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Search for Optimal Coupling Reagent in Multiple Peptide Synthesizer

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Abstract: *Ten different coupling reagents and their combinations were tested in parallel in the synthesis of four model peptide sequences. Significant differences were found between uronium and phosphonium salt-based reagents and carbodiimide. Diisopropylcarbodiimide was identified as an optimal reagent based on the purity of the product, stability of the reagent, and convenience of handling on plate-based multiple parallel centrifugation synthesizer.* © 2006 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 84: 340–347, 2006

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Keywords: *coupling reagents; model peptide sequences; reagents; plate-based multiple parallel centrifugation synthesizer; difficult sequences; reagent stability; mixed coupling reagents*

INTRODUCTION

Synthetic peptides are becoming more accessible as a commodity due to the progress of synthetic technology allowing parallel synthesis of up to several hundreds of sequences. Additionally, new coupling reagents are being introduced at a rapid pace. However, these novel reagents, despite their faster reaction kinetics, may not be that advantageous in multiple parallel synthesizers where other limitations prevail. First of all, in these machines, the individual amino acids must be distributed to the particular reagent compartments (e.g., wells of microtiterplates) and this distribution may take longer than a complete condensation reaction. Second, it is more difficult to keep the exact molar ratio during multiple deliveries of small volumes of reagents and therefore a reagent that can be applied in excess without the danger of side reactions (e.g., guanylation of amino group in the case of uronium salts) can be advantageous. Third, the reagent may have to be prepared hours before being used and must be stable during the course of the whole synthesis. Fourth, peptide sequences vary in their "difficulty" of synthesis and it is a challenge to predict reaction kinetics. Detection of incomplete coupling by real-time noninvasive monitoring is also an advantage; however, simpler ways of monitoring such as observing color changes are not possible with some reagents. Therefore, because in plate-based parallel synthesis the whole plate has to wait for the slowest coupling to finish, the cycle time is driven by these slow sequences.

We have designed and constructed a number of automated peptide and oligonucleotide synthesizers based on the tilted plate centrifugation technology.[1–4] We were interested in finding the most appropriate reagent for this application, providing high-quality peptides at the most economical cost.

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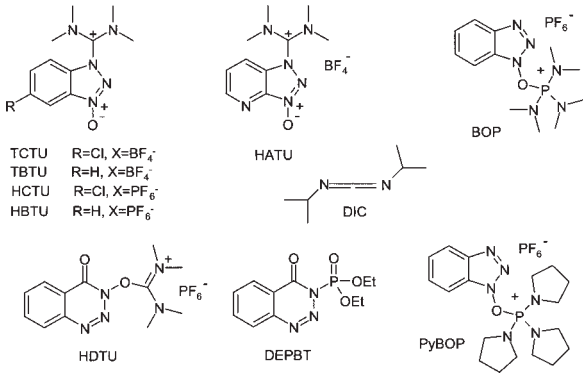

FIGURE 1 Structures of coupling reagents used in this study.

RESULTS

The reagents tested in our experiment were 2-(*1H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TCTU),[5] 2-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU),[5] 2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HDTU),[6] 2-(1*H*-benzotriazole-l-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU),[7] 2-(1*H*-benzotriazole-l-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[8] 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate (HATU),[9] 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4-(3*H*)-one (DEPBT),[10] benzotriazol-l-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP),[11] benzotriazol-l-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate (PyBOP),[12] and N,N′-diisopropylcarbodiimide (DIC).[13] Their structures are shown in Figure 1. Reagents were tested in the synthesis of four sequences of decapeptides chosen for their degree of difficulty. Difficulty was evaluated by the Peptide Companion software.[14] Every sequence was synthesized in duplicate to eliminate the possibility of variation between wells of the microtiterplate. Twelve reagents were used simultaneously on one plate. To avoid using "regulated substance" restrictions and including paperwork, we used for removal of the 9-fluorenylmethoxycarbonyl (Fmoc)-protecting group 4-methylpiperidine, which is, as we have shown earlier,[15] identical in its performance to piperidine.

