Peptide Research

Comparative Multiple Synthesis of Fifty Linear Peptides: Evaluation of Cotton Carrier Vs. T Bag–Benzhydrylamine Resin

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ABSTRACT

Parallel simultaneous synthesis of fifty linear peptides has been carried out in order to compare in detail two promising methodologies of simultaneous multiple peptide synthesis (SMPS): the "T bag" method, utilizing 4-methyl-benzhydrylamine resin (MeBHA), and synthesis on derivatized Fmoc-Gly-O-cotton fabric strips. The basic set of experiments, which utilizes identical Fmoc/Bu^t strategy for both approaches, shows that the peptides synthesized on cotton are superior in purity to those synthesized using T bags. In experiments utilizing Boc/Bzl strategy in T bags, the purities of peptides were higher than in the case of peptides synthesized in T bags by Fmoc/Bu^t strategy, and comparable with the purities achieved in synthesis performed on cotton. The lower yields on cotton are caused by mechanical losses in the given experimental arrangement.

INTRODUCTION

The methodology of solid-phase peptide synthesis (SPPS) (1,2,12,26) has evolved over recent years into several approaches of multiple peptide synthesis, i.e., preparation of several peptides of varying sequence simultaneously, utilizing common synthetic protocol. Thus, considerable saving of time necessary for the synthesis is achieved. Many techniques of this kind have been reported. They differ widely in the nature of the solid support [classical polymeric supports (3,15–19,21, 22), cellulose (6,9,13,14,24), polyethylene (4)], in the experimental arrangement [columns filled with polymer beads (15,16), T bags (3,17,18), injection syringes (21,22), plastic pins (7) or sheets (4,8), cotton strips (10,11,25), paper discs (6,13)] and in the strategy of the synthesis (mostly Boc/Bzl vs. Fmoc/Bu^t chemical protocols). Some of these syntheses were partially or fully automated and several designs of multiple peptide synthesizers have appeared. However, a detailed mutual comparison of these grossly varying approaches has yet to be reported.

Our interest in cotton as support for SPPS led us to perform a detailed comparison of peptide synthesis on this carrier with another multiple peptide synthesis approach. We selected the combination of MeBHA with the T bag approach of Houghten (17,18). The selection is based on the fact that the treatment of polymeric resin, encapsulated in separate polypropylene mesh T bags, resembles closely the handling of cotton strips and, moreover, such a choice makes it possible to use common reaction conditions and vessels when executing simultaneous multiple synthesis on both carriers.

Using the approaches mentioned, we synthesized fifty linear peptides (Table 1) including ACP (65-74), its omission analogs, adipokinetic hormone II, allatostatin I, alytesin, fragments of calcitonin, gastrin I, MSH-α and some peptides serving as substrates for HIV-1 proteinases. We intended to perform the comparison as rigorously as possible, i.e., to keep all the bags and cotton fabric strips in one reaction or wash bath, with the exception of the condensation step (also, during the condensation, the T bag and corresponding cotton carrier were together). At first glance, practical execution of such a synthetic plan looked easy; however, in reality it was necessary to compromise somewhat both approaches in order to make the comparison feasible. The first restriction comes from the necessity of using an identical strategy for both carriers. This forced us to use a base-labile Fmoc protecting group and a TFA-labile linker, because cotton-based synthesis can use acid-labile temporary protecting groups only with some limitations (cotton does not survive hydrogen fluoride treatment and its stability towards repeated exposures to TFA is limited). It is to be noted here that most of the syntheses using T bags utilized Boc strategy (17–19), TFA deprotection and HF cleavage, while the use of this method in combination with Fmoc strategy is rare (3). For comparison, several peptides from our set (see Table 1, peptide numbers 1, 27, 32–50) were synthesized in T bags using both Fmoc/Bu^t and Boc/Bzl methodologies. The second restriction comes from mechanical and adsorption properties of both carriers. While sufficient conversion within the coupling step and sufficient efficiency of washes can be achieved with cotton soaked with the proper solvent (preferably using high concentrations of reaction components), the T bag method requires vigorous shaking. Vigorous shaking when applied to cotton strips causes mechanical losses by bruising. This problem had to be accepted because we wanted to maintain the use of

