A COMPARATIVE STUDY OF BOP AS A COUPLING AGENT USING SIMULTANEOUS MULTIPLE PEPTIDE SYNTHESIS*

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A parallel simultaneous synthesis of multiple peptides was carried out in which the coupling agents BOP and DIPCI were compared. For peptides lacking Asn, no differences in average yield or RP HPLC purity were found. For peptides having Asn in their sequences, however, the yield and purity were significantly lower for BOP when compared to DIPCI. The addition of HOBt to BOP mediated couplings did not lead to improved results. In fact, BOP condensations without HOBt gave, on average, slightly purer peptides. Comparing the results for peptides prepared with BOP dissolved in DMF, NMP, or NMP + 20% DMSO, NMP afforded the best and DMF the worst results, but the differences were not significant. Peptides prepared using BOP, combined with in situ neutralization, were obtained both in higher yield and purity when compared with classical neutralization. In all BOP mediated couplings, peptides with Asn were obtained in lower yield and purity than peptides without Asn. FAB MS and amino acid analysis revealed that the side products are peptides with deleted Asn.

The BOP** reagent introduced by Castro et al.[3] found wide application both in classical solution[4] and SPPS[5 – 9]. It was found superior in comparison with other condensation reagents in terms of yields, purity, reaction speed, etc.[7,10 – 13]. BOP reagent was suc-

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**The symbols and abbreviations obey the published recommendations[2]. In addition we use the following abbreviations: AcOH, acetic acid; B, amide; BOP, benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; Bu^t, *tert*-butyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DIPCI, diisopropylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; FAB MS, fast atom bombardment mass spectrometry; J, 4-nitro-phenylalanine; HOBt, hydroxybenzotriazole; MeBHA, methylbenzhydrylamine; NMP, 1-methyl-2-pyrrolidone; RP HPLC, reverse phase high performance liquid chromatography; SMPS, simultaneous multiple peptide synthesis; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid.

cessfully used for difficult cyclizations[7,14,15] and fragment condensations[16,17]. Another advantage over other reagents is the possibility to skip the neutralization step and make the condensation in the in situ neutralization mode[4,6,8,9,18] saving time and solvents. In this way the formation of pyroglutamic acid from amino-terminal Gln is suppressed as is the formation of dioxopiperazines, when the third amino acid is condensed to the dipeptide-resin. From different bases tested, DIEA was found to be the best and is used most often[4 – 7,19]. Therefore we used it in all of our experiments.

The aim of this study was to compare the efficiency of BOP reagent with DIPCI, compare the influence of different solvents and HOBt on BOP mediated couplings with in situ neutralization and also compare the classical and in situ neutralization. For these reasons, the SMPS (tea bag)[1,20,21] method is the best choice, because all bags in the experiment are treated together with the exception of the condensation step being studied.

EXPERIMENTAL

The resin used through all experiments was of *p*-methylbenzhydrylamine . HCl type, 0.54 meq/g, 100 – 200 mesh (Advanced Chem Tech), DIPCI (Aldrich), DIEA, sequalog grade (Schweizerhall), BOP (Richelieu Biotechnologies). DMF (0.05% water), DCM (0.004% water) and DMSO (0.05% water), all from Fisher Scientific. NMP (<0.005% water), dimethyl sulfide (<0.005% water), 1,2-ethanedithiol and *p*-cresol were from Aldrich. All Boc-amino acids were from Bachem. Protecting groups used: Asp, Glu, Ser and Thr (benzyl); Cys (*p*-methoxybenzyl); Arg (*p*-toluenesulfonyl); Trp (formyl); Tyr (2-bromobenzyloxycarbonyl); Lys (2-chlorobenzyloxycarbonyl); His (2,4-dinitrophenyl); Met (O).

The coupling protocol was as follows: 1) DCM, 30 s; 2) 55% TFA in DCM, 30 min; 3) DCM, 1 min; 4),5) isopropyl alcohol, 1 min; 6),7) DCM, 1 min; 8), 9), 10) 5% DIEA in DCM, 2 min; 11), 12) DCM, 1 min; 13) coupling with DIPCI in DCM, 60 min; 14),15) DMF, 30 s; 16) DCM, 1 min.

All BOP mediated couplings (with the exception of the carboxy-terminal amino acid) were done using in situ neutralization, i.e. washings 8 – 12 were omitted, except that in experiment D2, classical neutralization[5] was used. For BOP condensations in DMF, NMP or NMP + 20% DMSO, one wash with the given solvent was added prior to the condensation step. The condensation time with BOP was 30 min. In all experiments all bags were treated together with the exception of steps 13 – 15, to assure identical conditions. The volume for condensation was 3 ml/bag; all other steps were done with 5 ml/bag.

In the set of experiments A1 and A2 (see Table I), bags containing 50 mg of resin were used. The DIPCI mediated coupling was made with 6-fold excess of activator and Boc-amino acids in DCM, i.e., the concentration of DIPCI and Boc-amino acids during condensation was 0.054 mol l^{-1}. The BOP mediated coupling was made with 6-fold excess of BOP and Boc-amino acids and 18-fold excess of DIEA, i.e. the concentration of BOP and Boc-amino acids during the condensation was 0.054 mol l^{-1} in DMF.