Before the large-scale comparative synthesis, we evaluated the coupling times needed to achieve a reasonable level of completion for the different coupling agents. Example of results obtained with short coupling times (2–8 min) and three reagents is given in Figure 2. Obviously, the "fastest" reagent is HBTU, which provides reasonable coupling efficiency even at 2 min. Using this coupling time, DIC provided almost no correct product. However, at 8 min, performance of DIC was comparable to both TBTU and HBTU. We have decided to use double coupling and 30 min coupling time in our comprehensive test to take advantage of the slower reacting DIC reagent. [In the case of DIC, the activated species is an active ester of N-hydroxybenzotriazole (HOBt). DIC without addition of HOBt would result in racemized product.] Coupling speed is actually not an advantage in synthesizers where distribution of amino acids into the reaction compartments may take several minutes per plate.

The configuration of the plate and high performance liquid chromatography (HPLC) results of the parallel comparative synthesis are shown in Figure 3. Products of synthesis were cleaved in parallel from the resin using mixture K,[16] and precipitated by ether. Side products were analyzed by HPLC [ultraviolet (UV) monitoring at 217 nm] and 20–30 reproducible peaks were identified in each trace. In our analysis, we concentrated on the peaks with content higher than 0.1%. By liquid chromatography–mass spectroscopy (LC-MS), we were able to identify some of them. Identities of these side products are given in parentheses in Figures 6, 8, 10, and 12. Distribution of major contaminants is shown in Table I.

In the synthesis, the sequence LHRH (EHWSYGWLPG) did not show any significant problems. Assignment of some of individual peaks is shown in

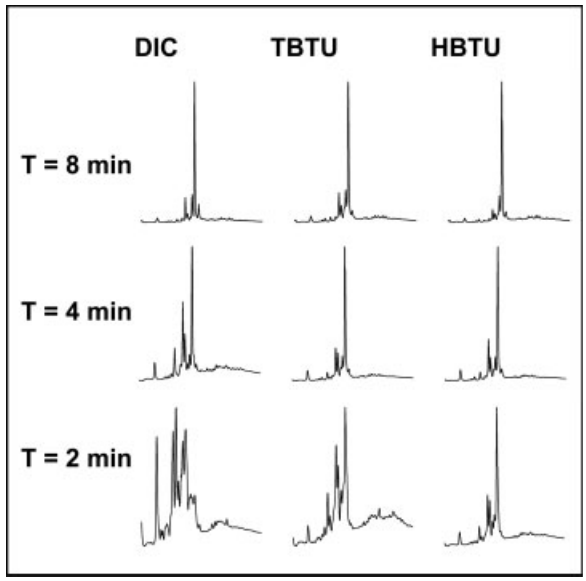

FIGURE 2 HPLC traces of ACP 65–74 synthesized using DIC, TBTU, and HBTU and coupling times of 2, 4, and 8 min.