Table 1. List of Peptides Synthesized in This Study

1.	VQAAIDYING	ACP(65-74)
2.	VQAAIDYIDG *[a]	
3.	VEAAIDYIDG *[a]	
4.	VEAAIDYING *[a]	
5.	VQAAIDYIN *[a]	
6.	VQAAIDYIG *[a]	
7.	VQAAIDYNG *[a]	
8.	VQAAIDING *[a]	
9.	VQAAIYING *[a]	
10.	VQAADYING *[a]	
11.	VQAIDYING *[a]	
12.	VAAIDYING *[a]	
13.	QAAIDYING *[a]	
14.	E*LNFTPNWGT	Adipokinetic Hormone (AKH)
15.	E*LNFSTGW	Adipokinetic Hormone II[e]
16.	E*LNFSAGW	Adipokinetic Hormone II[f]
17.	APSGAQRLYGFGL	Allatostatin 1
18.	E*GRLGTQWAVGHLM	Alytesin
19.	MLGTYTQDFNKF	Calcitonin (human) (14-25)
20.	HTFPQTAIGVGAP *[b]	
21.	TFPQTAIGVGAP *[b]	
22.	FPQTAIGVGAP *[b]	
23.	PQTAIGVGAP *[b]	
24.	QTAIGVGAP *[b]	
25.	TAIGVGAP *[b]	
26.	AIGVGAP *[b]	
27.	E*GPWLEEEEEAYGWMDF	Gastrin I (human)
28.	LEEEEEAYGWMDF *[c]	
29.	EAYGWMDF *[c]	
30.	YGWMDF *[c]	
31.	AcSYSMEHFRWGKPV	MSH-α
32.	MEHFRWGKPV *[d]	
33.	PLIMAVVN	
34.	AAAMSSAI	
35.	PAVSLAMT	
36.	VVAMPVVI	
37.	PYVGSGLY	
38.	FQAYPLRE	
39.	PLFAGISD	
40.	ATVLTVAL	
41.	GHRPLDKC	
42.	GGGVRGPRVC	
43.	AGNALMDGASQ	
44.	YVATRDNCI	
45.	NYKGSWYSMR	
46.	ASQLMGEN	
47.	EFPSRGKSSSY	
48.	KKREEAPSLR	
49.	ARPAKAAATQ	
50.	ASTGKTFPG	

*Analog of: [a]ACP(65-74); [b]calcitonin (human); [c]gastrin I (human); [d]MSH-α; [e]*Schistocerca gregaria*; [f]*Locusta migratoria*. E*: Pyroglutamic acid.

common reaction and wash baths, to ensure identical conditions for both methodologies. However, it is necessary to keep in mind that the yields achieved on cotton carrier are, for this reason, slightly lower. It was shown (27) that the coupling rate of activated amino acid on cotton depends on the concentration of the activated species and not on its excess (once the reasonable excess—usually three molar—is available in solution volume). This fact allowed us to use different amounts of both carriers in the same reaction vessel without the danger of biasing the results by using different excesses of reactants for each carrier.

EXPERIMENTAL

Modification of the Carriers for Fmoc-Based Synthesis

For T bag synthesis we used polypropylene mesh (Chicopee Industries, Gainsville, GA) bags loaded with 200 mg of MeBHA resin (0.5 mmol/g, 100–200 mesh; Advanced Chemtech, Louisville, KY). As cotton carrier, 1-sq.-inch pieces of cotton textile band, modified with Fmoc-Gly-OH (0.15 mmol/g, 3 μmol/cm^2), were used (11). *N*-Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)benzhydrylamine (Q-1660; Bachem, Bubendorf, Switzerland) (5) served as the TFA-labile linker. The Fmoc-Gly-O-cotton was separately deprotected with 20% piperidine in DMF (30 min), washed with DMF (3 × 2 min) and dichloromethane (4 × 2 min). The resin, sealed in T bags, was first neutralized with 10% diisopropylethylamine (DIEA) in dichloromethane (2 × 3 min) and washed with dichloromethane (3 × 1 min). All subsequent steps were applied to both carriers simultaneously. After one wash with DMF, the linker was condensed with DIC and HOBt in the presence of bromophenol blue as the monitoring agent (22,23,25). We used molar ratios of linker:DIC:HOBt:bromophenol blue:combined free NH_2 groups on the resin and cotton (3:3:3:0.001:1). The components were applied in DMF solution (3 ml per one T bag–cotton strip pair). After vigorous overnight shaking, the batch was washed 3 times with DMF and 3 times with dichloromethane. The substitution ratios of both modified carriers (resin: 0.46