In all other experiments, 100 mg bags were used. In the set of experiments B1 and B2 (see Table II) the molar ratio of DIPCI and Boc-amino acids was 6 : 6 (in DCM) and BOP–Boc-amino acid–DIEA were 6 : 6 : 12 (in DMF), i.e., the concentration of activator and amino acid during condensation was 0.108 mol l^{-1}.

In the set of experiments C1 – C4 (see Table III) the molar ratios were BOP–Boc-amino acid–HOBt–DIEA 6 : 6 : 0 : 12 (C1; in DMF); 6 : 6 : 6 : 12 (C2; in DMF); 6 : 6 : 6 : 12 (C3; in NMP);

6 : 6 : 6 : 12 (C4; in NMP + 20% DMSO). The concentration of BOP and Boc-amino acids during condensation was 0.108 mol l^{-1}.

In the set of experiments D1 and D2 (see Table IV), the same molar ratios and concentrations were used as in C1. In experiment D1, in situ neutralization was used; i.e. steps 8 – 12 were omitted. In experiment D2 classical neutralization was used; i.e. all 16 steps of the washing protocol were done.

All condensations of Boc-Asn and Boc-Gln mediated both by DIPCI and BOP were made with equimolar amounts of HOBt. The DIPCI activation of this amino acids was in DCM–DMF 1 : 1 (v/v). In DIPCI experiments, Boc-Arg(Tos), Boc-Leu, Boc-His(DNP) and Boc-Trp(For) were first dissolved in DMF and then coupled at a final DCM–DMF ratio of 9 : 1.

Before the "low-high HF" treatment[22], in all peptides without His, the Boc group was first deprotected by 55% TFA–DCM. In peptides with His, the DNP group was deprotected by 5% thiophenol in DMF (3 × 1 h), followed by alternating washes of isopropyl alcohol and DCM, 10 times each and then the Boc group was split off.

The "low HF" deprotection was made with all bags in every experiment together in 60% dimethyl sulfide, 25% HF, 10% 1,2-ethanedithiol and 5% *p*-cresol (v/v), 7 ml/bag, 2.5 h at 0 °C, vigorous shaking. The bags were washed 3 times with DCM, followed with alternating washes of isopropyl alcohol and DCM (10 times each), 3 times DMF and 4 times DCM.

The "high HF" was made in a 24 reaction vessel apparatus[23], HF : anisole 9 : 1 (v/v), 7 ml/bag, 90 min at −15 °C and 30 min at 0 °C. After blowing off HF by nitrogen and 45 min evacuation at 0 °C, the scavenger was extracted by ether (3 × 10 ml, shaker, 5 min) and the peptide was extracted by 2 × 5 ml of 10% AcOH (50 mg bag) or by 2 × 10 ml 10% AcOH (100 mg bag) and lyophilized. The yield of crude peptides was not corrected for salt (HF, TFA) or solvent (AcOH, water) content. RP HPLC was carried out with the crude products. After the first lyophilization, the lyophilizate was dissolved in 10% AcOH (10 – 15 ml), 30 μl aliquot was diluted with 300 μl of 5% AcOH, and 20 μl of this solution was applied to VYDAC Protein & Peptide C18 column (catalog No. 218TP54, 250 × 4.6 mm). The Beckman system (110A pump, 421 controller, Palo Alto, CA) was used. Peptides were detected at 215 nm using a Hitachi 100-20 spectrophotometer and Shimadzu C-R3A recording integrator (Shimadzu Scientific, Columbia, MD). The solvent system consisted of 0.05% TFA/water (eluent A) and 0.05% TFA/acetonitrile (eluent B) with a linear gradient of 5% B to 50% B in 45 min and with a solvent flow rate of 1.0 ml/min. FAB MS was done on ZAB-EQ equipment (VG Analytical, Manchester, England), in a glycerine matrix, bombardment by xenone beam 8kV. Samples for amino acid analysis were hydrolyzed with 6 M HCl at 110 °C for 24 h. The hydrolyzates were analyzed on a Beckman-Spinco 120 B amino acid analyzer.

RESULTS AND DISCUSSION

In the first set of experiments (see Tables I and V) we compared DIPCI in DCM using classical neutralization[24] with BOP in DMF using in situ neutralization[4,18]. Molar ratio of DIPCI and Boc-amino acids was 6 : 6 and the ratio of BOP, Boc-amino acids and DIEA was 6 : 6 : 18 (in relation to free amino groups on resin). The gain of weight of peptides on resin in the DIPCI mediated experiment A1 was 9% better for all peptides, 12% better for peptides containing Asn and 3% better for peptides without Asn in comparison with the BOP experiment A2. The yield of crude peptides after "low-high HF" (refs[22,25]) deprotection in the DIPCI experiment was 8% better for all peptides and 12% better for peptides with Asn in comparison with the BOP experiment. Peptides

TABLE I

Comparison of coupling efficiency (*w*, increase of weight on resin, %; *y*, yield of crude peptide, %; *p*, HPLC purity, %) of DIPCI in DCM (experiment A1) with BOP in DMF (experiment A2)

