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Automated Solid-Phase Peptide Synthesis: Use of 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium Tetrafluoroborate for Coupling of *tert*-Butyloxycarbonyl Amino Acids

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2 - (1*H* - Benzotriazol - 1 - yl) - 1,1,3,3, - tetramethyluronium tetrafluoroborate (TBTU) has been adapted for use as a coupling reagent for *tert*-butyloxycarbonyl (Boc) amino acids in automated solid-phase peptide synthesis. When compared to the existing preformed symmetrical anhydride procedure employing dicyclohexylcarbodiimide (DCC), the use of TBTU in the presence of 1-hydroxybenzotriazole (HOBt) provides a more efficient coupling procedure for Boc-amino acid derivatives. Overall cycle times using TBTU/HOBt coupling reagents (30 min) compare favorably to those of the DCC-mediated procedure (approx 65 min). Dimethylformamide can be used as the sole solvent for both activation and coupling reactions. Implementation of TBTU/HOBt coupling conditions does not require replumbing of any lines of the Applied Biosystems Model 430A instrument and necessitates changes to only three reagent bottle positions. The variable coupling efficiencies of Boc-asparagine following activation with TBTU/HOBt (as low as 89%) can be overcome by protection of the amide function of Boc-asparagine with the 9-xanthyl group. Examples of the synthesis and characterization of a number of peptides ranging in length from 13 to 29 residues are given.

Benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP),[1] "Castro's reagent," was designed 16 years ago as an effective coupling reagent for peptide synthesis in solution (1). In comparison to other peptide coupling reagents such as DCC, BOP couplings are essentially racemization-free and exhibit superior kinetics (2,3). More recently, BOP has been considered an extremely efficient peptide coupling reagent in both 9-fluorenylmethyloxycarbonyl (Fmoc)-polyamide- and Boc-polystyrene-mediated solid-phase peptide syntheses (SPPS) (2,3).

Since it has been established that the manufacture of BOP, as well as its utilization in SPPS, involves the formation of hexamethylphosphoric triamide—a respiratory toxin (and possible carcinogen) (4)—the use of the BOP reagent in peptide synthesis is not acceptable to many researchers because of its toxicity. This has prompted a search for new coupling reagents in synthetic peptide chemistry (5–7).

In 1978, Dourtoglou *et al.* (5) reported the synthesis of an analogue of BOP in which the phosphorus atom was replaced by a carbon atom. The corresponding *O*-benzotriazolyl-*N,N,N',N'*-tetramethyluronium hexafluoro phosphate salt (HBTU), like the BOP reagent, was found to be an excellent coupling reagent for peptide synthesis in solution; the amount of racemization of the synthetic peptides was very low, the reaction time was very fast (~15 min), and the yields obtained were not less than 87% (6). More recently, HBTU and a number

[1] Abbreviations used: TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; HBTU, *O*-benzotriazolyl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; DCM, dichloromethane; DMF, dimethylformamide; PSA, preformed symmetrical anhydride; HF, hydrogen fluoride; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; PAM, phenylacetamidomethyl; hGRF, human growth hormone releasing factor; ACT, activation vessel; CONC, concentration vessel; RV, reaction vessel.

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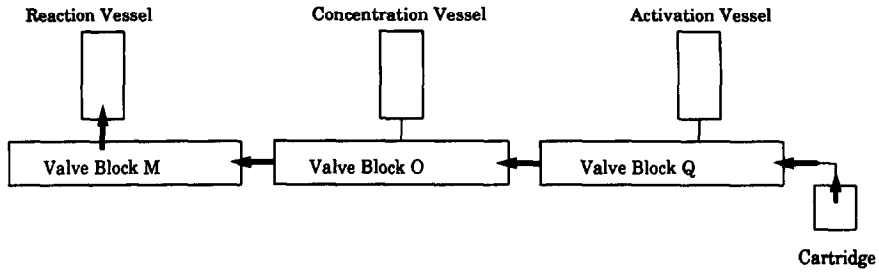

FIG. 1. A schematic diagram of the principal components of the Applied Biosystems Model 430A peptide synthesizer.

of analogues thereof, developed by Knorr and co-workers (7), have been used as coupling reagents in SPPS both in shaker and in continuous-flow peptide synthesizers. It has been demonstrated that coupling efficiencies can be further improved by the addition of HOBt in the activation procedure (8).

TBTU, the tetrafluoroborate homologue of HBTU, was shown recently by Knorr *et al.* to have kinetic properties comparable to those of HBTU, demonstrating that the counterion of these compounds has no significant influence on the coupling rate or racemization of the peptides during synthesis (8). In contrast to the BOP reagent, the by-products generated from the TBTU reaction are relatively harmless. These by-products (tetrafluoroborate, HOBt, and tetramethylurea) are completely soluble both in water and in organic solvents (7).

Recently, Fmoc-based chemistry utilizing HBTU/HOBt (9) and TBTU/HOBt coupling procedures (10) were adopted for use on the Applied Biosystems Model 430A peptide synthesizer. A semiautomated multiple peptide synthesis procedure using the "T-bag" method (11), Fmoc chemistry, and TBTU activation has been developed in the laboratory of Jung and co-workers (12). In this paper we describe the optimization of a TBTU/HOBt coupling procedure for automated SPPS employing Boc-based chemistry.