mmol/g; cotton: 0.13 mmol/g) were determined spectrophotometrically (11) at 301 nm utilizing absorption of the fulvene chromophore from the Fmoc group after deprotection. The measurements were carried out on a Spektromom instrument (MOM, Budapest, Hungary). The given substitution value is an average from three estimations, which did not differ by more than 5%.

Synthesis of Peptides — Fmoc Strategy

The Fmoc-protected amino acid derivatives were obtained from Bachem and were used without further purification. For the side-chain protection, we used Bu^t for Asp, Glu, Ser, Thr and Tyr; Mtr for Arg; Trt for Cys and His; and Boc for Lys. The following synthetic protocol was used:

1. N-terminal deprotection with 20% piperidine in DMF (30 min);
2. DMF wash (3 × 2 min);
3. dichloromethane wash (4 × 2 min);
4. coupling step with Fmoc-AA and DIC/HOBt monitored with bromophenol blue (molar ratio Fmoc-AA:DIC:HOBt:bromophenol blue:carrier substitution 6:6:6:0.001:1; 6 ml of DMF solution per one T bag–cotton strip pair), (overnight);
5. DMF wash (1 × 2 min);
6. dichloromethane wash (2 × 1 min);
7. ethanol wash (1 × 2 min);
8. dichloromethane wash (2 × 2 min);
9. DMF wash (1 × 2 min).

Steps 4–6 were carried out in separate vessels. When the T bags or cotton strips exhibited green to blue coloration, they were subsequently acetylated prior to further cycling (0.47 ml acetic anhydride, 0.7 ml triethylamine, 5 ml DMF per one T bag–cotton strip pair, 15 min, followed by washes [1× DMF, 3× ethanol, 2× DMF]). All steps were carried out with vigorous mechanical shaking. Final deprotection and cleavage of peptides from the carriers was performed with mixture K (82.5% TFA, 5% phenol, 5% water, 2.5% 1,2-ethanedithiol, 5% thioanisole) (20). We used 2 ml of the reagent per one cotton strip or 100 mg of the peptide on the resin (the resin was taken out of the T bag). The reaction was carried out in a polyethylene syringe equipped with a Teflon sintered disc, internal volume 5 ml. Two series of deprotecting experiments were carried out. In one of them the peptides not containing Arg(Mtr) were exposed for 1 h, those containing Arg(Mtr) for 3 h. In the second series, the respective reaction times were 2 h and overnight. After completion, the solution of free peptide was squeezed off and precipitated in 30 ml of dry ether. The support was extracted with 1 ml of TFA (3 min) on a shaker, the extract was added to the first portion and the precipitate was washed 4 times with 30 ml of ether (30 s in an ultrasonic bath followed by centrifugation). Finally the peptides were dissolved in 2 × 7 ml of 15% acetic acid and lyophilized. To test whether low yields of peptides from the cotton carrier were caused by the solubility of the peptide in ether under these conditions, we dissolved 3.2 mg of pure peptide 26 in 0.5 ml mixture K, precipitated it with 7.5 ml of ether and added 0.25 ml TFA. The precipitate was centrifuged and resuspended four times in 7.5 ml of ether. The pellet was dissolved in 5 ml of 15% acetic acid and lyophilized. We obtained 1.0 mg (31%) of peptide 26.

