C, taken up in 5 ml water, extracted with 5 ml ether, filtered pre-purified on CAE and lyophilized, yielding 3.0–6.8 mg (43%–58%) (C), 1.8–61 mg (42%–56%) (P) and <1 mg (PP) product. Amino acid analyses: XI Ala 0.86, Leu 1.72, Val 0.81, Glu 0.92, Asp 0.86, Gly 1.0, Arg 1:1; XII Leu 1.82, Val 0.9, Glu 1.0, Asp 1.0, Gly 1.0, Arg 1.0; XIII Leu 1.0, Val 1.1, Glu 1.2, Asp 1.2, Gly 1.0,

Arg 1.0; XIV Leu 1.18, Glu 0.8, Asp 1.02, Gly 1.0, Arg 1.2; XV Leu 0.91, Asp 1.0, Gly 1.0, Arg 1.05; XVI Asp 1.08, Gly 1.0, Arg 1.07.

Synthesis of Peptides XVII–XXI Used for Immunological Studies

Peptides XVII and XVIII were synthesized on 10-cm^2 cotton carriers, which were successively acylated with Fmoc-Gly, HPP-OTcp and the C-terminal amino acids Fmoc-Phe and Fmoc-Asp(OBu^t), respectively, as described above. The obtained substitutions were 1.8 (XVII) and 1.3 (XVIII) μmol/cm^2, respectively. The couplings were carried out according to coupling cycle b, mode G with Fmoc-amino acids [side-chain protection: Ser(Bu^t), Tyr(Bu^t), Glu(OBu^t), Arg (Pmc)]. After the last coupling, the carriers were separately treated with TFA/phenol/thioanisole/water/ethanedithiole (82.5:5:5:5:2.5) mixture (2 h, room temperature) and washed with TFA. The combined cleavage and wash solutions were evaporated at 30°C, taken up in water, extracted with ether, filtrated, pre-purified on Sep-Pak C_{18} cartridges and lyophilized, yielding 6.7 mg (33%) and 4.2 mg (44%) product. FAB-MS $(M+H)^+$: 1148 (XVII); 728 (XVIII).

Peptides XIV-XXI were synthesized as described for peptides XVII and XVIII with the only exception that Fmoc-Ahx was coupled instead of HPP-OTcp. (Substitution of the carrier: 2.3 μmol/cm^2.) After the last coupling, the carriers were shaken together in 10 ml acetic anhydride/pyridine/dimethylformamide (1:2:4) mixture (30 min, room temperature), washed with DMF and dichloromethane and dried. Afterwards the carriers were treated with a TFA/dichloromethane/triisobutylsilane (19:80:1) mixture (1 h, room temperature), washed successively with dichloromethane, 10% diisopropylethylamine/dichloromethane and dichloromethane and dried.

RESULTS AND DISCUSSION

Modification of the Carrier—Attachment of the Starting Amino Acid

Cellulose is a polymer with a molecular weight of up to several millions consisting of glucopyranose rings linked by 1,4-β-glycosidic bonds and forming exclusively unbranched chains (for review see Reference 13). Cellulose is practically insoluble in almost all organic solvents and does not swell in water. The glycosidic bonds are more stable than those of starch, in which the glucopyranose rings are connected by α-glycosidic bonds, but they can be cleaved by acidic hydrolysis, e.g., by hydrochloric or phosphoric acid. Each glucopyranose ring of cellulose has one primary and two secondary OH-groups that are connected by strong hydrogen bridges in which preferably the primary OH-groups are involved. In this case the less reactive secondary OH-groups are more easily accessible to chemical modification than the primary ones. Cellulose esters are formed upon reaction with acid chlorides or anhydrides in the presence of catalysts at which mono-, di- and triesters are formed statistically. Complete triesters are generated only under drastic conditions (elevated temperature).

Table 5. Comparison Between DMAP- and NMI-Catalyzed Acylations of Cotton Carrier

Fmoc-AA	Substitution (μmol/cm^2)[a] DMAP b	DMAP c	NMI c
Gly	1.3	2.7	3.4
Val	0.5	0.9	1.1
Phe	0.6	1.3	1.8
Asp(OBu^t)	0.7	1.2	1.8
Thr(Bu^t)	0.6	1.1	1.2
His(Trt)	0.8	1.4	2.1

Reaction time: 15 h; DMAP: 0.3 eq; NMI: 2 eq (relative to Fmoc-AA/DIC/HOBt)

[a]1 μmol/cm^2 = 0.05 mmol/g
[b]without TFA/DCM pretreatment
[c]after TFA/DCM pretreatment

Because of the lower nucleophilicity of the hydroxyl group compared with the amino group, the usual activations of the carboxylic component in peptide synthesis are not sufficient for its acylation. The method mostly used for the acylation of OH-groups in peptide synthesis is the carbodiimide/HOBt-mediated coupling with dimethylaminopyridine (DMAP) as catalyst (5). The problems, hereby, are the racemization risk for the activated species (3) as well as deprotection of Nα-Fmoc-protected amino acids by the strong base DMAP (2,3). Considering these drawbacks, several alternatives to the DMAP-catalyzed acylation have been suggested recently, such as activation by dichlorobenzoyl chloride (36), carbodiimide/HOBt-activation with reduced HOBt input (19) and alkylation by the cesium salt of the starting amino acid after conversion of the hydroxymethyl groups into halides (31). Frank and Döring (16) used mesitylene sulfonyl nitrotriazolide (MSNT) as an activation reagent for the acylation of OH-groups of Whatman 3MM paper with a methoxytrityl-protected acid labile handle (MeOTrt-O-CH_2-C_6H_4-O-$(CH_2)_5$-COOH), and obtained substitutions of up to 3 μmol/cm^2 or 0.18 mmol/g, respectively.