Peptide	Sequence	Experiment A1			Experiment A2		
		w	*y*	*p*	*w*	*y*	*p*
1	DQESCKGRCTEGF***N***VB	86	66	53	57	52	31
2	EGF***N***VDKKCQCDELCB	78	83	65	79	92	62
3	CCTDYTAECKPQVTRB	86	96	54	80	99	54
4	PQVTRGDVFTMPEDEB	83	96	81	83	94	65
5	MPEDEYTVYDDGEEKB	83	94	35	80	93	39
6	DGEEK***NN***ATVHEQVGB	61	69	65	57	67	32
7	HEQVGGPSLTSDLQAB	71	83	62	73	76	83
8	SDLQAQSKG***N***PEQTPB	64	74	52	60	75	28
9	PEQTPVLKPEEEAPAB	82	91	65	79	88	79
10	EEAPAPEVGASKPEGB	75	86	66	71	81	65
11	SKPEGIDSRPETLHPB	85	109	61	83	110	67
12	ETLHPQRPQPPAEEEB	71	95	73	75	96	80
13	PAEEELCSGKPSTLHB	85	70	62	80	105	85
14	PSTLHRLK***N***GSLFAFB	81	93	78	73	82	46
15	SLFAFRGQYCYELDEB	83	80	62	79	86	38
16	GYPKLIRDVWGGIEGPB	70	87	55	68	80	57
17	GIEGPIDAAFTRI***N***CB	86	93	73	71	76	48
18	TRI***N***CQGKTYLFKGSB	79	100	57	70	89	54
19	LFKGSQYWRFEDGVLB	80	92	42	78	88	51
20	SGTV***N***PAPI***N***IASHISSISARTGDB	71	73	50	58	64	54
21	SATV***N***PAPI***N***IASHISSISARTGDB	75	71	60	61	67	50
22	SGAV***N***PAPI***N***IASHISSISARTGDB	75	75	55	62	64	48
23	SGTA***N***PAPI***N***IASHISSISARTGDB	74	81	55	61	66	46
24	SGTVAPAPI***N***IASHISSISARTGDB	73	71	57	63	57	50
25	SGTV***N***AAPI***N***IASHISSISARTGDB	74	79	59	60	66	47
26	SGTV***N***PAAI***N***IASHISSISARTGDB	71	80	42	60	60	41
27	SGTV***N***PAPAIASHISSISARTGDB	71	74	54	61	66	50
28	SGTV***N***PAPI***N***AASHISSISARTGDB	75	81	56	60	63	49
29	SGTV***N***PAPI***N***IFSHISSISARTGDB	73	75	54	58	58	31
30	SGTV***N***PAPI***N***IAAHISSISARTGDB	75	75	64	63	63	66

TABLE I
(*Continued*)

Peptide	Sequence	Experiment A1			Experiment A2		
		w	*y*	*p*	*w*	*y*	*p*
31	SGTV*N*PAPN*I*ASHASSISARTGDB	75	83	63	63	64	41
32	SGTV*N*PAPN*I*ASHIASISARTGDB	73	78	63	63	64	46
33	SGTV*N*PAPN*I*ASHISAISARTGDB	75	82	64	58	62	51
34	SGTV*N*PAPN*I*ASHISSASARTGDB	73	80	52	58	57	33
35	SGTV*N*PAPN*I*ASHISSIAARTGDB	73	81	64	61	63	50
36	SGTV*N*PAPN*I*ASHISSISAATGDB	67	68	59	52	51	53
37	SGTV*N*PAPN*I*ASHISSISARAGDB	68	66	64	56	51	60
38	SGTV*N*PAPN*I*ASHISSISARTADB	70	74	59	57	60	43
39	SGTV*N*PAPN*I*ASHISSISARTGAB	73	80	59	57	63	44
40	TVVAVGPGRWDEDGEB	80	87	71	79	87	74
41	DVAEGDTVIYSKYGGB	75	78	77	69	78	70
42	GEEYLILSARDVLAVB	85	73	69	70	71	61
43	AFGIKL*N*VQRB	85	95	91	81	86	36

without Asn were obtained in the same yield by both methods. The RP HPLC purity of crude peptides in the DIPCI experiment was 9% better for all peptides and 15% better for peptides with Asn in comparison with the BOP experiment. The purity of peptides without Asn was 2% better in the BOP experiment. Whereas the difference between the purity of peptides with and without Asn in the DIPCI experiment is negligible, in the BOP experiment peptides with Asn are 19% worse than peptides without Asn.

Incorporation of Asn into the peptide chain is not without problems. The reaction proceeds more slowly in comparison with other Boc and Fmoc amino acids and in dependence on the condensation reagent and final deprotection and workup, varying amounts of β-Asp peptide, aspartoyl peptide, cyclic imide, β-aspartamidino peptide and cyano alanine can be formed besides the desired α-Asn peptide[8,24,26–28]. The formation of cyano alanine using Boc/Bzl protection and final HF cleavage is not so deleterious, because the cyano group is rehydrated during HF deprotection and the following work-up[24,26,29]. This side reaction is more harmful using Fmoc/Bu^t protection[28], because the TFA used for final deprotection rehydrates the cyano group only very slowly[28,29]. Using BOP reagent, some authors did not find any problems incorporating Asn[5,11,13].