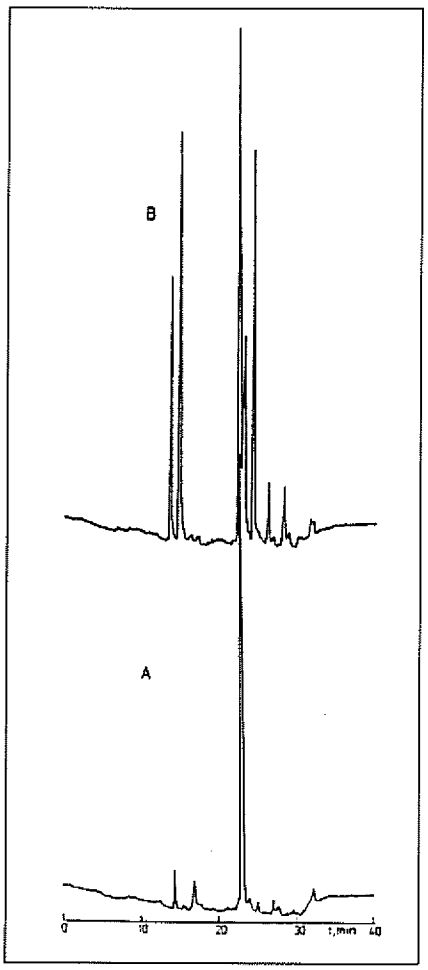

Figure 1. HPLC traces of deprotected crude peptide 5 (VQAAIDYIN)—Fmoc/Bu^t strategy. A) Cotton carrier; B) T bag.

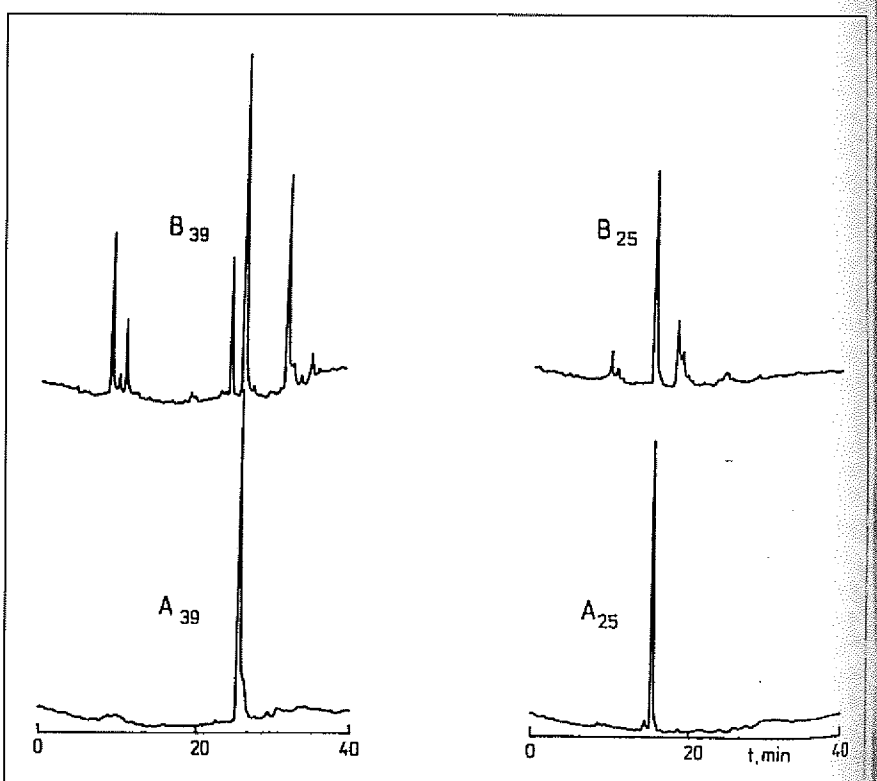

Figure 2. HPLC traces of deprotected crude peptide 25 (TAIGVGAP) and 39 (PLFAGISD)—Fmoc/Bu^t strategy. A) Cotton carrier; B) T bag.