MATERIALS AND METHODS

Materials

2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate was obtained from Richelieu Biotechnologies (St-Hyacinthe, Quebec, Canada). N^{α}-Boc-N^{β}-L-asparagine (xanthyl) was purchased from Peninsula Laboratories (Belmont, CA). All other Boc-L-amino acids, Boc-L-amino acid-4 (oxymethyl) phenylacetamidomethyl (PAM) resins (0.5–0.7 mmol amino acid/g), trifluoroacetic acid (TFA), diisopropylethylamine (DIEA), ethanolamine, methanol, dichloromethane (DCM), and *N,N*-dimethylformamide (DMF) were purchased from Auspep (Parkville, Victoria, Australia). The amino acid side-chain protections were as follows: benzyl (Ser and Thr); cyclohexyl ester (Asp and Glu); 4-methylbenzyl (Cys); toluenesulfonyl (Arg); benzyloxymethyl (His); 2-chlorobenzyloxycarbonyl (Lys); 2-bromobenzyloxycarbonyl (Tyr); formyl (Trp); xanthyl (Asn). 1-Hydroxybenzotriazole and DCC were obtained from Peptide Institute (Osaka, Japan). Hydrogen fluoride (HF) was obtained from Commonwealth Industrial Gases (Preston, Victoria, Australia). Ethyl ether and acetonitrile (Chromar grade) were from Mallinckrodt (Clayton, Victoria, Australia). *p*-Cresol was obtained from Fluka (Buchs, Switzerland). Fractogel TSK-HW40(S) was purchased from Merck (Darmstadt, Germany). Deionized water, obtained from a tandem Milli-RO and Milli-Q system (Millipore, Bedford, MA), was used for all high-performance liquid chromatography (HPLC) solvents. All other chemicals were reagent grade.

Solid-Phase Peptide Synthesis

SPPS was carried out at a 0.25-mmol synthesis scale on an Applied Biosystems Model 430A peptide synthe-

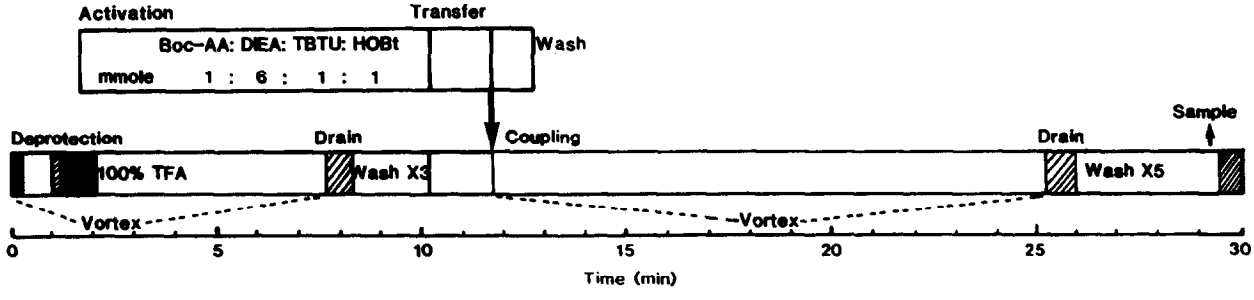

FIG. 2. A schematic outline of the TBTU/HOBt coupling cycles.

TABLE 1

List of Reagents and Solvents for TBTU/HOBt and DCC/HOBt Cycles[a]

Bottle position	TBTU/HOBt cycles	DCC/HOBt cycles
1	—	DIEA
2	TFA	TFA
3	70% ethanolamine/ methanol	70% ethanolomine/ methanol
4	—	—
5	DIEA	—
6	—	Methanol
7	0.5 M TBTU/HOBt in DMF[b]	0.5 M HOBt in DMF
8	—	0.5 M DCC in DCM
9	DMF	DCM
10	DMF	DMF

[a] For Applied Biosystems Model 430A peptide synthesizer. This instrument has not been reconfigured to accommodate the "multiple chemistry" option.

[b] The 0.5 M TBTU/HOBt reagent was prepared by dissolving 32 g TBTU and 13.5 g HOBt in 200 ml DMF and was immediately positioned on the instrument. No sedimentation or reduction in coupling efficiency was observed with this reagent over a 6-week period.

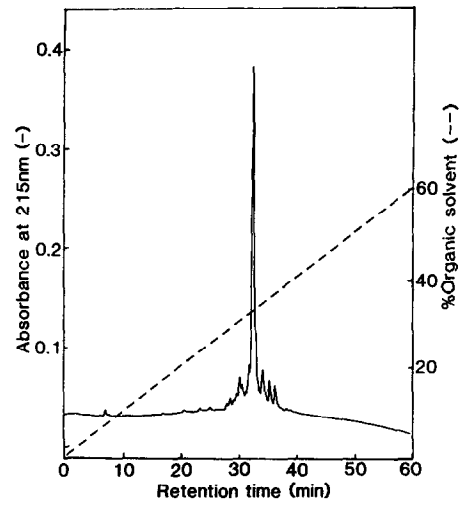

FIG. 4. Reversed-phase HPLC analysis of a crude 15-min coupling product from the synthesis of $hGRF_{1-29}$. Chromatographic conditions: Column, Brownlee RP-300 (4.6 × 100 mm). The column was developed with a 60-min linear gradient from 0–100% B, where solvent A was 0.1% (v/v) aqueous TFA and solvent B was 60% acetonitrile/40% water containing 0.09% (v/v) TFA. Flow rate, 1 ml/min. Column temperature, 45°C. Sample load, 30 μg.

sizer (Software Version 1.40), fitted with a standard size reaction vessel and using PAM resins and Boc-amino acids.

Figure 1 depicts the three principal components of the instrument, namely (i) an activation vessel (ACT) for the activation of Boc-amino acids, (ii) a concentration vessel (CONC) for solvent exchange, and (iii) a reaction vessel (RV) for the amino acid coupling process. Each component has an associated valve block through which reagents and solvents can enter the respective vessels and be transferred from one vessel to the next. The software that governs the operation of this instrument, in broad terms, comprises three separate cycles for controlling the ACT, CONC, and RV processes. During chemical synthesis, for the addition of one amino acid the instrument automatically synchronizes these three cycles in such a manner that reagent and solvent transfers are coordinated from the ACT to the CONC and from the CONC to the RV.