TABLE II

Comparison of coupling efficiency (*w*, increase of weight on resin, %; *y*, yield of crude peptide, %; *p*, HPLC purity, %) of DIPCI in DCM (experiment B1) with BOP in DMF (experiment B2)

Peptide	Sequence	Experiment B1			Experiment B2		
		w	*y*	*p*	*w*	*y*	*p*
1	APE*N*KAFVLSSVDELB	86	71	59	75	68	37
2	LIEKQAPE*N*KAFVLSB	92	100	70	80	85	46
3	LKQIRLIEKQAPE*N*KB	86	100	90	69	75	70
4	AFVLSSVDELEQQRDB	86	93	74	86	91	60
5	EQQRDEIVSYLCDLAB	79	45	68	73	58	60
6	QIFSKIDRPEASRIAB	85	94	38	87	98	49
7	SQEPQRMSR*N*FVRYVB	98	98	51	92	85	58
8	PTDAPVSPTTLYVEDB	91	88	60	85	78	63
9	VSPTTLYVEDISEPPB	73	42	73	70	54	61
10	SRLLDLVFLLDGSSRB	89	75	66	81	63	65
11	LHDFYCSRLLDLVFLB	82	61	53	81	67	82
12	PSELRRIASQVKYAGB	82	98	64	73	63	54
13	RIASQVKYAGSQVASB	83	80	52	70	75	52
14	VKYAGSQVASTSEVLB	83	83	64	75	86	72
15	AVVEYHDGSHAYIGLB	83	71	74	83	70	69
16	SFPASYFDEMKSFAKB	93	80	57	82	79	51
17	KSFAKAFISKA*N*IGPB	82	87	83	75	67	53
18	A*N*IGPRLTQVSVLQYB	86	67	68	78	63	54
19	SVLQYGSITTIDVPWB	88	70	71	87	63	71
20	IDVPW*N*VVPEKAHLLB	93	94	76	88	80	32
21	KAHLLSLVDVMQREGB	80	96	64	67	79	48
22	QEPGGLVVPPTDAPCB	98	98	53	95	91	42
23	CQEPGGLVVPPTDAPB	75	64	90	70	81	93
24	CDLAPEAPPPTLPPDB	87	62	87	79	63	91
25	DLAPEAPPPTLPPDCB	93	105	54	89	96	57
26	PTLGDEGDTDLYDYYB	88	95	76	86	81	73
27	CPGLLTPTPKLEKLSB	92	94	75	85	100	87
28	QVDGTLPVLGTLDLCB	89	73	49	88	89	46
29	CQVDGTLPVLGTLDLB	89	78	45	83	86	48
30	CTLGDEGDTDLYDYYB	88	80	49	82	82	57

TABLE II
(*Continued*)

Peptide	Sequence	Experiment B1			Experiment B2		
		w	*y*	*p*	*w*	*y*	*p*
31	TLGDEGDTDLYDYYCB	92	86	78	88	83	57
32	CGDTDLYDYYPEEDTB	90	99	41	80	83	42
33	GDTDLYDYYPEEDTCB	93	96	44	88	94	55
34	***N***SITLT***N***LPPGTEYVB	92	76	67	80	46	36
35	T***N***LTPGTEYVVSIVAB	86	87	72	75	82	58
36	GTEYVVSIVAL***N***GREB	88	99	77	82	92	77
37	VSIVAL***N***GREESPLLB	93	96	51	85	79	60
38	L***N***GREESPLLIGQQSB	82	105	56	80	106	63
39	ESPLLIGQQSTVSDVB	82	61	58	76	53	56
40	IGQQSTVSDVPRDLEB	92	96	80	83	98	69
41	TVSDVPRDLEVVAATB	80	83	63	73	80	56
42	PRDLEVVAATPTSLLB	92	98	77	83	92	76
43	VVAATPTSLLISWDAB	88	94	50	82	85	55
44	***NN***EDSYVPSAEQILB	85	96	60	79	94	49
45	***NNN***EDSYVPSAEQILB	83	96	62	78	81	38
46	AFGIKL***N***VQRB	94	101	94	88	100	38

Others found variable amounts of nitrile which could not be completely suppressed by addition of HOBt[17,28,30].

In all experiments, we used 2 – 3 control peptides (only one of them is shown in tables) of the sequence AFGIKLNVQR-amide, from which we prepared also all omission analogs. In the DIPCI experiment, we obtained high yield of pure controls, but in the BOP experiment (see Table I) the purity was low and other product in about 38 – 40% yield was present in both controls. RP HPLC comparison of the side product with all omission analogs showed that the side product had the same retention time as the control with missing Asn (AFGIKLVQR-amide). Using preparative RP HPLC, we separated the side products and analyzed them by FAB MS and amino acid analysis. The molecular ions for side products were by 114 units lower in comparison with calculated molecular weights for desired control peptides, and in their amino acid analyses Asn was missing. Besides the controls, we analyzed also Asn containing peptides

Nos 1, 6, 8 and 14 (Table I) and found 15 – 35% side products with missing Asn. Therefore, the main side products in BOP mediated couplings were peptides with missing Asn.