Synthesis of Peptides — Boc Strategy

The protected amino acid drivatives were purchased from Bachem and used without further purification. The following protecting groups were utilized: Bzl for Asp, Glu, Ser and Thr; 2-BrZ for Tyr; Tos for Arg; 2-ClZ for Lys; MeBzl for Cys; DNP for His and For for Trp. We used T bags loaded with 100 mg of MeBHA resin (0.5 mmol/g). The synthetic protocol was as follows:

1. N-terminal deprotection, 55% TFA in dichloromethane (30 min);
2. dichloromethane wash (1 min);
3. isopropyl alcohol wash (2 × 1 min);
4. dichloromethane wash (2 × 1 min);
5. neutralization, 5% DIEA in dichloromethane (3 × 2 min);
6. dichloromethane wash (2 × 1 min);
7. coupling step, Boc-AA:DIC:free amino groups (molar ratio 6:6:1), 4 ml of dichloromethane per one T bag (Boc-Gln and Boc-Asn were condensed with an equimolar amount of HOBt in 4 ml of DMF), (1 h);
8. DMF wash (2 × 30 s);
9. dichloromethane wash (2 × 1 min).

Steps 7 and 8 were performed in separate vessels. The deprotection procedure was carried out in several steps: First, in the peptides containing His, the DNP group was cleaved by 5% thiophenol in DMF (3 × 1 h), followed by alternating washes of isopropanol and dichloromethane, 10 times each. The Boc group was cleaved with 55% TFA in dichloromethane (30 min). Final deprotection and cleavage of the peptide from the support was performed by means of the "high HF" procedure (27,28) (HF:anisole, 9:1, v/v, 7 ml per content of one T bag, 1 h at 0°C). The peptides containing Trp(For) were deprotected with HF:anisole:1,2-ethanedithiol (9:0.5:0.5) (28,29). After blowing out HF with nitrogen, the scavengers were extracted with 3 × 10 ml of ether. The peptide was extracted with 2 × 10 ml of 10% AcOH and lyophilized.

All products of these syntheses were analyzed by HPLC (Spectra-Physics, San Jose, CA) (pump, SP 8800; detector, SP 8450; integrator, SP 4290; autosampler, SP 8780) on a Vydac C_{18} column (The Separations Group, Hesperia, CA) (4.6 mm × 250 mm) at 222 nm and linear gradient 0%–100% MeOH/0.05% TFA/60 min (flow rate: 1 ml/min). The selection of a proper HPLC peak of the peptide product in a

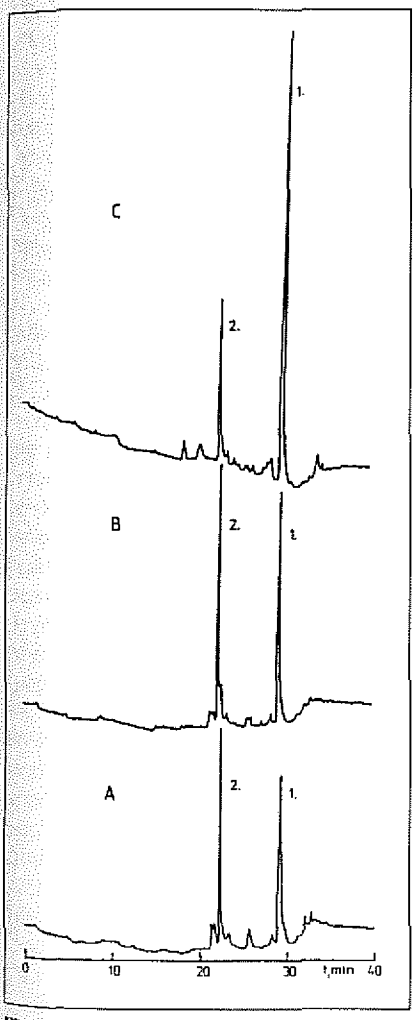

Figure 3. HPLC traces of deprotected crude peptide 36 (VVAMPVVI), comparison of Fmoc and Boc strategies. A) Cotton carrier, Fmoc/Bu^t; B) T bag, Fmoc/Bu^t; C) T bag, Boc/Bzl. 1 = Main product peak; 2 = impurity containing methionine S-oxide.