We have used 3-cm-wide cotton strips for all investigations and syntheses described in this paper. For the comparative syntheses, Whatman 540 paper or HPA Polypropylene membrane of MilliGen were used. A pretreatment of the cellulose carrier with diluted TFA in DCM effects a slight irreversible swelling of the carrier, thus apparently increasing the number of OH-groups available for acylation (see Table 5). This treatment was supposed to have the same effect as the other methods used for the activation of cotton in the textile industry (13). However, we have demonstrated that the short exposure (30 s) to liquid ammonia, used as a convenient method of activation, did not increase the substitution level; only 70% of the substitution level reached on untreated cotton was achieved.

Up to now, Fmoc-amino acid chlorides (9) as highly reactive species have been used mainly for rapid syntheses in a biphasic system (7). Their attachment to Whatman 540 paper was accomplished according to the reaction of cyanuric chloride with cellulose OH-groups described by Hunger et al. (22). The obtained substitution of the carrier was determined by photometric measurement of the dibenzofulvene-piperidine-adduct formed upon cleavage of the Fmoc-group by piperidine/dimethylformamide (DMF) and amounts from 1–2 μmol/cm^2 or 0.08–0.16. mmol/g, respectively. However, the applicability of this reaction is

Table 6. Substitutions of Cotton Carriers after NMI-Catalyzed Acylation with Fmoc-Gly/DIC/HOBt—Dependence on Reaction Time and NMI Amount (Relative to Fmoc-Gly/DIC/HOBt)

Reaction time (h)	Substitution (μmol/cm²)[a]			
	0.5 eq NMI	1 eq NMI	2 eq NMI	4 eq NMI
0.5	0.4	0.6	0.7	0.7
1	0.7	0.8	1.1	1.2
2	0.8	1.0	1.4	1.4
4	1.4	1.7	2.6	2.6
15	2.1	2.8	3.5	3.4

[a] 1 μmol/cm² = 0.05 mmol/g

Table 7. Substitutions of the Cellulose Carrier after Double Acylations with Fmoc-Gly/DIC/HOBt/NMI

Reaction time (h)	Substitution	
	(μmol/cm²)	(mmol/g)
1 × 0.5	0.67	0.05
2 × 0.5	1.38	0.11
1 × 1	1.1	0.09
2 × 1	2.15	0.17
1 × 2	1.38	0.11
2 × 2	2.6	0.21
1 × 4	2.8	0.22
2 × 4	3.4	0.27

limited to bifunctional and polar amino acids. More hydrophobic amino acids as valine or leucine yielded only substitutions of 0.05–0.1 μmol/cm².

Acylations of cellulose OH-groups by dichlorobenzoyl chloride or carbodiimide/HOBt-activation with reduced HOBt input, mentioned above, yield substitutions of <0.1 μmol/cm² and are therefore not suited for this purpose. These findings agree with results obtained by Blankemeyer-Menge et al. (8).

The DMAP-catalyzed carbodiimide/HOBt-coupling of Fmoc-amino acids onto cellulose OH-groups yields high substitutions (see Table 5), but it is accompanied by the drawbacks mentioned above. However, for the introduction of the amino group, which will be used for the easy attachment of the linker molecule onto the cellulose carrier, the side reactions usually accompanying this method (racemization, double acylation) are irrelevant, because the amino acid being coupled to the carrier is not going to become part of the prepared sequence. Moreover, the obvious choice for this purpose is glycine. We have observed a positive influence of ultrasonic bath application for the ester bond formation. After 20 h of shaking the DMF solution of Fmoc-Gly together with DMAP (0.3 equivalents), DIC and HOBt (all 0.5 M solutions), we obtained the same results as when all of these components were introduced into the cotton strip in a volume of DMF equal to the absorption capacity of cotton (1.1 ml/g) and letting it sit for 20 h between two layers of glass to prevent its drying. In both cases the achieved substitution was approximately 0.1 mmol/g (0.097 and 0.103 mmol/g). When the ultrasonic bath was applied for 1 h, instead of shaking, the substitution achieved was 0.122 mmol/g.

NMI is known as a catalyst for the acetylation of hydroxyl groups also on solid phase (10,20), and it can also be used as acylation catalyst in oligonucleotide synthesis (29). The reaction mechanism is analogous to that of DMAP catalysis. First, the *N*-methylimidazolium ion is formed, which then transfers the acyl component onto the OH-function. Because of NMI's much lower pK-value of 7 compared with DMAP, it should be better suited, at least regarding the stability of the Fmoc-group. Indeed, in a solution of Fmoc-Ala in a mixture of NMI/DMF 1:1 (v/v), no free alanine was detected by thin-layer chromatography (TLC) even after a 20-h exposure. Up to now, NMI has not been used in peptide synthesis. Only recently, NMI has been reported as the catalyst for the esterification of cellulose OH-groups with Fmoc-amino acids using MSNT as activation reagent (8).

Table 5 shows that similar or even higher substitutions can be obtained after NMI-catalyzed carbodiimide/HOBt-coupling of Fmoc-amino acids onto cotton carriers compared with the use of DMAP. It could also be shown that the obtained substitutions depend on both the amount of NMI used and the reaction time (see Table 6). Maximum substitution was achieved using 2 equivalents of NMI (related to Fmoc-AA/DIC/HOBt) and 15-h reaction time.

Racemization of the activated amino acids is also suppressed using NMI instead of DMAP. We have not observed amounts of racemization greater than 2.3% (in the case of Cys(Bu^t)—with DMAP this amino acid is racemized by 5%). Protected histidine [His(Trt)] was racemized to the same extent as Asp(OBu^t)—1.2% in the presence of NMI (with DMAP, the corresponding values, determined by gas chromatography, were 6.7% and 2.7%, respectively). However, the racemization risk can be further decreased by using two shorter acylations instead of a long one as shown earlier (8). Results of repeated acylations of a cellulose carrier (paper) are given in Table 7.