In the second set of experiments (see Tables II and V), we decreased the molar excess of base from 18 to 12 (see Experimental). The gain of weight of peptides on resin with DIPCI activation was 6% better in comparison with BOP. The yield of crude peptide in the DIPCI experiment B1 was roughly 6% higher when compared with the BOP experiment B2. The RP HPLC purity of crude peptides in the DIPCI experiment was 7% higher for all peptides and 17% higher for peptides with Asn. Peptides without Asn had comparable purity in both experiments. We analyzed again the control peptides and peptides Nos 1, 17, 20, 34 and 45 (Table II) and found 15 – 38% of side products with missing Asn.

The data obtained from the first and second set of experiments make it clear, that for peptides without Asn, DIPCI and BOP are fully comparable in terms of yield of crude peptide and HPLC purity. (However, the reaction time was only 30 min for BOP in comparison with 60 min for DIPCI.) Peptides with Asn were obtained in lower yield and with much lower purity using BOP reagent in comparison with DIPCI (see Table V).

There are many examples in the literature that show improved coupling reaction (i.e. speed, suppression of side reactions including racemization, etc.) by the addition of HOBt to carbodiimide[8,24,27], active ester[8,24,26] and BOP mediated couplings[5,8,14,19,28]. Therefore, we compared BOP couplings made with and without HOBt.

In the set of experiments C1 (see Tables III and V), the molar ratios were BOP : Boc-amino acid : HOBt : DIEA : free amino groups 6 : 6 : 0 : 12 : 1. Only when Boc-Asn and Boc-Gln were condensed, HOBt was added in equimolar ratio to the Boc-amino acid. In the set of experiments C2, HOBt was added to all condensation steps and the molar ratio was BOP : Boc-amino acid : HOBt : DIEA : free amino groups 6 : 6 : 6 : 12 : 1. Both experiments were done in DMF. The yields of peptides on resin and the yields of crude peptides were very similar in both experiments. The HPLC purity for all peptides was roughly 3 – 4% better in experiment C1 (without HOBt). In both C1 and C2, peptides without Asn were roughly 28% more pure than peptides with Asn. In general, the difference between these two experiments was very small. Taking into account both yield and purity, experiment C1 (without HOBt) was slightly better. These results are in disaccord with those of Hudson[10], who found "dramatic improvement" in rate and magnitude of activation using BOP and HOBt in comparison with BOP only. Also Rule et al.[12] claimed improvement by the addition of HOBt to BOP. At first view, our results seem surprising, but they can be simply explained. HOBt is a weak acid[29,31] and it consumes DIEA. This lowers the basicity of the reaction media. Because BOP couplings proceed best when the base is in two- to threefold excess over BOP (refs[4,30,32]), addition of HOBt can slow down the reaction. Also, due to the lower

TABLE III

Dependence of coupling efficiency (*w*, increase of weight on resin, %; *y*, yield of crude peptide, %; *p*, HPLC purity, %) of BOP on the ratio BOP–Boc-AA–HOBt–DIEA. Experiment C1, 6 : 6 : 0 : 12, (in DMF); experiment C2, 6 : 6 : 6 : 12, (in DMF); experiment C3, 6 : 6 : 6 : 12, (in NMP); experiment C4, 6 : 6 : 6 : 12, (in NMP–DMSO 8 : 2)

Peptide	Sequence	Experiment C1			Experiment C2			Experiment C3			Experiment C4		
		w	*y*	*p*	*w*	*y*	*p*	*w*	*y*	*p*	*w*	*y*	*p*
1	***N***VSDKITFMC***N***DHYIB	83	76	34	82	91	25	83	77	43	82	77	43
2	***N***DHYILKGS***N***RSQCLB	81	85	34	77	94	40	79	87	58	79	89	74
3	RSQCLEDHTWAPPFPB	54	59	70	54	67	85	56	67	53	56	67	58
4	APPFPICKSRDCDPPB	70	86	81	69	92	80	72	83	85	71	86	81
5	DCDPPG***N***PVHGYFEGB	70	81	79	70	87	53	73	92	72	73	82	81
6	GYFEG***NN***FTLGSTISB	75	68	30	73	73	26	76	73	33	75	70	35
7	GSTISYYCEDRYYLVB	87	89	65	87	87	58	90	92	43	90	65	71
8	RYYLVGVQEQQCVDGB	66	76	45	65	68	56	71	75	61	69	77	69
9	QCVDGEWSSALPVCKB	91	95	54	84	92	38	90	94	38	89	89	52
10	LPVCKLIQEAPKPECB	86	104	85	85	109	78	86	99	78	85	78	83
11	PKPECEKALLAFQESB	79	78	84	75	84	70	83	96	80	81	89	75
12	AFQESK***N***LCEAME***N***FB	77	58	32	71	55	50	80	71	30	83	56	34
13	AME***N***FMQQLKESGMTB	95	104	35	92	92	44	97	108	56	94	102	64
14	ESGMTMEELKYSLELB	93	65	51	86	69	30	94	85	59	94	83	66
15	YSLELKKAELKAKLLB	83	100	87	80	100	80	85	87	94	85	97	87

TABLE III
(*Continued*)