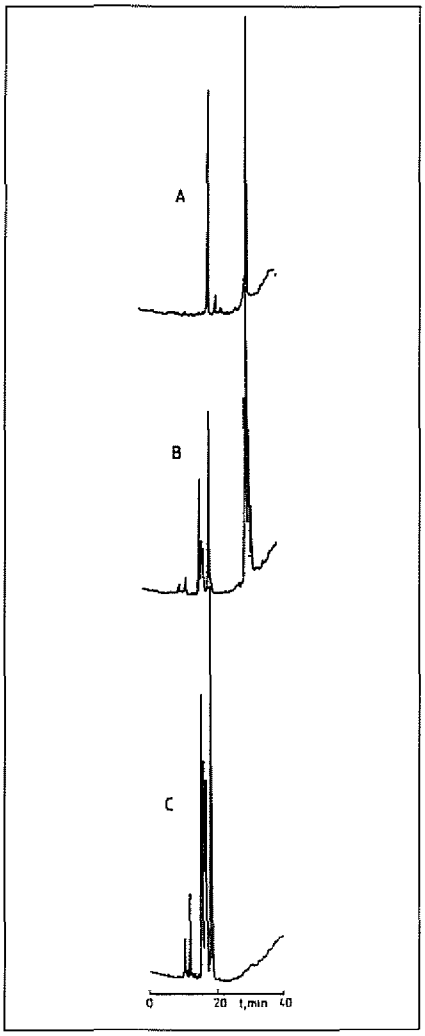

Figure 4. The effect of prolonged final cleavage time on the purity of peptide 38 (FQAYPLRE). A) Cotton carrier, Fmoc/Bu^t, standard conditions; B) T bag, Fmoc/Bu^t, standard cleavage time; C) T bag, Fmoc/Bu^t, prolonged cleavage time.

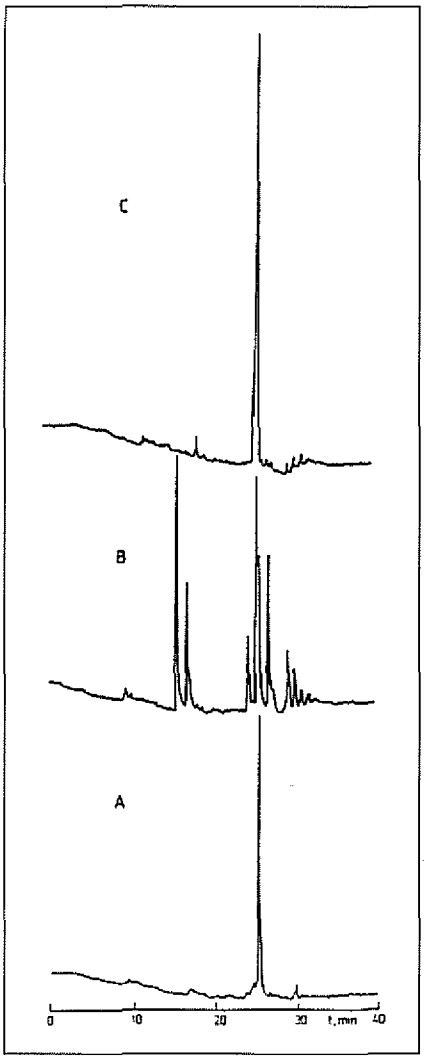

Figure 5. HPLC traces of peptide 1 (VEAAIDYIDG), comparison of Fmoc and Boc strategies. A) Cotton carrier, Fmoc/Bu^t strategy; B) T bag, Fmoc/Bu^t; C) T bag, Boc/Bzl.

crude reaction mixture was simplified by the use of the ELEM/PREDICT program (CSPS, Prague, Czechoslovakia). Amino acid analyses were carried out after acidic hydrolysis (6 M HCl, 110°C, 20 or 70 h) on a Durrum 500 device (Durrum, Palo Alto, CA) and on an amino acid analyzer (T 339; Mikrotechna, Prague, Czechoslovakia). FAB MS spectra were measured on a ZAB EQ spectometer (VG Analytical, Manchester, UK).