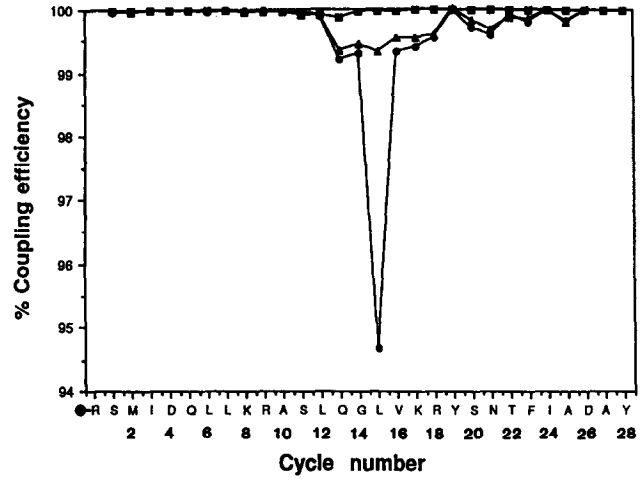

FIG. 3. Coupling yields for the synthesis of human growth hormone releasing factor (residues 1–29). Syntheses were performed at 0.25-mmol scale on Arg(toluenesulfonyl)-OCH_2-PAM resin using various TBTU/HOBt coupling times. Coupling efficiencies were determined by quantitative ninhydrin monitoring (see Materials and Methods). Peptide sequence: Y-A-D-A-I-F-T-N-S-Y-R-K-V-L-G-Q-L-S-A-R-K-L-L-Q-D-I-M-S-R. Coupling times: 2.5 min (●), 10 min (▲), and 15 min (■).

DCC-Mediated Preformed Symmetrical Anhydride (PSA) Couplings

Standard Applied Biosystems Model 430A cycles were employed for single PSA couplings for all amino acid additions except for glutamine, asparagine, and arginine, where double-coupling active ester (HOBt) cycles were used.

TBTU-Mediated Active Ester Couplings

The automated protocol for adopting the TBTU/HOBt coupling method for use on the Model 430A peptide synthesizer is shown in Fig. 2. The rationale upon which this automated protocol was based is described below.

TABLE 2

Comparison of Coupling Yields for the Synthesis of Peptide 270 Using TBTU/HOBt and DCC/HOBt Cycles[a]

Amino acid	Coupling yields (%) TBTU/HOBt cycles	DCC/HOBt cycles[b]
1. His	—	—
2. Asn[c]	99.99	99.81 (99.93)
3. Asp	99.95	99.96
4. Asn	99.99	99.86 (99.97)
5. Leu	99.87	99.82
6. Lys	99.92	99.80
7. Asp	99.89	99.80
8. Lys	99.93	99.99
9. Ile	99.99	99.90
10. Gln	99.99	99.78 (99.99)
11. Pro	99.99	99.92
12. Val	nd	nd
13. Pro	99.99	99.81
14. Pro	nd	nd
15. Phe	nd	nd
16. Leu	99.95	99.50
17. Arg	99.99	99.38 (99.71)
18. Gln	99.88	99.62 (99.74)
19. Ile	99.85	99.55
20. Arg	99.96	99.36 (99.70)
21. Leu	99.53	99.52
22. Tyr	99.57	99.54

Note. Peptide 270: Tyr-Leu-Arg-Ile-Gln-Arg-Leu-Phe-Pro-Pro-Val-Pro-Gln-Ile-Lys-Asp-Lys-Leu-Asn-Asp-Asn-His.

[a] Coupling yields were determined by quantitative ninhydrin analysis (17). nd, not determined.

[b] Values shown in parentheses are for double coupling using HOBt active esters (see Materials and Methods).

[c] Asparagine side chain unprotected for standard DCC/HOBt cycles, but protected with xanthyl for TBTU/HOBt cycles.

Activation cycle. Active ester formation is performed in the Boc-amino acid cartridge. Briefly, 1 ml DIEA (6 mmol) is added, as a timed delivery, to 1 mmol Boc-amino acid followed by 2 ml of 0.5 M TBTU/HOBt (1 mmol each) in DMF, via a 2-ml metered loop, and the mixture is dissolved. Six equivalents of base are used: three equivalents are necessary to generate the carboxylate salt of the Boc-amino acid, to ionize the HOBt, and to neutralize the trifluoroacetate anion of the C-terminal protected peptide resin salt. Since it has been demonstrated that both coupling kinetics and yield depend strongly on base concentration (13), an excess of DIEA (three equivalents) was incorporated into the present protocol.

It has been reported elsewhere that the occurrence of sequence-dependent coupling problems in SPPS can be largely overcome by the use of DMF in the coupling reaction (14). In the standard DCC/HOBt cycles of the Model 430A instrument, this is achieved by introducing a buffer transfer step (in the CONC vessel) that permits DCM to be replaced by DMF. The use of DMF as the sole solvent for both activation and coupling in the TBTU/HOBt cycles presented here overcomes this time-consuming step. Furthermore, the use of DMF, in addition to reducing the cost of peptide synthesis, reduces the potential environmental damage caused by the halogenated solvent DCM.