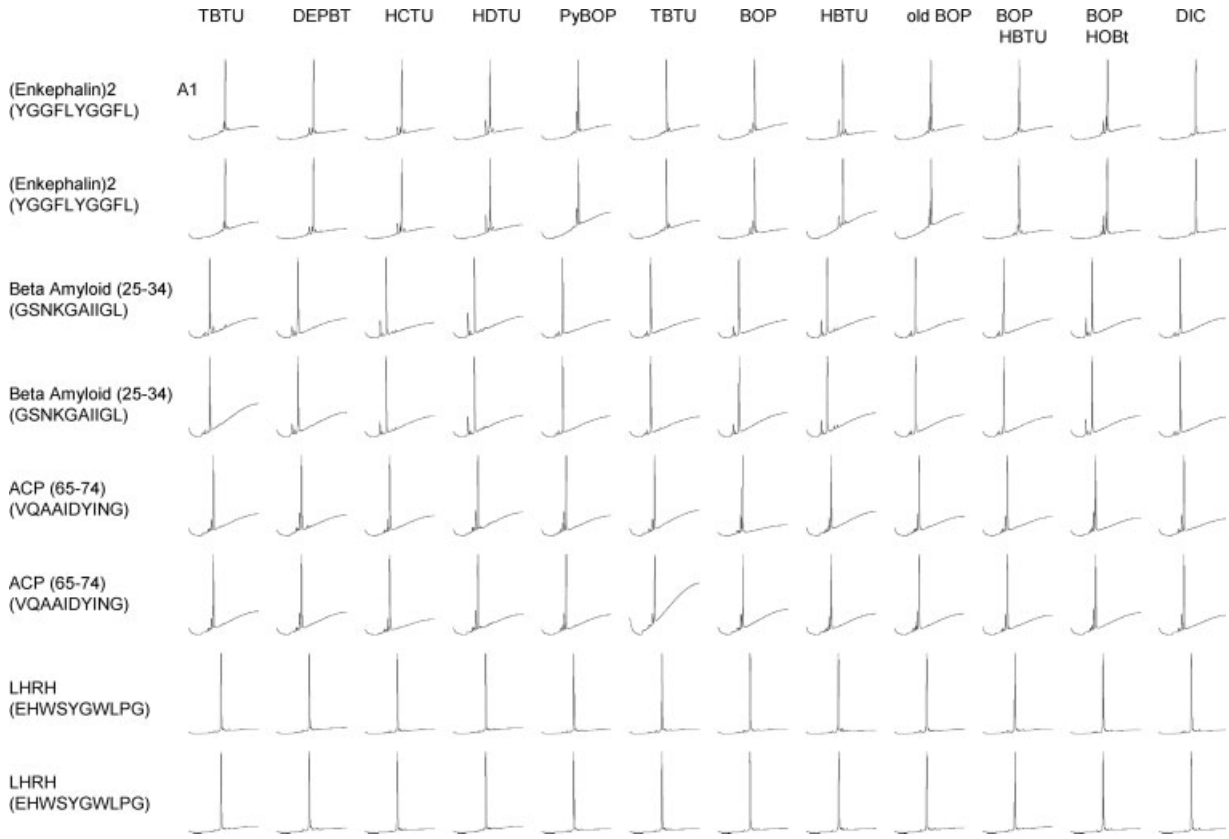

FIGURE 3 Plate layout and HPLC results.

Figure 4. The first peak (1), which was present only in syntheses utilizing uronium salts, is probably the guanylated peptide.[17] The second peak (2) is composed of two components with molecular weights corresponding to peptides missing both glycine and serine or tyrosine, leucine, and proline. The third peak (3) eluted earlier than the major product (front shoulder on the product peak) is missing histidine. Because peak numbers 5 and 7 contain peptides of the molecular weight corresponding to the product with additional *tert*-butyl group—probably attached to one of the tryptophans—we assumed that one of these peaks have the amino acid of the D configuration. Because the molecules of diastereomeric LHRH were eluted later (peak numbers 6 and 8), we can speculate that later peak number 7 is the one containing the D-amino acid. Peak number 8 is also missing a glycine.

Even though the purities, based on HPLC, were very similar with all tested reagents (83–90%), the best were achieved with DIC (Figure 5). Distribution of side products are shown in Figure 6. Surprisingly, an old solution of BOP reagent (stored at room temperature in a brown bottle for five weeks) produced the same (or better) results than the freshly prepared reagent. The use of uronium salts (with the exception of TCTU) resulted in a slightly higher racemization than use of BOP, DIC, or DEPBT. TBTU was the only uronium salt not producing guanylation product. (The extent of guanylation was not considered significant. The highest content observed was 0.5%.)

One of our test sequences was linear dimer of leucine enkephalin (YGGFLYGGFL). We have discovered that the synthesis of this sequence was more difficult than anticipated. YGGFL was the test peptide for establishing the performance of the new algorithm for the newly designed synthesizer because it can be prepared in nearly 100% purity with minimal effort and does not require extensive care during cleavage and deprotection. If this sequence showed problems during testing, then it is likely that no "real-life" peptide could be synthesized successfully. In the instrument test, we ran the same synthesis twice consecutively on the same sample of the resin to give the dimer. We discovered, however, that the dimer contains significant impurities even though the synthesis of monomer did not show any problems. As a result, this peptide was used as one of the test sequences.