For the investigation of the chemical stability of the amino acid cellulose ester, glycine-paper (1.6 μmol/cm²) and glycine-cotton (3.54 μmol/cm²) were treated either with 20% piperidine/DMF or with 25% TFA/DCM for 3 h each. Afterwards, the carriers were acylated with Fmoc-Gly/DCC/HOBt, and the substitutions measured are given in Table 8.

The data can be interpreted as a loss of 0.6% (paper) and 0.9% (cotton) of peptide per one synthetic cycle, respectively, when using Fmoc-amino-acids (deprotection by piperdine) and a loss of 0.5% (paper) and 0.6% (cotton) per one cycle, respectively, when using Boc-amino acids (deprotection by TFA). Considering the fact that these segmental carriers have not been designed for the synthesis of longer peptides, these losses can be tolerated. Also the mechanical stability of the carriers is sufficient during both the acidic and alkaline treatment. Electron microscopic pictures of the carriers before and after the treatment did not show any difference in fiber structure.

The further modification of the carrier for peptide synthesis depends on the purpose for which the peptides are prepared. If a final cleavage and isolation of the peptides are intended, the insertion of a selectively cleavable handle is advisable. For this purpose, acid labile handles of *p*-alkoxybenzyl ester type (for the synthesis of peptide acids) and methoxy-substituted benzhydrylamines (for the synthesis of peptide amides) in combination with the orthogonal Fmoc/Bu^t strategy are best suited. Albericio and Barany (1) described the synthesis of 3-[4-(hydroxymethyl)phenoxy]propionic acid 2,4,5-trichlorophenyl ester (HPP-OTcp), its esterification by the starting amino acid and its subsequent coupling to aminomethyl resins in the presence of HOBt. To avoid the preparation of Fmoc-AA-HPP-OTcp, an alternative route for the introduction of the handle was chosen: after the cleavage of the Fmoc-group from the Fmoc-AA-cellulose carrier, the free amino groups were acylated with HPP-OTcp in the presence of HOBt. The following acylation of the OH-group of the handle with the starting amino acid was carried out analogously to the acylation of cellulose OH-groups with Fmoc-amino acids described above, that means preferably by NMI-catalyzed carbodiimide HOBt-activation. During this reaction, additional cellulose OH-groups are acylated besides the handle OH-groups (10%–15% of the resulting substitution), but the formed cellulose esters are not attacked during the final cleavage of the peptide from the carrier by TFA. Nevertheless, this additional acylation of cellulose OH-groups can be avoided by acetylation of the Fmoc-AA-cellulose carrier in the presence of NMI prior to the attachment of the handle molecule.

The Coupling Step

We have used two different arrangements for the coupling step. The classical way of performing the coupling is the shaking of carrier pieces in the solution of activated amino acid. However, to speed up the coupling process, increasing the concentration of the activated components is desirable. We have found that the best way is to soak a rather concentrated solution of activated amino acid (0.3–0.5 M) into the carrier and let it sit without shaking,

Table 8. Amino Acid Loss after Prolonged Treatment with Deprotection Reagents

Material	Treatment	Substitution (μmol/cm^2) [a]	Content (%)
Gly-paper	0	1.6	100
Gly-paper	20% piperidine, 3 h	1.46	91
Gly-paper	25% TFA, 3 h	1.5	94
Gly-cotton	0	3.54	100
Gly-cotton	20% piperidine, 3 h	3.16	88
Gly-cotton	25% TFA, 3 h	3.30	93

[a] 1 μmol/cm^2 = 0.08 mmol/g (paper) or 0.05 mmol/g (cotton)

Table 9. Solvent Content in Carriers after Different Treatments

Material	Dry weight (mg)	DMF content after Soaking (mg)	(%)	Compression (mg)	(%)	Centrifugation (mg)	(%)
Cotton	160	182	114	38	24	10	6.2
Polystyrene	153	268	175	[a]		101	66

[a] Not determined

ensuring that it does not get dry before the complete coupling is achieved. In this connection, we can point out another positive feature of cotton as a carrier—it is relatively easy to eliminate the liquid from the carrier. In the case of other carriers of the bead type (polystyrene, polyamide), we can use filtration, but in addition, we can use other techniques for the solvent removal. The carrier can be compressed together with dry porous material or it can be centrifuged. In Table 9, you can see the efficiency of solution elimination from the carriers using different treatments. Simple calculation can show the comparison of two carriers and washing protocols. The filtration technique with polystyrene uses 10 ml of the solvent per 1 g of the carrier per wash, and (assuming that complete equilibrium is always achieved) after 3 washes, 0.54% of the original solute may still remain in the carrier (consumption: 30 ml of solvent). Cotton carrier (1 g) using centrifugation should contain only 0.38% of the originally present solute after just one wash (consumption of 1.1 ml of solvent). The efficiency of solvent elimination by centrifugation was demonstrated by the synthesis of model peptide ACP 65-74. In this synthesis, only one wash between coupling and deprotection and between deprotection and coupling was used. The product prepared in this way was shown to be of the same quality as the product synthesized using the standard protocol.

For detecting completeness of couplings, we used the bromophenol blue monitoring suggested by Krchňák et al. (23,25). This noninvasive test is based upon blue coloration of the carrier because of the presence of free amino groups after the addition of a small amount of the indicator to the acylation mixture. Decolorization of the carrier detects absence of free amino groups (completeness of coupling). Particularly for peptide syntheses in μmol scale, this analytical method is recommended because in this case the conventional invasive tests such as the ninhydrin reaction consume a substantial part of the peptide carrier after each coupling.