Another advantage of DMF is that BOP couplings are favored in this polar aprotic solvent rather than DCM (3). Moreover, TBTU, at the concentration employed (i.e., 0.5 M), is only sparingly soluble in DCM and *N*-methylpyrrolidone (data not shown). Solubility problems encountered with arginine and histidine residues in the standard activation protocol can be overcome us-

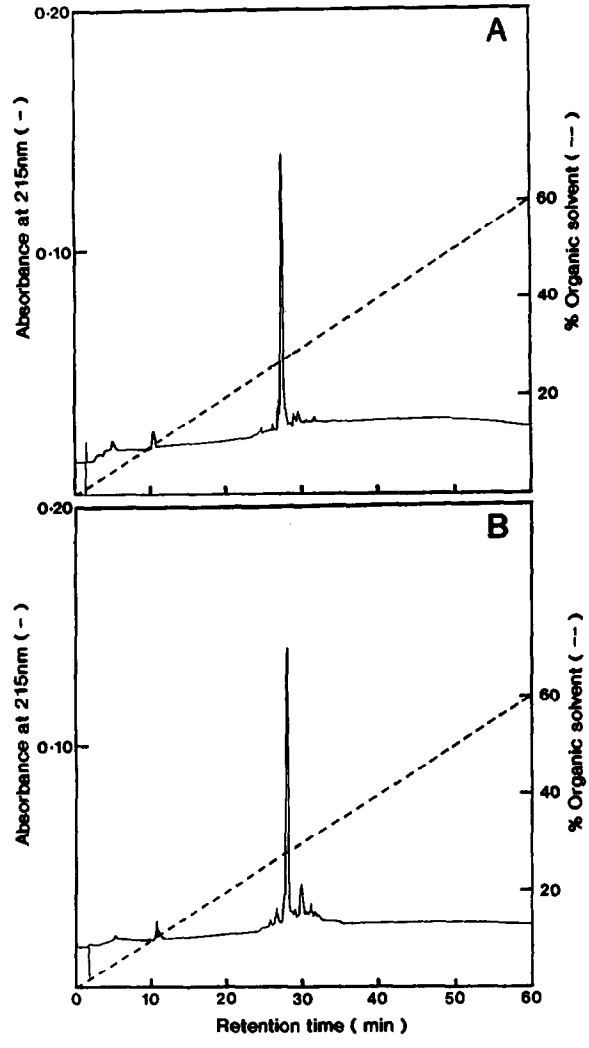

FIG. 5. Reversed-phase HPLC analysis of the crude products from the synthesis of the 22-amino acid residue peptide 270. Synthesis of peptide 270 (Y-L-R-I-Q-R-L-F-P-P-V-P-Q-I-K-D-K-L-N-D-N-H) was performed using a 0.25-mmol scale for both TBTU/HOBt coupling cycles (A) and DCC/HOBt coupling cycles (B). Chromatographic conditions were the same as those described in the legend to Fig. 4. Sample load, 10 μg.

TABLE 3

Comparison of Amino Acid Compositions of Crude Peptide 270 Synthesized by TBTU/HOBt and DCC/HOBt Coupling Procedures[a]

Amino acid	Coupling procedure used	
	TBTU/HOBt (residues/mol)	DCC/HOBt (residues/mol)
Asx	4.3	4.2 (4)
Glx	2.0	2.0 (2)
Pro	3.2	3.0 (3)
Val	1.0	0.9 (1)
Ile	1.8	2.0 (2)
Leu	3.0	3.1 (3)
Tyr	1.0	1.0 (1)
Phe	1.0	1.0 (1)
His	1.2	1.0 (1)
Lys	2.0	2.0 (2)
Arg	1.9	1.9 (2)

[a] Peptides were hydrolyzed with 6 N HCl for 24 h at 110°C, *in vacuo*. Values shown in parentheses are the expected residues per mole of synthetic peptide.

ing TBTU/HOBt cycles. Boc-Arg (tosyl) and Boc-His (benzyloxymethyl), like all other amino acids tested, are easily dissolved.

Total time for amino acid dissolution and activation is 8.5 min. The activated amino acid is then transferred directly to the reaction vessel, via valve blocks **Q**, **O**, and **M** (bypassing both activation and concentration vessels) (see Fig. 1), followed by two rinses (2 × 1 ml) of the cartridge and one rinse (1 ml) of the **Q** and **O** and **M** valve blocks with DMF.

Total activation cycle time is 11 min.

Concentration cycle. In contrast to the standard preformed symmetrical anhydride (PSA)-mediated coupling procedure, where the insoluble by-product dicyclohexylurea must be removed in the ACT vessel, by filtration and dissolution with methanol, the by-products of the TBTU/HOBt coupling procedure—tetrafluoroborate and tetramethylurea—are completely soluble in water and in organic solvents (7). Hence, the ACT and CONC vessels are not required and the CONC cycle in the Model 430A software is reduced solely to a synchronous role, facilitating delivery from the ACT to the RV.

Total cycle time is 2.5 min.

Reaction vessel cycle. The reaction vessel cycle pattern includes deprotection, neutralization, coupling, and resin sampling steps. With the exception of the neutralization step, this follows the same general pattern as the standard Model 430A Boc-amino acid RV cycles. It has been shown that a fourfold reduction in the time required for Boc removal can be achieved by the use of neat (100%) TFA (15). After an initial wash with 3 ml of neat TFA (1.0 min), the RV is drained and complete removal of the Boc groups is achieved by adding 6 ml of neat TFA and mixing for 6 min. The RV is drained and the resin is successively washed (three times) with DMF to remove residual TFA.

In contrast to standard Model 430A activation cycles for Boc-amino acids, the TBTU/HOBt activation is performed in the presence of a large excess of DIEA. This eliminates the need for a separate step to neutralize the deprotected peptidyl resin prior to coupling the next amino acid. Thus, in the TBTU/HOBt cycles, neutralization is achieved immediately upon delivery of the activated amino acid from the amino acid cartridge.

The coupling time for TBTU/HOBt active esters is 15 min. Following coupling, the RV is drained and the peptidyl resin is washed (five times) with DMF. Resin samples, in the range 3–5 mg, are taken during the last DMF wash.