RESULTS AND DISCUSSION

We used bromophenol blue monitoring (23) throughout the whole Fmoc/Bu^t experiment. With cotton, only a few strips were green to green-blue after the coupling step. With T bags, nearly all were green to blue and only rarely white or yellow. This suggested that the couplings proceeded better on cotton than on T bags. Numerical evaluation and monitoring data of our basic set of experiments (Fmoc/Bu^t strategy) are shown in Table 2. All syntheses were successful at least to the point that the desired peptide was identified with certainty by FAB MS and amino acid analysis and we were able to evaluate its yield and HPLC purity. The average gain of weight of protected peptide on resin was 82%. This is in good accord with our data obtained earlier (19) where we found values between 76% to 88% using Boc/Bzl strategy. It was not possible to calculate the yield of protected peptide on cotton, owing to partial mechanical disintegration during vigorous shaking in the presence of T bags. (Average weight gain was determined in the repeated syntheses of peptides 20–26 and it was found to be 67%.) The yields of crude peptides achieved using cotton as a carrier are, in general, lower by 20% than using the T bag–resin combination because of the above reasons and also because of the fact that, for precipittion of the product, much higher excess of ether was used in the case of cotton synthesis. On the other hand, the purity of peptides prepared on cotton is significantly (on average by 19%) higher. The relatively low yields of crude free peptides from both T bags and cotton are undoubtedly caused by extensive ethereal washes that were used to remove the scavengers. We have shown with the pure

Table 2. Yields and Purities of Peptides Synthesized Using the Fmoc/Bu^t Strategy, With Some Comparisons to the Boc/Bzl Strategy (See Footnotes)

	Yield of Crude Peptide (%)		HPLC Purity (%)[a]		Bromophenol Blue Monitoring[b]	
No.	T Bag	Cotton	T Bag	Cotton	T Bag	Cotton
1	41(45[c])	11	37- (98[c])	90++	1.4	0.5
2	45	31	26-	87++	1.6	0.6
3	60	42	31-	80++	1.4	0.5
4	51	24	35-	56+	1.3	0.5
5	51	26	43-	93++	1.9	0.6
6	44	43	39-	83++	1.9	0.6
7	57	37	38-	89++	1.8	0.6
8	50	17	51-	70+	1.8	0.8
9	55	27	68-	86+	1.8	0.8
10	61	34	57-	91++	1.6	0.8
11	53	26	36-	87++	1.6	0.8
12	49	18	34-	83++	1.3	0.6
13	60	25	40-	69+	1.3	0.6
14	36	10	47-	93++	1.6	0.5
15	46	15	61-	98++	1.6	0.4
16	28	3	62-	92+	1.8	0.4
17	37	32	30-	57+	1.5	0.6
18	46	12	53+	39-	1.5	0.9
19	52	31	41-	69+	1.8	0.5
20	73	53(63[d])	69+(77[e])	53+(50[e])	0.6	0.5
21	67	58(63[d])	76+	59+	0.7	0.4
22	68	56(65[d])	76+	66+	0.7	0.4
23	68	64(64[d])	71+	62+	0.7	0.4
24	63	63(77[d])	74+	77+	0.7	0.3
25	71	66(79[d])	77+	82+	0.8	0.4
26	34	24(65[d])	60+(59[e])	68+(68[e])	0.6	0.4
27	35(69[c])	18	31-(60c)	56+	0.9	0.5
28	31	15	45-	60+	1.0	0.5
29	32	23	56-	73+	1.3	0.6
30	42	29	80+	97++	1.2	0.7
31	55	22	32-	51+	1.1	0.6
32	63(70[c])	32	50-(58[c])	72+	1.7	0.8
33	36(42[c])	33	55-(89[c])	52-	2.3	0.5
34	66(40[c])	45	62+(73[c])	50-	2.0	0.6
35	55(58[c])	37	64+(73[c])	57-	2.4	0.5
36	45(62[c])	24	52-(86[c])	59-	2.4	0.5
37	55(74[c])	30	65+(99[c])	75+	1.8	0.5
38	80(90[c])	57	37-(71[c])	79+	2.3	0.4
39	66(68[c])	62	59+(98[c])	85++	1.5	0.6
40	65(31[c])	55	85++(87[c])	90++	2.4	0.4
41	98(73[c])	47	30-(68[c])	64-	0.3	0.1
42	30(91[c])	28	25-(51[c])	25-	0.0	0.0
43	46(95[c])	29	29-(51[c])	50+	1.5	0.3
44	90(75[c])	48	52+(33[c])	81+	0.4	0.3
45	57(89[c])	23	34-(53[c])	46+	1.8	0.6
46	80(86[c])	62	42-(49[c])	93+	1.5	0.8
47	66(70[c])	43	36-(65[c])	63+	2.0	0.6
48	64(93[c])	38	32-(78[c])	46+	1.7	0.6
49	57(75[c])	41	38-(58[c])	44-	2.3	0.5
50	71(91[c])	39	56-(69[c])	83++	1.9	0.6
A.V.	53.6(71[c])	33.5	51.3(70[c])	70.4		