To ensure the highest sensitivity and speed of the coupling, we have applied the indicator in a separate step prior to the coupling. After removal of the solvent (centrifugation or compression), the solution of activated amino acid was applied. For this method, the indicator solution had to be applied in a solution containing a small amount of *N*-hydroxybenzotriazole; if this slightly

acidic compound was not present, the indicator was eliminated from the carrier together with the redundant solvent. The same phenomenon was observed recently by Flegel and Sheppard (15) during the synthesis performed on the polyamide carrier.

Comparative Syntheses Using Fmoc/Bu^t and Boc/Bzl Strategies

Both the chemical and mechanical stability of the cellulose carriers basically allows for the use of Boc-amino acids as well as Fmoc-amino acids. However, the deprotection of benzyl-protected side chains is problematic. The strong acids such as hydrogen fluoride usually used for that purpose cannot be applied to cellulose supports since they cleave the glycosidic bonds. Exposure of cellulose to water-free hydrogen fluoride leads to its complete degradation with an equilibrium between α-D-glucopyranosyl fluoride and several oligosaccharides being established (33). As an alternative deprotection reagent, boron tris(trifluoroacetate) is described (34), which is said to deprotect even Arg(NO_2).

To test the applicability of Boc-amino acids for peptide synthesis on cellulose carriers, model peptide Ala-Pro-Phe-Ala-Val-Gly consisting exclusively of bifunctional amino acids was synthesized first. The use of the acid labile handle is not possible in combination with Boc-amino-acids. However, the starting amino acid can be also attached directly to the cellulose with final cleavage of the peptide from the carrier by alkaline hydrolysis of the cellulose ester. Therefore, the following syntheses also served for comparison between acid- and alkaline-cleaved peptides.

Three cotton carriers (A,B,C) were acylated with Fmoc-Gly and another one with Boc-Gly by NMI-catalyzed carbodiimide/HOBt-activation. After the deprotection, one Fmoc-Gly carrier (A) was furnished with the acid labile handle, the OH-groups of which were again acylated with Fmoc-Gly. Another Fmoc-Gly carrier (B) was treated with acetic anhydride in the presence of NMI to acetylate the remaining reactive OH-groups of the cellulose. In previous experiments, it had been shown that acetylation of cellulose OH-groups is not possible using the conventional acetic anhydride/pyridine mixture, but in the presence of NMI instead of pyridine, the reaction proceeds to the degree that afterwards no OH-groups are accessible anymore to acylation with amino acids, as described above.

On all four carriers, the peptide Ala-Pro-Phe-Ala-Val-Gly was synthesized using Boc- (D) and Fmoc- (A,B,C) amino acids, respectively. Figure 1 shows HPLC traces of the alkaline (1 M NaOH: B,C,D) and acid (TFA/DCM: A)-cleaved peptides. Obviously, the peptide quality does not depend strongly on the used temporary amino-protecting group (C,D). Also the manner of cleavage has no remarkable influence on the purity of the products (A,B). In case of direct synthesis on cellulose, it is, however, necessary to acetylate nonacylated cellulose OH-groups after coupling of the first amino acid; otherwise, additional acylations and consequently formation of deletion sequences is possible (interpretation of the side peaks in C and D which do not appear in A and B).

The next syntheses were set up to test the applicability to the Boc/Bzl strategy on cellulose carriers including deprotection of benzyl-protected side chains. On the other hand, these syntheses served as a model for the involvement of so-called "problematic" amino acids like tryptophan and histidine into the investigated synthetic method. The influence of particular amino acids on the peptide quality should be examined. For this purpose, the complete alanine replacement set (II–VIII) of a heptapeptide "difficultin" (I) consisting exclusively of trifunctional amino acids was synthesized following both Fmoc/Bu^t and Boc/Bzl strategies.