For a 0.25-mmol scale synthesis, the total time required to add one amino acid residue onto the protected peptide chain is 30 min (compared to approximately 65

TABLE 4

Peptides Synthesized on the Model 430A Peptide Synthesizer using TBTU/HOBt Coupling Conditions[a]

Peptide	Number of amino acids	Amino acid sequence	Coupling efficiencies	
			Average (%)	Overall (%)
259	20	KMPYEPCLPQYPHINGSVKT	99.76	96.5
260	20	KVFTHDLLPPGPPSNGGPRT	99.93	98.9
261	15	PQFVQNINIENLFRY	99.81	98.0
267	24	CGETGPGKAGEQGETGPGKAGEQG	99.69	93.7
270	22	YLRIQRLFPPVPQIKDKLNDNH	99.91	98.3
271	15	SSLRRHRRRRPEVKY	99.88	98.5
272	15	EHRVKRGLTVAVAGA	99.78	97.3
273	13	SGPLKAEIAQRLE	99.73	97.1
276	16	VKKRCSMWIIPTDDEA	99.72	96.2

[a] 0.25-mmol scale syntheses on PAM resin.

TABLE 5

Comparison of Coupling Yields for Boc-asparagine in TBTU/HOBt- and DCC/HOBt-Mediated Coupling Reactions[a]

Coupling conditions	Cycle number 1 Asn	2 Asp	3 Asn	4 Leu
DCC/HOBt (Boc-Asn)	99.93	99.96	99.97	99.82
TBTU/HOBt (Boc-Asn)	89.64	99.95	95.90	99.89
TBTU/HOBt (Boc-Asn (Xan))	99.99	99.95	99.99	99.87

[a] The amino acid sequence of peptide 270 is given in Table 2. 0.25-mmol scale syntheses on His(benzyloxymethyl)-OCH_2-PAM resin. Coupling yields (%) were determined by quantitative ninhydrin analysis (17).

min for the standard PSA Boc-amino acid cycles on the Model 430A instrument).

Implementation of TBTU/HOBt cycles on the Applied Biosystems Model 430A peptide synthesizer. Two new cycle functions were created to facilitate delivery of the activated amino acid from the cartridge to the RV—an ACT cycle function "CARTRIDGE TRANSFER" (opening valves 26, 35, 37, and 44) and a CONC cycle function "TRANSFER" (opening valves 0, 2, 11, 15, and 23).

In addition to the software changes described above, only one new reagent (0.5 M TBTU/HOBt in DMF) was required to implement the TBTU/HOBt cycles. Changes in reagent and bottle positions required to adopt these cycles are listed in Table 1. No replumbing of the instrument was required.[2]

Specific details of the synthesis cycles can be obtained from the authors upon request.

Ninhydrin Assay

The Kaiser ninhydrin assay for amino groups (16), as modified by Sarin *et al.* (17), was used to monitor the coupling reactions during SPPS.

Hydrogen Fluoride Cleavage

Cleavage and deprotection of the peptides were carried out using anhydrous HF in a Teflon apparatus obtained from Peptide Institute using established procedures (18). Briefly, to 0.5 g of the dried resin, 1 ml of *p*-cresol and 9 ml of HF were added. After 1 h at −5°C the HF was removed *in vacuo* and the resin washed (three times) with ethyl ether. The peptide was extracted (three times) with 10% (v/v) aqueous acetic acid and then lyophilized.

Isolation of Peptides

All crude peptides were initially desalted by size-exclusion chromatography. Briefly, crude peptides (~100 mg) were dissolved in 3–4 ml of 10% (v/v) aqueous acetic acid and applied directly to a column (40 × 2.5 cm) of Fractogel TSK-HW40(S) previously equilibrated with 10% (v/v) aqueous acetic acid. The column was devel-

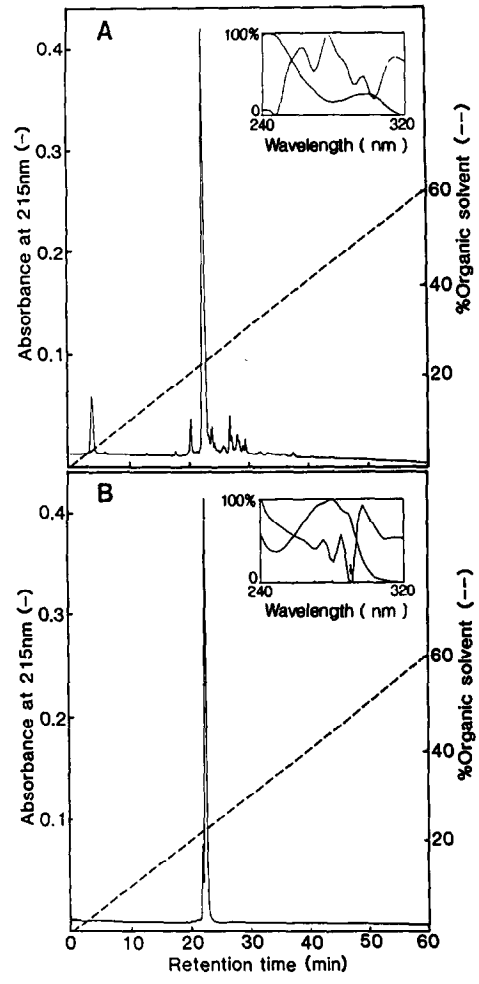

FIG. 6. Reversed-phase HPLC analysis of the tryptophan-containing 16 amino acid residue peptide 276. Following HF treatment of the peptidyl resin, the crude product was extracted, lyophilized (see Materials and Methods), and then analyzed by RP-HPLC (A). The absorption spectrum, obtained using a diode-array detector, is shown in the inset: Absorbance has been normalized to relative absorbance on a scale 0–100%. The crude product was treated with aqueous 0.1 M piperidine to remove the *N*-formyl group (see Materials and Methods), chromatographed on a Fractogel TSK-HW40 (S), and then analyzed by RP-HPLC (B). The absorption spectrum is shown in the inset. The position of a characteristic tryptophan extremum (290 ± 2 nm) (26,27) is indicated by the arrow. Chromatographic conditions were the same as those outlined in the legend to Fig. 4.