The HPLC of this model peptide synthesized by HCTU is given in Figure 7, and profile of major impurities is shown in Figure 8. The most striking as-

Table 1 Content of Four Model Peptides and Major Contaminants in the Crude Product. Quantitation by HPLC at 217 nm

Peptide (Peak No.)	Difficulty[a]	TCTU	TBTU	HCTU	HDTU	HBTU	BOP HBTU	PyBOP	BOP	BOP (Old)	BOP HOBt	DIC	DEPBT
LHRH (EHWSYGWLPG)	0.96, 0.89	88.4	83.7	87.8	84.5	88.1	86.2	88.0	84.8	88.0	87.1	89.0	89.9
LHRH–des-His (3)		1.9	2.5	1.8	2.9	2.9	2.1	1.3	1.5	1.3	0.7	0.7	1.5
LHRH–diastereomer (6)		1.7	2.3	2.3	2.4	2.8	1.4	1.2	1.2	1.1	1.3	0.7	1.0
(Enkephalin)2 (YGGFLYGGFL) (4)	1.00, 1.00	77.3	84.6	77.3	69.9	70.4	82.6	71.0	78.0	73.0	65.6	84.8	73.3
(Enkephalin)2–des-Tyr (3)		10.3	2.4	5.4	2.5	2.8	5.4	19.4	10.1	15.0	14.1	1.7	5.8
(Enkephalin)2–des-Leu (2)		3.8	4.2	9.3	14.4	15.2	4.6	4.1	6.6	4.5	13.0	4.9	9.4
β-Amyloid (25–34) (GSNKGAIIGL) (3)	1.35, 1.18	86.4	85.5	75.3	65.1	75.4	87.8	87.6	84.4	88.2	75.3	89.5	79.7
β Amyloid (25–34)–des-Ile (1)		2.6	4.3	13.5	20.0	14.6	3.8	2.9	8.9	2.8	17.5	3.5	9.2
ACP (65–74) (VQAAIDYING) (6)	1.40, 1.20	75.1	77.6	77.8	67.2	75.0	74.8	69.6	73.7	71.4	71.9	72.7	66.5
ACP (65–74)–des-Val (5)		9.9	11.6	10.7	15.3	11.2	13.8	14.6	14.5	14.5	15.5	14.5	14.3
ACP (65–74)–des-Ile (2)		2.6	3.2	3.0	4.2	2.7	3.2	2.7	3.2	3.0	2.8	3.3	3.8
ACP (65–74)–des-Ile (3)		1.5	2.2	1.7	2.0	2.1	2.5	1.5	2.3	2.0	1.6	2.2	2.7

[a]Most difficult coupling (first value) and average value for difficulty of couplings (second value); scale: <0.8 easy, 0.8–1.2 normal, >1.2 difficult.

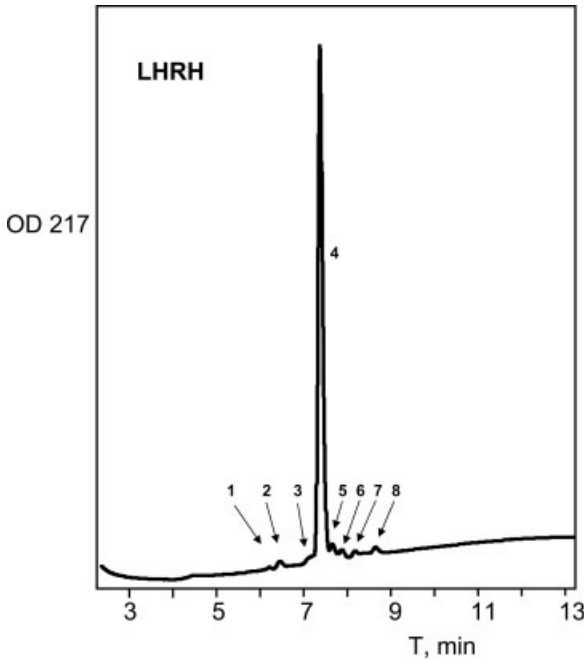

FIGURE 4 HPLC of LHRH synthesized using TCTU.