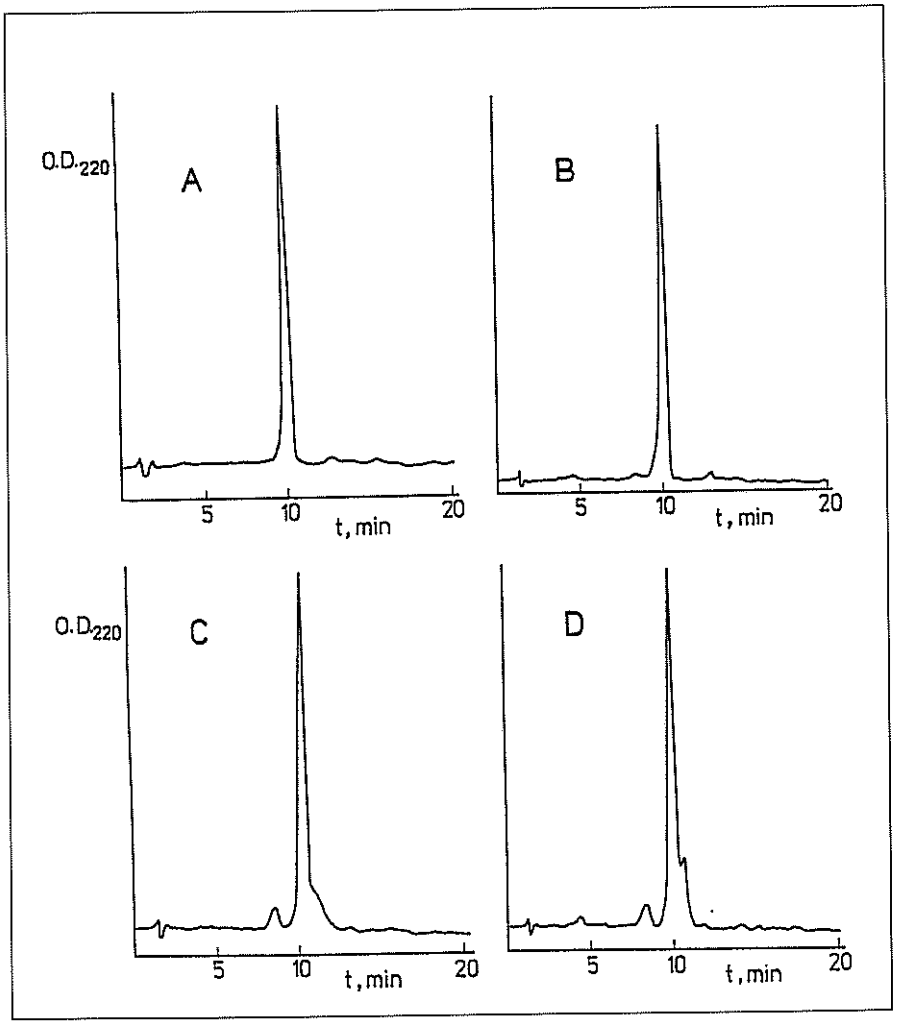

Figure 1. HPLC chromatograms of Ala-Pro-Phe-Ala-Val-Gly (Vydac C_{18}, 20 × 0.4 cm, gradient 20%–60% CH_3CN/0.05% TFA; detection: 222 nm). A: Synthesis performed with Fmoc-AA on acidolytically cleavable handle; B: Fmoc-AA, cleavage by 1 M NaOH, acetylation after attachment of the first amino acid onto cotton; C: Fmoc-AA, cleavage by 1 M NaOH, no acetylation; D: Boc-AA, cleavage by 1 M NaOH, no acetylation.

I, Arg-Trp-Thr-Lys-Asp-His-Tyr
II, Arg-Trp-Thr-Lys-Asp-His-Ala
III, Arg-Trp-Thr-Lys-Asp-Ala-Tyr
IV, Arg-Trp-Thr-Lys-Ala-His-Tyr
V, Arg-Trp-Thr-Ala-Asp-His-Tyr
VI, Arg-Trp-Ala-Lys-Asp-His-Tyr
VII, Arg-Ala-Thr-Lys-Asp-His-Tyr
VIII, Ala-Trp-Thr-Lys-Asp-His-Tyr

The syntheses were carried out simultaneously on cotton carriers, which had been first acylated with HPP-OTcp and then with the starting amino acids for set A. For set B, the C-terminal amino acids were coupled directly to the cellulose, and the carrier was acetylated afterwards. The amino acids were coupled as HOBt esters [derivatives: A: Fmoc-Ala, Fmoc-Tyr(Bu^t), Fmoc-His(Trt), Fmoc-Asp-(OBu^t), Fmoc-Lys(Boc), Fmoc-Thr(Bu^t), Fmoc-Trp, Boc-Arg(HCl); B: Boc-Ala, Boc-Tyr(Bzl), Boc-His(Boc), Boc-Asp(OBzl), Boc-Lys(Z), Boc-Thr(Bzl), Fmoc-Trp, Boc-Arg(HCl)].

The completeness of couplings was followed by the bromophenol blue monitoring (23,25). After coupling of the N-terminal amino acids, the peptides of set A were removed from the carrier acidolytically (TFA/DCM). The peptide carriers of set B were cleaved by 1 M NaOH after treatment with 1 M BTFA/TFA.

Figure 2 shows the HPLC chromatograms of two peptides prepared by Fmoc/Bu^t and Boc/Bzl strategies. The comparison of A and B peptides clearly indicates the superiority of the cleavage conditions. The heterogeneity of the B peptides can be explained by incomplete deprotection as well as by resulting side reactions during the alkaline cleavage, e.g., aspartimide formation during alkaline treatment of Asp(OBzl)-containing peptides (37).

Figure 3 shows the comparison of HPLC chromatograms of several peptides synthesized using Fmoc protection strategy (set A). Interestingly, there are no significant differences in purity, thus demonstrating the feasiblity of peptide synthesis on cellulose carriers: all types of trifunctional amino acids, except for cysteine which was not tested in this set, can be used in this synthetic method. As to be expected, the peptide not containing tryptophan (A VII) shows the highest purity because the alkylation reactions during the cleavage described for tryptophan-containing peptides cannot occur here.

Comparative Syntheses on Resin and Planar Carriers

For comparison of peptides synthesized on conventional and cellulose carriers, model peptides IX and X (Acyl Carrier Protein 65-74) were synthesized at the same time on a BHA polystyrene resin and on an Ala-paper or Gly-cotton carrier placed in the same reaction vessel. The syntheses were carried out on the HPP-OTcp handle using HOBt esters. In the first case, Fmoc-Val, Fmoc-Leu, Fmoc-Lys(Boc), Fmoc-Pro, Fmoc-Ser(Bu^t), Fmoc-Phe and Fmoc-Asp(OBu^t) were coupled successively to the carrier. After the last coupling, the carriers were separated and the peptides cleaved and isolated separately.

IX, Ala-Asp-Phe-Ser-Pro-Lys-Leu-Val
X, Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly

The HPLC chromatograms (Figure 4) of both products contain the same main component, which is identical with the desired sequence according to FAB-MS and the same side products as well. However, the content of the principal side product is considerably higher in the peptide synthesized on the resin than in the one synthesized on paper. According to FAB-MS, this component lacks the serine residue. In this synthesis the ninhydrin test was used as the test for the complete coupling on the resin. This reaction is much less sensitive to the secondary amino group of proline than to the primary amino groups of the other amino acids. Consequently, a false negative ninhydrin test after the proline