[2] The Applied Biosystems Model 430A peptide synthesizer used in this study had not been previously modified by the manufacturer to accommodate the "multiple chemistry upgrade configuration" option.

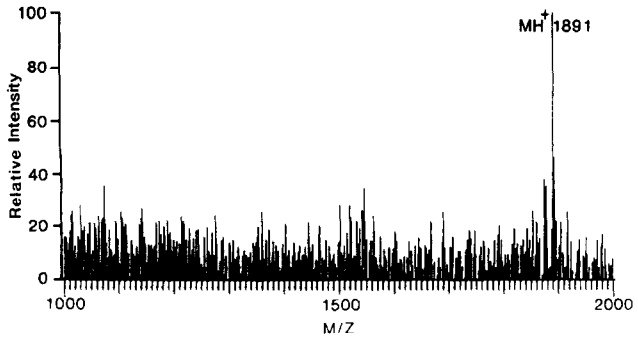

FIG. 7. Mass spectrometric analysis of synthetic peptide 276 following size-exclusion chromatography.

oped at a flow rate of 1.5 ml/min and the column eluant was monitored by absorbance at 280 nm.

Peptide-containing fractions from the Fractogel TSK-HW40(s) column were assessed for purity by analytical reversed-phase HPLC on either (i) a Hewlett–Packard liquid chromatograph Model 1050 equipped with a Model 3396 integrator or (ii) a Hewlett–Packard liquid chromatograph Model 1090 fitted with a Model 1040 diode-array detector as described elsewhere (19). The column used was a Brownlee RP-300 (30-nm pore size, 7-μm particle diameter) octylsilica packed into a stainless steel 100 × 4.6-mm internal diameter cartridge (Applied Biosystems, Foster City, CA).

Selected peptide fractions were pooled and then dried by lyophilization.

Deprotection of N^{im}-Formyltryptophan

N^{im}-Formyltryptophan-containing peptides (100 mg) were dissolved in 3–5 ml of 0.1 M piperidine and stirred for 1 h at 25°C, to remove the formyl group, and then desalted by size-exclusion chromatography on a column (40 × 2.5 cm) of Fractogel TSK-HW40(S).

Analysis of Synthetic Peptides

Following size-exclusion chromatography, the amino acid composition of the synthetic peptides was determined on a Beckman amino acid analyzer (Model 6300) fitted with a Model 7000 data analysis system. Samples were hydrolyzed *in vacuo* at 110°C for 24 h with either 6 M HCl containing 0.1% (w/v) phenol or 4 M methanesulfonic acid containing 0.2% (w/v) tryptamine (20). In the latter case, the hydrolyzate (20 μl) was neutralized by the addition of 180 μl of 0.2 M sodium citrate buffer, pH 5.60, prior to loading onto the amino acid analyzer.

Automated amino acid sequence analysis of synthetic peptides was performed using an Applied Biosystems sequencer (Model 470A) fitted with a Model 120A online phenylthiohydantoin–amino acid analyzer modified to allow injection of total phenylthiohydantoin–amino acid derivative (21). Polybrene (22) was used as a carrier.

In some cases, the relative molecular mass (M_r) of the synthetic peptides was ascertained by mass spectrometry. Mass spectra were acquired on a JEOL JMSDX300 mass spectrometer fitted with a JEOL JMA-3100 data system.

RESULTS AND DISCUSSION

Optimization of TBTU/HOBt Cycles

It has been reported that poor couplings can occur for several consecutive residues at peptide chain lengths of 10–20 residues due, presumably, to the formation of β-sheet aggregates (14,23). In order to optimize the time required for coupling using the TBTU/HOBt cycles, a "difficult" 29-residue peptide (23), human growth hormone releasing factor ($hGRF_{1-29}$), was synthesized on Arg(toluenesulfonyl)–OCH_2–PAM resin using coupling times of 2.5, 10, and 15 min. The efficiency of amino acid coupling was determined, postsynthetically, by quantitative ninhydrin analysis of residual uncoupled amine content of peptidyl resin samples taken automatically at the end of each synthesis cycle. Figure 3 shows the calculated ninhydrin data for the synthesis of $hGRF_{1-29}$ using these three different coupling times. Although 2.5-min couplings were sufficient to ensure complete coupling of peptide chain lengths up to 12 residues, longer chain lengths required extended coupling times of 15 min. Thus, 15-min couplings were used throughout this study to ensure maximal coupling efficiencies. The overall quality of the crude 15-min coupling time product was evaluated, by RP-HPLC, after cleavage with anhydrous HF (see Materials and Methods) (Fig. 4). The average stepwise coupling yield, as calculated from

TABLE 6

Comparison of Reagent and Solvent Usage per Residue Addition for TBTU/HOBt- and DCC/HOBt-Mediated Coupling Protocols on the Model 430A Peptide Synthesizer[a]

	Coupling protocol	
Reagent	TBTU/HOBt	DCC/HOBt
Boc-amino acid	1 mmol	2 mmol
TBTU	1 mmol	—
DCC	—	1 mmol
HOBt	1 mmol	2 mmol
DMF	~150 ml	~120 ml
DCM	—	~200 ml
DIEA	1 ml	3 ml
TFA	9 ml	9 ml
MeOH	—	4 ml
Waste neutralizer	10 ml	10 ml

[a] Approximate reagent usage per synthesis cycle was based upon the synthesis of a 15-residue peptide.

the quantitative ninhydrin data, for the 15-min coupling synthesis was >99.9% with an overall chain assembly yield of 98%.