pect of this model peptide, aside from its excellent synthesis with DIC, is the difference between the uronium and phosphonium salts. A major side product when coupling with HCTU, HDTU, and HBTU (surprisingly not TCTU and TBTU, pointing to the significant role of counterion) is des-Leu sequence. Phosphonium salts generate a des-Tyr sequence. Addition of an excess of HOBt into BOP increases the content of des-Leu product, while mixing BOP and HBTU results in suppression of both deletion products.

A similar situation was observed in the β-amyloid synthesis (Figures 9 and 10). The poor performance of HCTU, HDTU, and HBTU as well as BOP with addition of HOBt is caused by the poor coupling of Ile, and again, mixing of BOP and HBTU resulted in a very well-performing coupling reagent. DIC provided the highest yield of product.

A classical test sequence is acyl carrier protein 65–74 (see Figures 11 and 12). The highest yield was provided by TBTU and HCTU. This test sequence was the only one in which DIC did not excel (72.7 vs. 77.8% for HCTU). The major side product in this case is the des-Val sequence. Its content was significantly lower with uronium reagents than with the rest (with the exception of HDTU). Mixing BOP and HBTU did not result in any improvement in the impurity profile.

Results from this experiment strongly suggests the use of DIC as an optimal coupling reagent. Uronium salts, especially TBTU, provided good results; how-

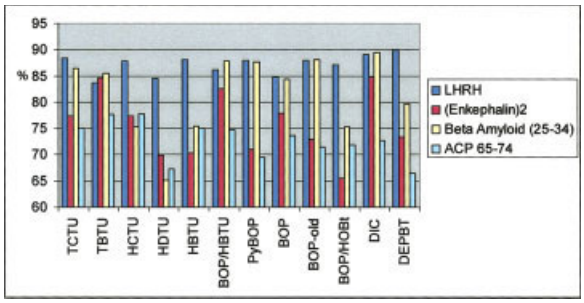

FIGURE 5 Comparison of HPLC purity of model peptides prepared by different reagents.

ever, their solubility is limited (0.6*M* solutions are close to saturation and some—HCTU or TCTU—cannot go over 0.44 or 0.33*M*). BOP has shown very good solubility and long-term stability in solution. PyBOP, on the other hand, decomposes very quickly. Figure 13 shows an HPLC of a model peptide synthesized with fresh PyBOP and with solutions 3 days old.

In the next experiment we explored an additional coupling reagent, HATU, and DIC at various concentrations with different additives, and alternative modifications of the BOP reagent protocols. As a control to link this experiment to the previous one, we used 1*M* DIC, TBTU, TCTU, and an old solution of BOP. HATU performed similarly to TBTU and provided a different impurity profile than HCTU, HDTU, and HBTU (see Figure 9). Concentrations of DIC from 0.5 to 2*M* did not make a significant difference, as did the addition of bromophenol blue, which aids in real-time determination of the end point in difficult couplings. Addition of HOBt into DIC solution increased the amount of des-Val-ACP (16.5 vs. 14.5%) in the crude product.

BOP results were the most interesting. The differences were observable on the enkephalin dimer and ACP sequence. Doubling the concentration of BOP

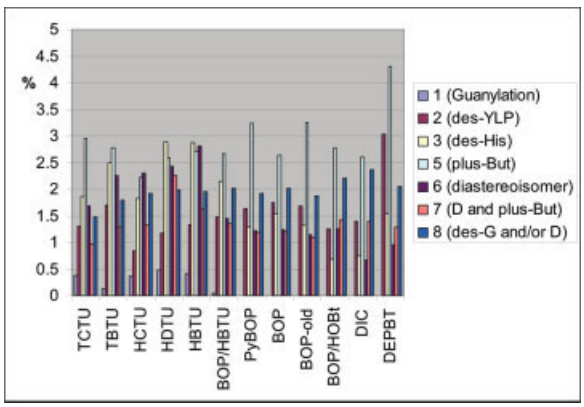

FIGURE 6 Distribution of side products in the synthesis of LHRH. (For peak assignment, see Figure 4.)