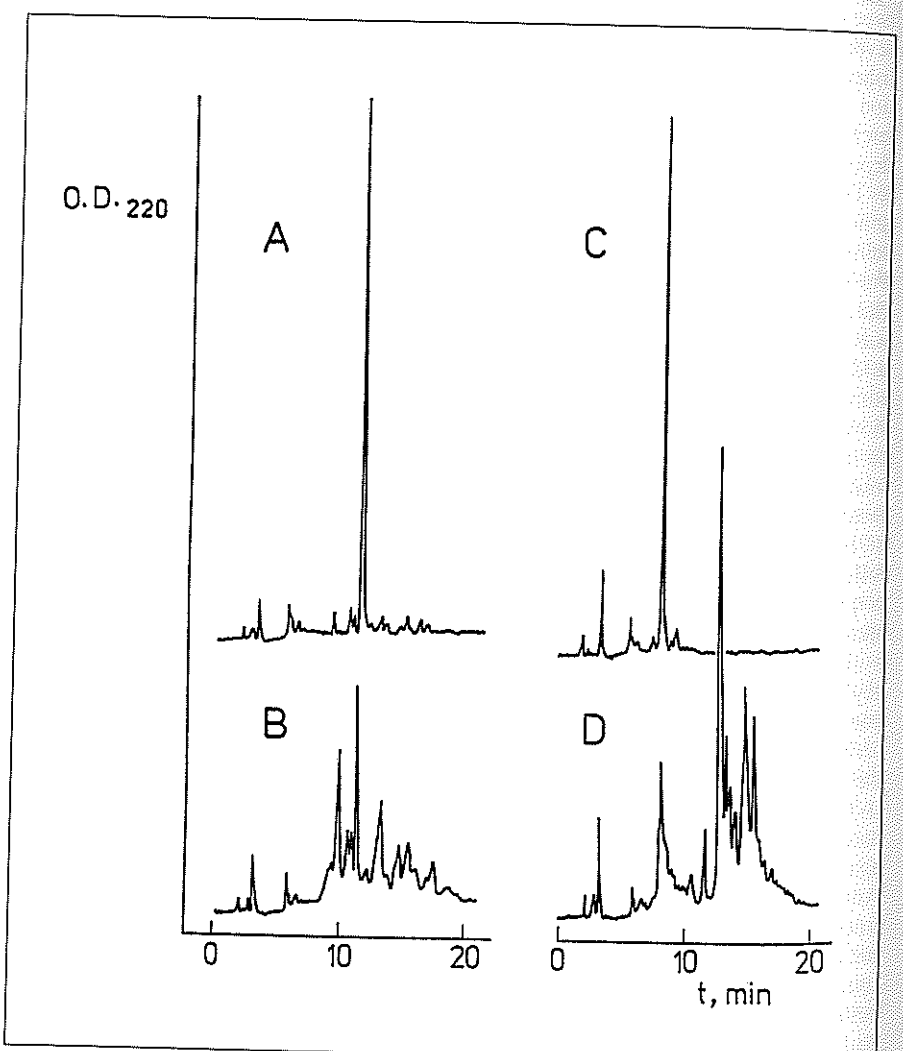

Figure 2. HPLC chromatograms of Arg-Trp-Thr-Lys-Asp-Ala-Tyr (A and B) and of Arg-Ala-Thr-Lys-Asp-His-Tyr (C and D) synthesized by Fmoc/Bu^t (A and C) or by Boc/Bzl (B and D) strategy (Ultrasphere ODS 4.6 × 25 cm, 0%–40% CH_3CN/0.05 M KH_2PO_4, pH 3, 20 min, 220 nm).

coupling is probably the cause of the formation of the des-Ser deletion sequence. It can be concluded, however, that the coupling of serine proceeded faster on paper.

As the second test peptide, we have chosen the sequence 65-74 of Acyl Carrier Protein (X), which is known to cause trouble during the synthesis. This synthesis was performed similarly: first, Fmoc-Gly-HPP-OTcp was coupled to both benzhydrylamine resin and Gly-cotton, and half of the cotton strip was placed into the shaker together with the resin. The second half of the cotton strip was placed between two pieces of glass, and the synthesis was performed with the successive wetting and drying (squeezing together with filter paper) technique. The following amino acid derivatives were coupled to all carriers: Fmoc-Asn-OH, Fmoc-Ile-OH, Fmoc-Tyr(Bu^t)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Ala-OH, Fmoc-Gln-OH, Fmoc-Val-OH.

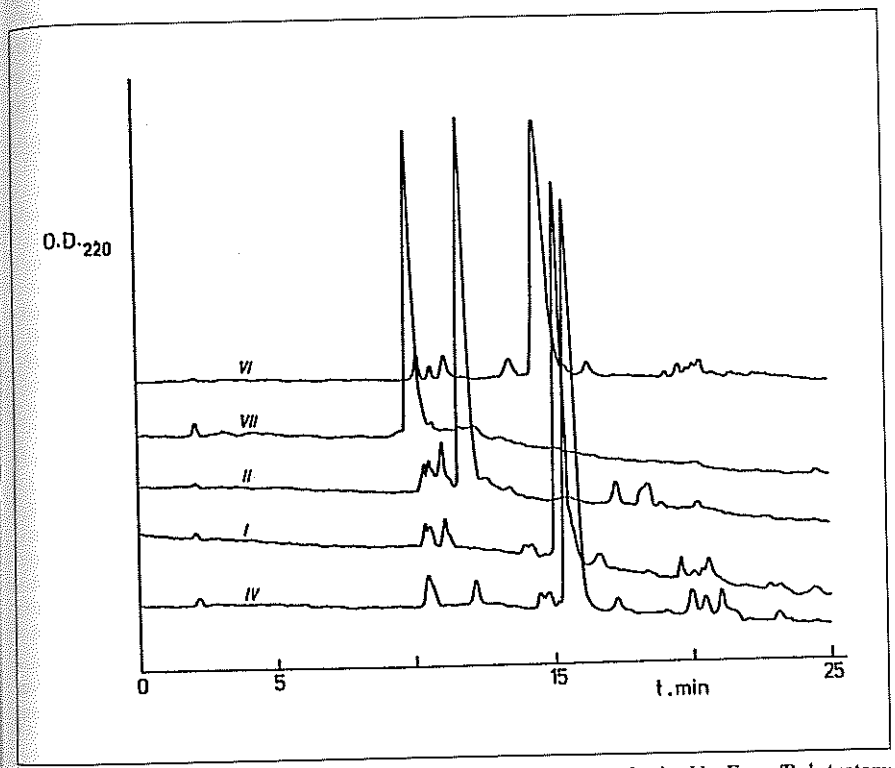

Figure 3. HPLC chromatograms of peptides I, II, IV, VI, and VII synthesized by Fmoc/Bu^t strategy (Vydac C_{18}, 25 × 0.4 cm, 0%–60% MeOH/0.05% TFA, 30 min).