Comparison of TBTU/HOBt and DCC/HOBt Activation Cycles

To demonstrate the superior kinetics of the TBTU/HOBt cycles over existing DCC/HOBt-mediated couplings, a 22-residue peptide (peptide 270) was chemically synthesized using both the TBTU/HOBt and the Applied Biosystems standard DCC/HOBt coupling procedures. A comparison of the coupling yields for both TBTU/HOBt- and DCC/HOBt-mediated syntheses is given in Table 2. The stepwise yields for both coupling protocols were >99% per residue with overall chain assembly yields of 98.3% and 96.3% for TBTU/HOBt and DCC/HOBt synthesis protocols, respectively. The yield of peptide resin obtained for synthesis using the TBTU/HOBt cycles (1.12 g) compared favorably with that obtained with the DCC/HOBt cycles (1.08 g).

After deprotection and cleavage from the resin, the peptides were extracted with 10% (v/v) acetic acid and then lyophilized. For both TBTU/HOBt and standard DCC/HOBt protocols, this resulted in a crude product with a 71–79% yield based on the theoretical yields corrected for resin sampling losses.

Analytical RP-HPLC profiles of the crude peptides, shown in Fig. 5, revealed a major peak and minor by-products for both TBTU/HOBt (Fig. 5A) and DCC/HOBt (Fig. 5B) peptide synthesis protocols. The amino acid compositions of crude peptide 270 prepared by both protocols were in excellent agreement with each other and with the theoretical composition predicted from the sequence (Table 3).

The general utility of the TBTU/HOBt coupling cycles in the Model 430A instrument has been demonstrated for a variety of synthetic peptides, listed in Table 4.

Coupling of Boc-asparagine

It is well recognized that when Boc-asparagine is activated with DCC (24), or Fmoc-asparagine with phosphonium reagents (e.g., Castro's reagent) (13), poor coupling yields in SPPS can occur. This is due to dehydration of the side-chain amide group to the corresponding nitrile.

In the case of DCC-mediated coupling, this problem can be overcome by the use of HOBt active esters generated *in situ* by the inclusion of HOBt in the activation reaction (24). This is illustrated for peptide 270 in Table 5. For TBTU/HOBt-mediated couplings, however, Boc-asparagine coupling yields were still very poor (Table 5). However, if the side-chain amide of asparagine is protected by the 9-xanthyl derivative, asparagine dehydration—and poor coupling yields—in TBTU/HOBt coupling reactions were not observed (Table 5). Although it has been reported that intramolecular dehydration can occur during glutaminyl bond formation—albeit, to a minor degree compared to the incidence of nitrile formation during aspariginyl bond formation (25)—it was not observed during this study; unprotected Boc-L-glutamine residues, when compared with other Boc-amino acids, were assembled with comparable coupling yields.

Deprotection of N^{α}-Boc-L-N^{im}-formyltryptophan

Since the indole ring of Boc-tryptophan is acid labile and also readily alkylated by carbonium ion reagents during cleavage of peptide from resin, the *N*-formyl derivative was used during chain assembly. The *N*-formyl group is acid stabile and survives HF cleavage conditions. The facile removal of the formyl group is illustrated for the 16-amino residue peptide 276 (sequence shown in Table 4). Analytical RP-HPLC profile of the crude N^{im}-formyltryptophan-containing peptide 276 is shown in Fig. 6.

Spectral analysis of the N^{im}-formyltryptophan-containing peptide, shown in the inset to Fig. 6A, reveals an absorption maximum at 300 ± 2 nm and a minimum at 280 ± 2 nm. This observation is consistent with the previously reported spectral analysis for formylated tryptophan (26). Subsequent analysis of the second-order-derivative spectrum reveals three characteristic extrema for N^{im}-formyltryptophan at 269 ± 2, 291 ± 2, and 302 ± 2 nm. Removal of the N^{im}-formyl group, by treatment of the peptide with aqueous base (see Materials and Methods), can be monitored by spectral analysis. Fully deformylated tryptophan (Fig. 6B, inset) exhibits the characteristic extremum for tryptophan at 290 ± 2 nm in the second-order-derivative spectrum (27,28).

Amino acid analysis of peptide 276 ($Asx_{2.0}$, $Thr_{1.0}$, $Ser_{1.0}$, $Glu_{1.0}$, $Pro_{1.0}$, $Ala_{1.1}$, $Val_{1.0}$, $Met_{0.9}$, $Ile_{1.4}$, $Lys_{1.8}$, $Trp_{0.7}$, $Arg_{1.1}$), with the exception of Cys (not determined), was in good agreement with the expected composition.

If the N^{im}-formyl group is removed prior to, or during, HF deprotection/cleavage, a scavenger such as indole or ethanedithiol must be included in the reaction mixture to prevent alkylation of tryptophan (29). However, in our experience, the inclusion of ethanedithiol in the reaction mixture has led to poor solubility and low extraction yields. Further confirmation of the authenticity of deprotected peptide 276 was obtained by mass spectrometry. The mass spectrum of peptide 276, shown in Fig. 7, yielded a protonated molecular ion of mass 1891 Da, which is consistent with the expected molecular mass of the peptide.[3]

[3] Peptide 276 was the Association of Biomolecular Resource Facilities test peptide for 1991. Mass spectrometric (MS) analysis of crude

SUMMARY

1. As for other uronium (e.g., HBTU) and phosphonium salts (e.g., BOP), the key advantage of the TBTU reagent in SPPS—over the traditional DCC-mediated symmetrical anhydride activation procedure—is the very short reaction time (~15 min) required for coupling (6). This factor, together with (i) the use of neat TFA, described by Kent *et al.* (15), for efficient Boc removal, (ii) the absence of insoluble by-products (e.g., dicyclohexlurea in the DCC-mediated cycle), and (iii) the ability to use a single solvent (e.g., DMF) with the TBTU/HOBt coupling reagents allows for single Boc-amino acid addition cycle times of 30 min. Such cycle times compare very favorably with the traditional DCC-mediated cycle times of ~65 min and approach the rapid (20- to 25-min) cycle times developed by Kent for the Model 430A instrument (personal communication). It is anticipated that modification of the "Kent" rapid cycles to incorporate the TBTU/HOBt activation protocol will further reduce overall cycle times.