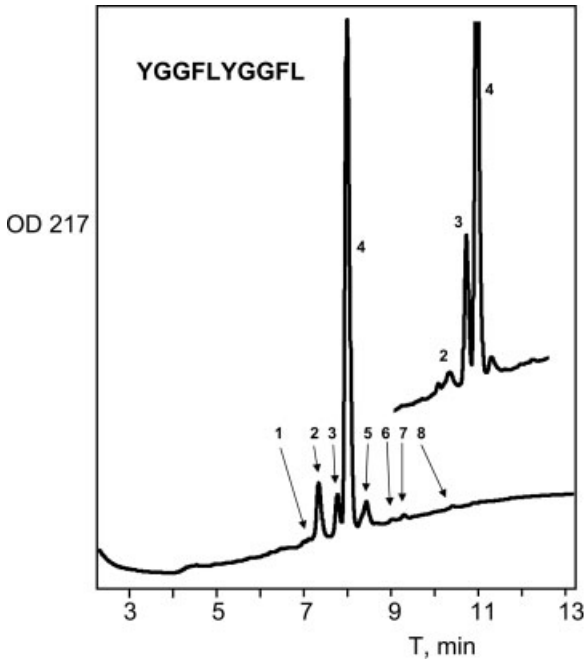

FIGURE 7 HPLC of YGGFLYGGFL synthesized by HCTU and PyBOP (insert). Note the difference between the impurities 2 and 3 (des-Leu and des-Tyr peptide).

and keeping the volume of the base constant resulted in increased amounts of the side product. In this case, the molar ratio of BOP to base was 1:1 vs. normal 1:2. Increasing the volume of base solution twice (molar ratio 1:4) did not improve the profile over the control; however, addition of a double volume of BOP solution (molar ratio of reagents 1:1) improved the coupling of Tyr in the enkephalin dimer synthesis significantly (10.1 vs. 3.7%), but did not improve coupling of Val in the ACP fragment.

CONCLUSION

We have shown that the use of only one model sequence for evaluation of synthetic reagents and pro-

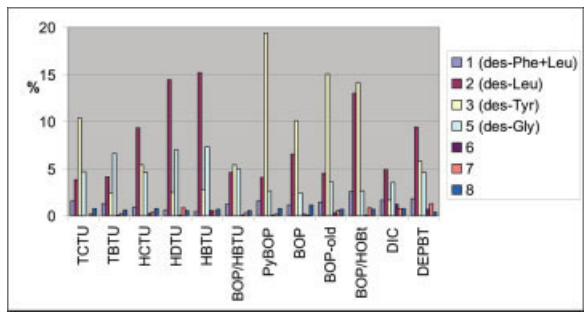

FIGURE 8 Distribution of side products in the synthesis of Leu-enkephalin dimer. (For peak assignment, see Figure 7.)