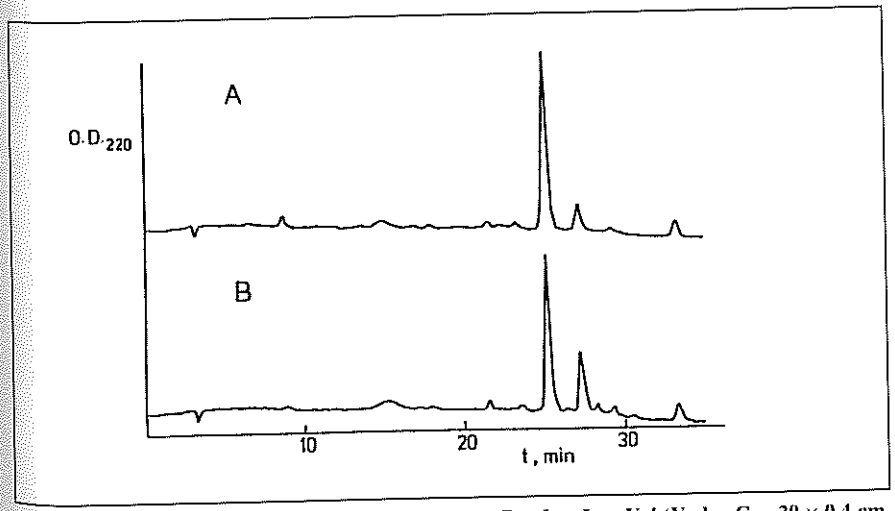

Figure 4. HPLC chromatograms of Ala-Asp-Phe-Ser-Pro-Lys-Leu-Val (Vydac C_{18}, 20 × 0.4 cm, 0%–70% MeOH/0.05% TFA, 35 min). A: synthesized on paper, B: synthesized on BHA resin.

The results of the separate cleavage of peptide X is shown in Figure 5. The best result was obtained on the cotton placed between two glass layers during the coupling steps; the peptide is basically without any side products. The same deletion peptide (des-Val analogue) was observed in products prepared in a shaker vessel on both carriers. Again, the wrong monitoring may be blamed for this result. In this case, however, the coupling of Fmoc-Val was faster on polystyrene resin.

XI, Arg-Gly-Asp-Leu-Gln-Val-Leu-Ala (VP1 145-152)
XII, Arg-Gly-Asp-Leu-Gln-Val-Leu
XIII, Arg-Gly-Asp-Leu-Gln-Val
XIV, Arg-Gly-Asp-Leu-Gln
XV, Arg-Gly-Asp-Leu
XVI, Arg-Gly-Asp

Another test case described here is the set of C-terminal-shortened peptides of the FMDV VP1 145-152 sequence. These compounds were synthesized to determine the shortest sequence exhibiting the protective function for FMDV-sensitive endothelium cells. The sequences XI–XVI were prepared on paper, cotton and polypropylene membranes overcoated by hydroxypropyl acrylate (11). All three carriers were acylated with Fmoc-Gly, yielding the following substitutions:

Cotton (C): 2.8 μmol/cm^2 or 0.14 mmol/g

Paper (P): 2.1 μmol/cm^2 or 0.17 mmol/g

HPA-polypropylene (PP): 0.4 μmol/cm^2 or 0.1 mmol/g

The relatively low substitution per area of the PP carrier is due to its lower specific weight (4 mg/cm^2) compared with the cellulose carriers used (cotton: 20 mg/cm^2; paper: 12 mg/cm^2).

The peptides XI–XVI were synthesized simultaneously on all three carriers by adding another carrier of each type with the acid labile handle and the starting amino acid to each subsequent coupling cycle. The peptides were cleaved from each carrier separately and compared by HPLC. As in Figure 6, there is no essential difference between the peptides synthesized on cotton (C), paper (P) and polypropylene (PP) carriers concerning the quality of the crude peptides.

Example of an Application of Cotton-Bound Peptides

For immunological investigations, e.g., for solid-phase immunoassays and for immunization with short synthetic peptides, carrier-bound peptides are needed. Conventionally, two different carriers for peptide synthesis and for the immunological application are used. However, the same carrier may be used for both purposes. The polyethylene rods of Geysen et al. (18) have been reported with only nanomole amounts of peptides being synthesized for carrier-bound testing of the peptides.

We also attempted to apply peptides synthesized on cotton carriers directly in solid-phase immunoassays. We have synthesized three loop sequences of α- and β-adrenergic receptors twice on the cotton carrier.

XVII, Gly-Arg-Trp-Glu-Tyr-Gly-Ser-Phe-Phe
XVIII, Arg-Glu-Leu-Val-Pro-Asp
XIX, Ac-Gly-Arg-Trp-Glu-Tyr-Gly-Ser-Phe-Phe-Ahx-Gly-Cotton
XX, Ac-Arg-Glu-Leu-Val-Pro-Asp-Ahx-Gly-Cotton
XXI, Ac-Ser-Thr-Leu-Lys-Pro-Pro-Asp-Ahx-Gly-Cotton

Peptides XVII and XVIII were synthesized on the acid labile handle and acidolytically cleaved from the carrier. The isolated crude peptides were of sufficient purity according to HPLC (69% and 71%, respectively), so that they could be used without purification.