2. A further advantage of the TBTU/HOBt cycles is that they are less expensive than the traditional DCC/HOBt cycles. This economic advantage is achieved by the reduced consumption of reagents and solvents (Table 6).

3. Implementation of the TBTU/HOBt activation chemistry on the Model 430A instrument can be readily achieved without any modification or replumbing of the instrument.

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REFERENCES

1. Castro, B., Dormoy, J-R., Evin, G., and Salve, C. (1975) *Tetrahedron Lett.* **14,** 1219–1222.
2. Hudson, D. (1988) *J. Org. Chem.* **53,** 617–624.
3. Fournier, A., Wang, C-T., and Felix, A. M. (1988) *Int. J. Pept. Protein Res.* **31,** 86–97.
4. Shott, L. D., Borkovec, A. B., and Knapp, W. A. (1971) *Toxicol. Appl. Pharmacol.* **18,** 499–506.
5. Dourtoglou, V., Ziegler, J. C., and Gross, B. (1978) *Tetrahedron Lett.,* 1269–1272.
6. Dourtoglou, V., Gross, B., Lambropoulou, V., and Zioudrou, C. (1984) *Synthesis,* 572–574.
7. Knorr, R., Trzeciak, A., Bannworth, W., and Gillessen, D. (1989) *in* Peptides 1988 (Jung, G., and Bayer, Eds.), pp. 37–39, de Gruyter, Berlin.
8. Knorr, R., Tzeciak, A., Bannworth, W., and Gillessen, D. (1989) *Tetrahedron Lett.* **30,** 1927–1930.
9. Fields, C. G., Lloyd, D. H., Macdonald, R. L., Otteson, K. M., and Noble, R. L. (1991) *Pept. Res.* **4,** 95–101.
10. Breipohl, G., and Knolle, J. (1990) *in* Solid Phase Synthesis (Epton, R., Ed.), pp. 217–226, SPCC (UK), England.
11. Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. USA* **83,** 5131–5135.
12. Beck-Sickinger, A. G., Dürr, H., and Jung, G. (1991) *Pept. Res.* **4,** 88–94.
13. Gausepohl, H., Kraft, M., and Frank, R. (1988) *in* Peptides 1988 (Jung, G., and Bayer, Ed.), pp. 241–243, de Gruyter, Berlin.
14. Meister, M. M., and Kent, S. B. H. (1983) *in* Peptides, Structure and Function (Hruby, V. J., and Rich, D. H., Eds.), pp. 103–106, Pierce Chem. Co., Rockford, IL.
15. Kent, S. B. H., Parker, K. F., Schiller, D. L., Woo, D. D., Clark-Lewis, I., and Chait, B. T. (1988) *in* Peptides, Chemistry and Biology (Marshall, G. R., Ed.), pp. 173–178, Escom, Leiden.
16. Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) *Anal. Biochem.* **34,** 595–598.
17. Sarin, V., Kent, S. B. H., Tam, J. P., and Merrifield, R. B. (1981) *Anal. Biochem.* **117,** 147–157.
18. Stewart, J. M., and Young, J. D. (1984) Solid Phase Peptide Synthesis, 2nd ed., pp. 85–88, Pierce Chem. Co., Rockford IL.
19. Simpson, R. J., Ward, L. D., Reid, G. E., Batterham, M. P., and Moritz, R. L. (1989) *J. Chromatogr.* **476,** 345–361.
20. Simpson, R. J., Neuberger, M. R., and Liu, T-Y. (1976) *J. Biol. Chem.* **251,** 1936–1940.
21. Begg, G. S., and Simpson, R. J. (1989) *in* Techniques in Protein Chemistry (Hugli, T. E., Ed.), pp. 79–88, Academic Press, New York.
22. Klapper, D. G., Wilde, C. E., and Capra, J. D. (1978) *Anal. Biochem.* **85,** 126–131.
23. Kent, S. B. H. (1985) *in* Peptides, Structure and Function (Deber, C. M., Hruby, V. J., and Kopple, K. D., Eds.), pp. 407–414, Pierce Chem. Co., Rockford, IL.
24. Mojsov, S., Mitchell, A. R., and Merrifield, R. B. (1980) *J. Org. Chem.* **45,** 555–560.
25. Gish, D. T., Katsoyannis, P. G., Hess, G. P., and Stedman, R. J. (1956) *J. Am. Chem. Soc.* **78,** 5956.
26. Culwell, A., and Sparrow, J. T. (April 28, 1987) Applied Biosystem User Bulletin 18.
27. Grego, B., Van Driel, I. R., Goding, J. W., Nice, E. C., and Simpson, R. J. (1986) *Int. J. Pept. Protein Res.* **27,** 201–207.
28. Grego, B., Nice, E. C., and Simpson, R. J. (1986) *J. Chromatogr.* **352,** 359–368.
29. Sharp, J. J., Robinson, A. B., and Kamen, M. D. (1973) *J. Am. Chem. Soc.* **95,** 6097–6108.
30. Smith, A. J., Young, J. D., Carr, S. A., Marshak, D. R., Williams, L. C., and Williams, K. R. (1992) *in* Techniques in Protein Chemistry III (Hogue-Angeletti, R., Ed.), Academic Press, San Diego, CA (in press).

ties test peptide for 1991. Mass spectrometric (MS) analysis of crude peptide 276 (code, 0276C), determined by electrospray MS, yielded a very intense peak with an observed M_r of 1891 Da (30). This peak, the desired product, represents >95% of the crude desalted peptide. Peptide 276, a substrate for casein kinase II and cyclic AMP-dependent protein kinase, was active in these biological assays (75.5 and 82.3%, respectively).