Peptide	Sequence	Experiment C1			Experiment C2			Experiment C3			Experiment C4		
		w	*y*	*p*	*w*	*y*	*p*	*w*	*y*	*p*	*w*	*y*	*p*
16	MTSSRIGTHTTPAB	91	113	78	86	126	78	89	117	78	90	113	82
17	CTVHP*N*HPPPSYGVB	85	94	54	85	110	32	84	92	44	83	99	40
18	SYGV*N*VTGLPG*N*LB	67	64	64	54	58	42	73	79	65	75	63	73
19	HRHIVRKRTLRRLLB	86	114	93	90	119	98	90	117	95	90	117	95
20	IGRG*N*FGEVFSGCB	84	98	58	83	108	37	85	95	51	86	100	47
21	LGEHHCTPSPPVDHGB	78	105	86	77	104	68	80	106	83	77	105	92
22	YPYDVPDYASLRSB	84	100	90	83	99	91	87	102	93	89	103	93
23	LGSGAFGTIYKGCB	87	108	82	87	91	81	90	109	84	87	100	87
24	LMCQCWRKDPEERPTFB	94	90	38	92	96	43	95	105	43	94	80	47
25	LMKLCWKKDPDERPTCB	96	103	51	98	116	60	97	112	61	93	90	63
26	LVADCLKKVREERPLFB	91	108	83	89	111	71	89	108	84	87	104	78
27	TLHSCWQQLYSPSPSAB	80	92	79	72	92	77	79	92	79	77	79	77
28	LGGGQYGEVYEGCB	89	105	81	87	107	81	91	95	87	89	90	90
29	AFGIKL*N*VQRB	80	89	38	83	91	39	86	94	40	85	90	42

pK_a of HOBt, the free amino group on the growing peptide chain will be partly protonated, which could retard reactivity towards activated amino acids. (For details see ref.[31].) These two influences combined can eliminate the advantage of HOBt addition when the amount of base is the same in both experiments, as it was in C1 and C2. The addition of HOBt to active esters has been studied[33], and it was found that addition of more than equimolar amounts of HOBt to the active component can be disastrous. Because BOP couplings proceed partly via HOBt esters (the HOBt comes of BOP), these conclusions apply also to BOP couplings. The added HOBt was a hydrate, so it can also hydrolyze the active ester[33].

The solvation of both the growing peptide chain and resin is very important for successfull SPPS (refs[8,26,31,34]). Dipolar aprotic solvents have the ability not only for good solvation of the resin, but they are also able to suppress intra- and interchain aggregation of the growing peptide chain, leading to better coupling efficiency in sequence-dependent difficult couplings. In the literature, NMP (refs[8,9,31]) and NMP containing 20% DMSO (refs[9,31]) were found to be excellent in comparison with other solvents. Therefore, in experiments C3 (NMP) and C4 (NMP + 20% DMSO), we compared the influence of the solvent (see Tables III and V). The yield of peptide on resin was the same in both experiments. The yield of all crude free peptides was 6% higher in experiment C3 (NMP) in comparison with experiment C4 (NMP + 20% DMSO). When comparing purity by RP HPLC, completely different results were obtained: the purity of all peptides and peptides both with and without Asn was 3%, 2% and 4% lower in C3 compared to C4, respectively. In both C3 and C4, peptides without Asn were 25 – 27% purer than peptides with Asn. When experiments C1 – C4 are compared, and both yield and purity of peptides are considered, the order of growing successfulness is C2, C1, C4, C3.

In the fourth set of experiments (see Tables IV and V), we compared the influence of neutralization mode on the yield and purity of peptides using BOP reagent. In experiment D1, the neutralization was done in situ[4,32]. In experiment D2, classical neutralization[5] was used. The gain of weight on resin for all peptides was 3% higher, and yield for all crude peptides was 2% higher using in situ neutralization. Differences in purity as determined by RP HPLC for peptides with versus without Asn were again remarkable, as in all previous experiments using BOP reagent. As compared with peptides containing Asn, purity for peptides without Asn using in situ neutralization was 23% higher, while purity of peptides without Asn using classical neutralization was 20% higher. Purity for all peptides was slightly better in the in situ neutralization versus the classical neutralization experiment. When considering both yield and purity of all peptides, in situ neutralization afforded better results; in situ neutralization also saved both time and solvents. In addition, the formation of pyroglutamic acid from amino-terminal Gln is suppressed[34]. All peptides in this study were prepared on MeBHA resin. The advantage of in situ neutralization would be even greater in SPPS on classical resin

TABLE IV
Dependence of coupling efficiency (*w*, increase of weight on resin, %; *y*, yield of crude peptide, %; *p*, HPLC purity, %) on neutralization method