Peptides XIX–XXI have been synthesized for carrier-bound use in ELISA tests. In this synthesis, ε-aminohexanoic acid (Ahx) was inserted instead of the handle to provide a certain distance between the carrier and the peptides; this is believed to be advantageous for recognition by the antibody. The amino groups of the N-terminal amino acids were acetylated and the side chains were deprotected by 20% TFA in dichloromethane containing 1% triisobutylsilane (17). As negative control, the modified carrier without peptide was used: Ac-Ahx-Gly-Cotton. The unspecific adsorption of antibodies by the carrier could be suppressed by the usual treatment with BSA. It could be shown by ELISA tests and bioassays (the results of which will be published elsewhere) that the cotton-bound peptides exhibit the same effects as do the corresponding free peptides.

CONCLUSIONS

The given data suggest that cotton can be successfully used for the solid-phase synthesis of peptides using the Fmoc/Bu^t strategy. Its modification is simple; it can be performed under mild conditions, as the modified carrier will remain stable. Properties of the carrier allow for the multiple or continuous modification of the synthesis and for different solvent handling. The synthesis does not have to be performed in a reaction vessel since the capillarity of the cotton can hold a sufficient amount of the solution. Elimination of the solvent by centrifugation is very efficient. These properties are promising from the point of view of construction of an automatic synthesizer (27). One possible application of peptides bound to the cotton carrier would be its use in the ELISA test.

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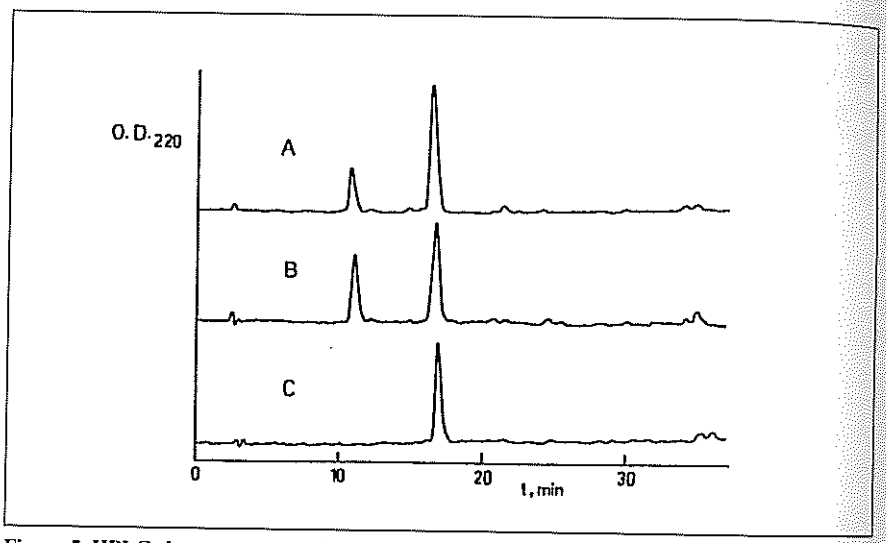

Figure 5. HPLC chromatograms of ACP_{65-74} synthesized in the shaker vessel A: on cotton, B: on polystyrene resin, or C: on cotton placed between two layers of glass (Vydac C_{18}, 20 × 0.4 cm, 0%–70% MeOH/0.05% TFA, 35 min).

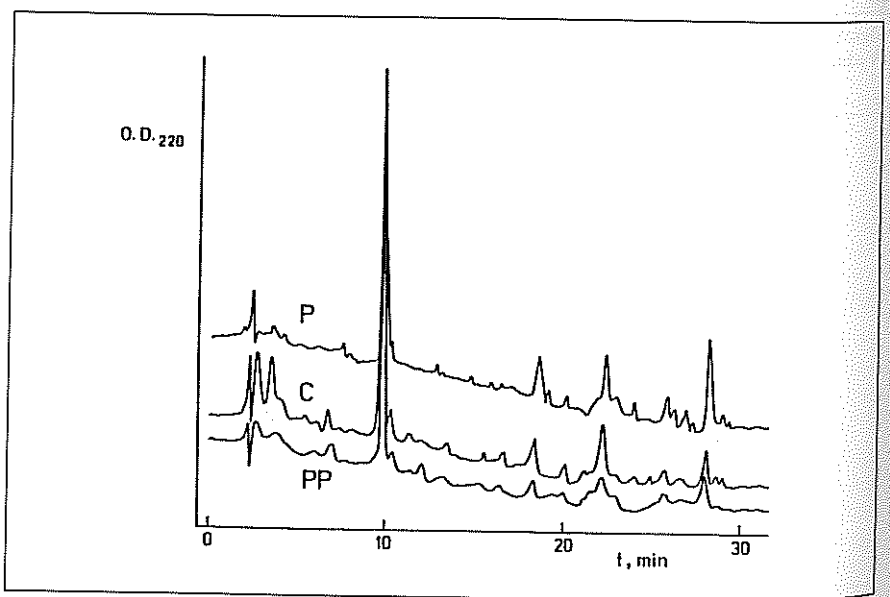

Figure 6. HPLC chromatograms of peptide XII prepared on cotton (C), paper (P) and polypropylene (PP) (Vydac C_{18}, 25 × 0.4 cm, 5 μm, 10%–70% CH_3CN/0.05% TFA, 30 min, 220 nm).

thors express their thanks to Dr. Daniels for the generous gift of polypropylene membrane.

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Address correspondence to:

Dr. Michal Lebl
Selectide Corp.
10900 N. Stallard Pl.
Suite 122
Tucson, AZ 85737