A.V.: average values. [a]The "+" signs: The main peak in HPLC trace is product; the "++" signs: the main peak in HPLC trace is product in high purity; the "-" signs: the HPLC trace indicates higher amount of substantial impurities. [b]The bromophenol blue monitoring was evaluated as numerical average of "the monitoring value" at the end of each step ("the monitoring value" was determined visually: 0 = white, 1 = green, 2 = green-blue, 3 = blue). [c]Value obtained in experiment using Boc/Bzl strategy. [d]Weight gain of the carrier. [e]Value obtained in the experiment in which both resin and cotton were placed inside the T bag.

peptide 26 example that the yield of precipitation and repeated treatment with ether may be as low as 31%. (The synthesis of peptide 1 in the optimal arrangement (Reference 25) afforded 58% of very pure product in comparison to 11% with the multiple arrangement used in this paper.) Of the fifty peptides used in the comparison, 20 compounds prepared on cotton (peptide numbers 1–3, 5–7, 9–12, 14–16, 25, 30, 39, 40, 44, 46 and 50) had purity better than 80%. The same is true for only two peptides (peptide numbers 30 and 40) synthesized on the resin in T bags. The pattern of HPLC peaks was, in most cases, closely analogous for both carriers (see Figures 1 and 2). The main by-products were identified as deletion peptides, peptides containing methionine S-oxide (Figure 3) or peptides containing an incompletely split Mtr group from Arg (Figure 4). Interestingly, there was no close parallelism between the results achieved with the alternative techniques; i.e., it was quite common that peptides difficult to synthesize in T bags were not so difficult to synthesize on cotton and vice versa. Only five peptides were found difficult simultaneously for both approaches. These were peptide numbers 33, 36, 41, 42 and 49. The T bag method resulted in peptides of better quality in only five cases (peptide numbers 18, 21, 33, 34 and 35).

Because HPLC of peptides containing Arg residues showed some peaks corresponding to incomplete removal of the Mtr group, we decided to prolong the reaction time in the final cleaving step (only for peptide numbers 33–50; 2 h for peptides without Arg(Mtr); overnight for peptides with Arg(Mtr)). For peptides with Arg(Mtr) (peptide numbers 38, 41, 42, 44, 45, 47, 48, 49), the average purity rose from 36% (3 h) to 46% (overnight), and the peaks with longer retention times disappeared (Figure 4). For peptides without Arg(Mtr), increase in reaction time from 1 to 2 h was without effect and the average purity in both cases was 57%. However, it is to be noted that this modification of deprotection conditions has been tested with peptides on resin only.

When the synthesis with the T bag–resin combination was carried out using the Boc/Bzl strategy (Table 2, Figures 3 and 5), the average yield of protected peptide on resin was 95%. This value is much higher than in the Fmoc/Bu^t experiment (see above). Both the yields and purity of products are better than with the Fmoc/Bu^t strategy using both T bags and cotton, and the values obtained are in accord with published data (19). Comparison of this approach with the cotton-based synthesis using Fmoc/Bu^t strategy suggests that the purity of peptides is comparable (approximately 70%), while the yields on cotton carrier are lower because of the mechanical losses of peptide from the cotton carrier and because of the different workup procedure.

We intentionally selected for the comparison peptides that proved in the past to be difficult to synthesize. Hence, it is not surprising that some of them were obtained in low yields or in a quality requiring further purification, especially when the reaction conditions were not optimized. But it can be safely deduced, from the above results, that peptides synthesized on cotton carrier were superior in purity with respect to peptides synthesized in T bags using Fmoc/Bu^t strategy, and at least comparable with those synthesized in T bags using Boc/Bzl strategy. With the current methodology, the yields on cotton were notably lower.

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