Peptide	Sequence	Experiment D1			Experiment D2		
		w	*y*	*p*	*w*	*y*	*p*
1	ATHQAYJVRKAB	97	101	90	97	103	82
2	ATHQAYJQRKAB	97	98	90	98	103	91
3	ATHQAYJARKAB	98	109	91	100	108	92
4	ATHQAYJIRKAB	98	101	90	96	98	83
5	ATHQAYJ***N***RKAB	95	95	86	96	102	89
6	ATHQVYJQRKAB	95	97	90	96	101	88
7	ATHQVYJARKAB	98	104	84	99	106	85
8	ATHQVYJIRKAB	97	100	88	93	97	88
9	ATHQVYJGRKAB	98	104	88	95	98	84
10	ATHQVYJSRKAB	97	105	88	96	104	86
11	ATHQVYJKRKAB	98	109	90	97	108	88
12	ATHQVYJERKAB	98	103	90	96	98	83
13	ATHQVYJ***N***RKAB	94	100	87	94	99	88
14	ATHQIYJARKAB	98	107	88	94	104	91
15	ATHQIYJQRKAB	98	102	90	95	98	81
16	ATHQIYJ***N***RKAB	93	98	89	90	96	86
17	ATHQVYPARKAB	100	120	96	83	98	90
18	ATHQVYPVRKAB	100	116	96	96	106	95
19	ATHQVYPIRKAB	100	109	94	98	106	94
20	ATHQAYPVRKAB	99	111	93	98	111	94
21	ATHQAYPARKAB	100	112	93	86	90	85
22	ATHQAYPIRKAB	100	115	91	97	109	91
23	ATHQVYJVQKAB	96	95	88	88	95	88
24	ATHQVYJVIKAB	105	93	90	95	92	90
25	ATHQVYJVYKAB	94	86	86	94	90	77
26	ATHQVYJVSKAB	97	96	88	95	92	89
27	ATHQVYJVEKAB	95	96	87	94	95	84
28	ATHQVYJVPKAB	95	91	90	94	88	88
29	ATHQVYJVKKAB	96	99	87	95	100	89
30	ATHQVYJVHKAB	103	99	90	99	98	91

TABLE IV
(*Continued*)

Peptide	Sequence	Experiment D1			Experiment D2		
		w	*y*	*p*	*w*	*y*	*p*
31	ATHQVYJVDKAB	87	88	88	89	88	89
32	ATYQVYJVQYAB	84	62	86	78	61	84
33	ATHQIYPARKAB	99	105	92	86	89	92
34	ATHQIYPVRKAB	98	109	94	96	103	94
35	ATHQIYPIRKAB	98	107	92	97	101	86
36	KARVLJEALB	92	100	86	89	94	87
37	KARVLJERLB	95	110	87	92	109	85
38	KARVLJEQLB	93	98	88	86	97	87
39	KARVLJEHLB	101	105	87	96	98	83
40	KARVLJEYLB	89	89	89	86	91	88
41	KARVLJEILB	92	98	88	90	98	87
42	KARVLJEDLB	89	93	85	87	90	85
43	KARVLJEPLB	95	87	88	93	88	88
44	KARVLJE*N*LB	79	77	75	79	73	69
45	KARVLJEKLB	94	110	89	90	106	84
46	KPRVLJEALB	90	99	88	89	95	85
47	KGRVLJEALB	95	102	88	92	98	84
48	KHRVLJEALB	96	99	86	97	98	86
49	RATLVTVVLHB	98	93	76	95	92	77
50	ATFQAYLAMTB	87	59	83	85	64	79
51	PPAVSPLREAB	95	99	90	88	95	87
52	AFGIKL*N*VQRB	87	89	40	84	87	38

with benzyl ester type anchor to the solid support, where dioxopiperazine formation during the condensation of the third amino acid is a real danger.

On the basis of data obtained from 444 peptides (not all peptides are listed in Tables I – IV), we recommend the use of BOP reagent with in situ neutralization for condensation of all amino acids, with the exception of Asn. Resulting peptides will be of reasonable high yield and purity, and both time and solvents will be saved. Boc-Asn should be condensed using other reliable methods, such as DCC or DIPCI/HOBt or pentafluorophenyl ester etc.

TABLE V

Comparison of DIPCI and BOP coupling efficiency in dependence on solvent polarity, HOBt addition and neutralization method. Summary of results from Tables I – IV

Parameter	Experiment									
	A1	A2	B1	B2	C1	C2	C3	C4	D1	D2
Weight gain of resin, %										
All peptides	76.2	67.4	87.6	81.2	82.3	80.3	84.0	83.1	94.6	91.8
Peptides with Asn	74.5	62.7	89.5	81.5	80.5	78.5	82.6	81.9	86.8	86.3
Peptides without Asn	79.6	76.5	86.6	81.0	83.4	81.4	84.9	83.8	96.0	92.9
Yield of crude peptide, %										
All peptides	82.2	74.3	85.5	80.0	90.2	92.8	93.5	87.7	98.1	95.7
Peptides with Asn	79.2	66.8	92.2	81.2	83.8	87.4	88.2	84.0	89.2	89.9
Peptides without Asn	87.8	88.8	81.9	79.4	94.2	96.3	96.9	90.1	99.8	96.8
HPLC purity of crude peptide, %										
All peptides	61.5	52.2	66.1	59.5	62.2	58.6	62.7	65.7	85.2	83.7
Peptides with Asn	61.0	45.8	72.1	55.2	45.3	41.1	47.3	49.3	66.2	66.8
Peptides without Asn	62.3	64.5	62.8	61.8	72.8	69.6	72.5	76.1	88.9	86.9
(Yield of crude peptides) × (purity of all peptides)	50.6	38.8	56.5	47.6	56.1	54.4	58.6	57.6	83.6	80.1