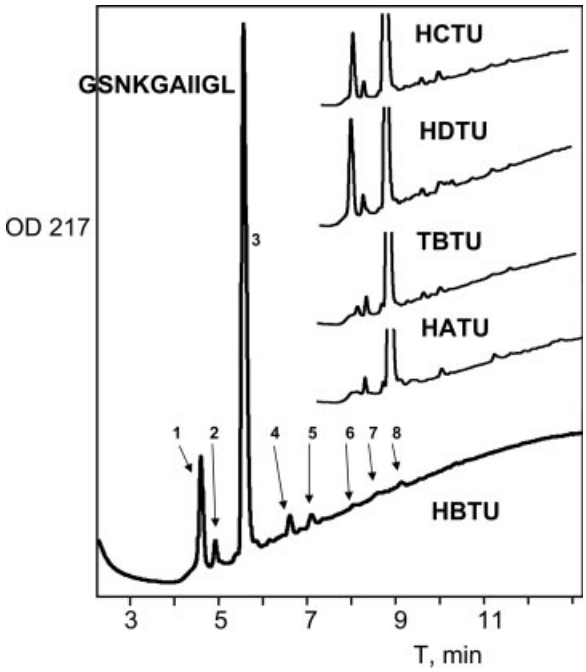

FIGURE 9 HPLC of β-amyloid 25–34 synthesized by HBTU. Inserts show detail of HPLC trace of the same peptide synthesized with HATU, TBTU, HDTU, and HCTU.

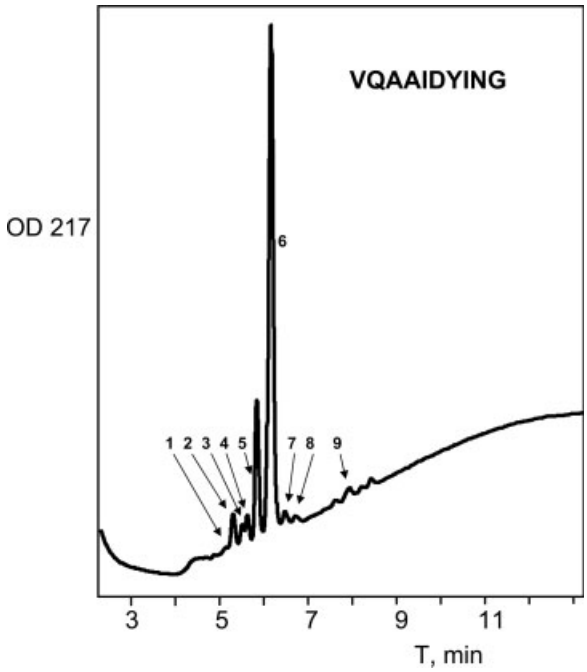

FIGURE 11 HPLC of ACP 65–74 synthesized by HDTU.

tocols can be misleading. Different reagents have different tendencies to generate deletion sequences. For example, hexafluorophosphate uronium salts are less effective in coupling Fmoc–Leu (and Fmoc–Ile), while phosphonium salts couple Fmoc–Tyr(But) less efficiently in the same sequence. Mixing of different types of coupling reagents (BOP and HBTU) can improve synthetic results significantly.

For the use in automatic synthesizers, the stability of the reagent solution is critical. We have shown that although most of the reagents are stable in a matter of days or even weeks (BOP), some reagents (PyBOP) must be used on a freshly prepared basis.

This study has shown the significant advantages of DIC as the coupling reagent in situations where the speed of the reaction is not critical. Because in the multiple parallel synthesizer the distribution of protected amino acids and coupling reagents takes a significant part of synthesis procedure, and because the machine has to wait for the slowest reaction (well) to finish, the faster coupling reagents are not providing a significant advantage. If, at the same time, their use is not providing superior purity of the product, their use should be avoided. However, the greatest advantage

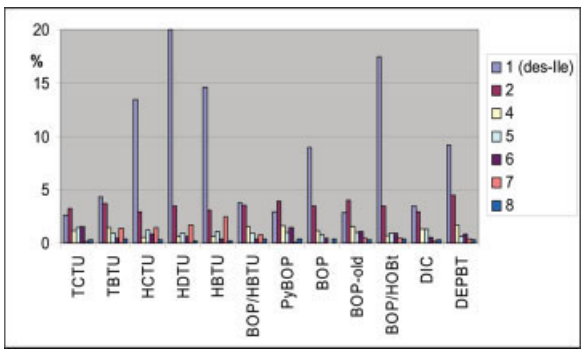

FIGURE 10 Distribution of side products in the synthesis of β-amyloid 25–34. (For peak assignment, see Figure 9.)