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REFERENCES

1. Jezek J., Houghten R. A. in: *Peptides 1990* (E. Giralt and D. Andreu, Eds), p. 74. ESCOM, Leiden 1991.
2. *Nomenclature and Symbolism for Amino Acids and Peptides*. Recommendations 1983. Eur. J. Biochem. *138*, 9 (1984).
3. Castro B., Dormoy J. R., Evin G., Selve C.: Tetrahedron Lett. *14*, 1219 (1975).
4. Le Nguyen D., Seyer R., Heitz A., Castro B.: J. Chem. Soc., Perkin Trans. 1 *1985*, 1025.
5. Briand J. P., Dorsselaer A. V., Raboy B., Muller S.: Pept. Res. *2*, 381 (1989).
6. Coste J., Dufour M. N., Le-Nguyen D., Castro B. in: *Peptides, Chemistry, Structure and Biology* (J. E. Rivier and G. R. Marshall, Eds), p. 885. ESCOM, Leiden 1990.
7. Felix A. M., Wang Ch. T., Heimer E. P., Fournier A.: Int. J. Pept. Protein Res. *31*, 231 (1988).
8. Fields G. B., Noble R. L.: Int. J. Pept. Protein Res. *35*, 161 (1990).
9. Forest M., Fournier A.: Int. J. Pept. Protein Res. *35*, 89 (1990).
10. Hudson D.: J. Org. Chem. *53*, 617 (1988).
11. Fournier A., Wang Ch. T., Felix A. M.: Int. J. Pept. Protein Res. *31*, 86 (1988).
12. Rule W. K., Shen J. H., Tregear G. W., Wade J. D. in: *Peptides 1988* (G. Jung and E. Bayer, Eds), p. 238. Walter de Gruyter, Berlin, New York 1989.
13. Seyer R., Aumelas A., Caraty A., Rivaille P., Castro B.: Int. J. Pept. Protein Res. *35*, 465 (1990).
14. Plaue S.: Int. J. Pept. Protein Res. *35*, 510 (1990).
15. Poncet J., Jouin P., Castro B. in: *Peptides, Chemistry, Structure and Biology* (J. E. Rivier and G. R. Marshall, Eds), p. 991. ESCOM, Leiden 1990.
16. Kneib-Cordonier N., Albericio F., Barany G. in: *Peptides, Chemistry, Structure and Biology* (J. E. Rivier and G. R. Marshall, Eds), p. 895. ESCOM, Leiden 1990.
17. Nokihara K., Hellstern H., Hofle G. in: *Peptides, Chemistry, Structure and Biology* (J. E. Rivier and G. R. Marshall, Eds), p. 1046. ESCOM, Leiden 1990.
18. Beyerman M., Bienert M.: Tetrahedron Lett. *33*, 3745 (1992).
19. Steinauer R., Chen F. M. F., Benoiton N. L.: Int. J. Pept. Protein Res. *34*, 295 (1989).
20. Arad O., Houghten R. A.: Pept. Res. *3*, 42 (1990).
21. Houghten R. A.: Proc. Natl. Acad. Sci. U.S.A. *82*, 5131 (1985).
22. Tam J. P., Merrifield R. B. in: *The Peptides. Analysis, Synthesis, Biology* (S. Udenfriend and J. Meienhofer, Eds), Vol. 9, Part C, p. 185. Academic Press, New York 1987.
23. Houghten R. A., Bray M. K., DeGraw S. T., Kirby C. J.: Int. J. Pept. Protein Res. *27*, 673 (1986).
24. Barany G., Merrifield R. B. in: *The Peptides. Analysis, Synthesis, Biology* (E. Gross and J. Meienhofer, Eds), Vol. 2, Part A, p. 1. Academic Press, New York 1980.
25. Tam J. P., Heath W. F., Merrifield R. B.: J. Am. Chem. Soc. *105*, 6442 (1983).
26. Atherton E., Sheppard R. C. in: *Solid Phase Peptide Synthesis. A Practical Approach* (D. Rickwood and B. D. Hames, Eds). IRL Press at Oxford University Press, Oxford 1989.
27. Bodanszky M.: *Principles of Peptide Synthesis*. Springer, Berlin 1984.
28. Gausepohl H., Kraft M., Frank R. W.: Int. J. Pept. Protein Res. *34*, 287 (1989).
29. Mojsov S., Mitchell A. R., Merrifield R. B.: J. Org. Chem. *45*, 555 (1980).

30. Gausepohl H., Kraft M., Frank R. in: *Peptides 1988* (G. Jung and E. Bayer, Eds), p. 241. Walter de Gruyter, Berlin, New York 1989.
31. Geiser T., Beilan H., Bergot B. J., Otteson K. M. in: *Macromolecular Sequencing and Synthesis. Selected Methods and Applications* (D. H. Schlesiger, Ed.), p. 199. Alan R. Liss, New York 1988.
32. Le Nguyen D., Heitz A., Castro B.: J. Chem. Soc., Perkin Trans. 1 *1987*, 1915.
33. Hudson D.: Pept. Res. *3*, 51 (1990).
34. Kent S. B. H., Alewood D., Alewood P., Baca M., Jones A., Schnolzer M. in: *Innovation and Perspectives in Solid Phase Synthesis. Peptides, Polypeptides and Oligonucleotides* (R. Epton, Ed.), p. 1. Intercept Limited, Andover 1992.

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