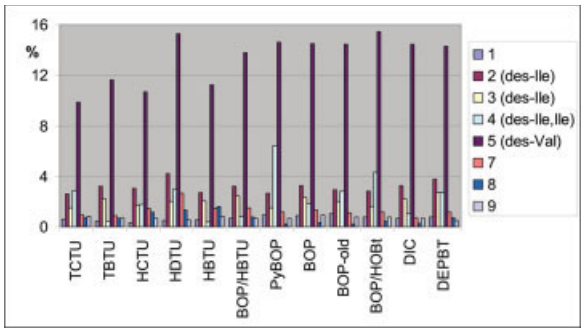

FIGURE 12 Distribution of impurities in ACP 65–74. (For peak assignment, see Figure 11.)

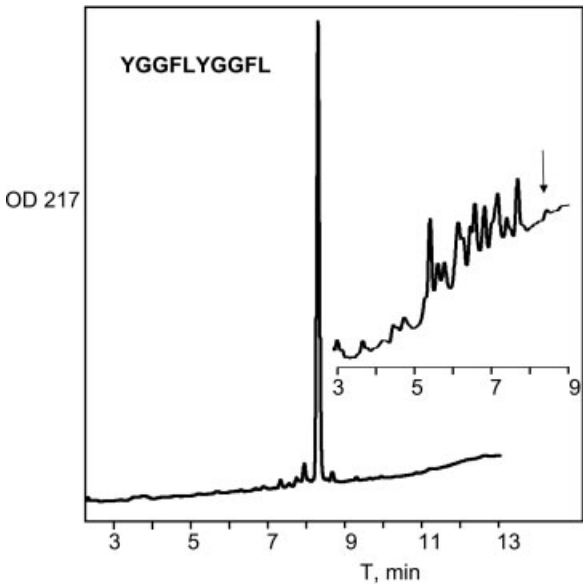

FIGURE 13 HPLC of Leu-enkephalin dimer synthesized using freshly prepared PyBOP solution and using solution left at room temperature for three days (inset trace). The arrow points to a retention time corresponding to correct product. (These experiments were performed independently from the described comparative study—coupling time was 50 min—and HPLC was run on the column of different batch.)

may be gained by a reagent that allows real-time monitoring of the coupling progress. Because the only available nondestructive monitoring is the use of bromophenol blue (which cannot be used in the case of reagents using base as an additive), DIC is the reagent of choice. Continuous monitoring allows the avoidance of excessive use of reagents (in the case of wells in which coupling is completed after first addition of reagent, a second coupling is performed only on the wells showing incomplete reaction after the first coupling), thus reducing cost of the synthesis. Continuous monitoring can also prevent synthesis of deletion peptides (the synthesizer could perform additional couplings using different reagents, if necessary, on the "stubborn" wells). In addition, DIC is the most inexpensive coupling reagent.

EXPERIMENTAL

Fmoc amino acids and Rink resin (0.4 mmol/g) were purchased from Novabiochem (EMD Biosciences, Inc., San Diego, CA, USA), coupling reagents were from Novabiochem (EMD Biosciences, Inc., San Diego, CA, USA), Matrix Innovation (Montreal, Canada), or Advanced ChemTech (Louisville, KY, USA). Solvents were from VWR International, Inc. (West Chester, PA, USA). 4-Methylpiperidine was from Sigma-Aldrich (Milwaukee, WI, USA).

Rink resin (300 mg) was added into mixture of dimethylformamide (DMF) and dichloromethane (DCM) (10 mL total) to form a nonsedimenting suspension that was distributed into the wells of a flat-bottomed polypropylene microtiterplate (Evergreen Scientific, Los Angeles, CA, USA). The plate was placed into a centrifugal synthesizer. An additional 100 μL of DMF was added into the plate wells (beads sedimented) and the plate was centrifuged with a tilt of 6°. The standard synthetic protocol was modified so that after pipetting individual Fmoc-protected amino acids (0.3*M* solution in 0.3*M* HOBt in DMF), the twelve individual reagents were added from additional storage tubes four reagents at a time. Delivery of each reagent (0.6*M* in DMF—0.44*M* in the case of HCTU and 0.33*M* TCTU) was followed by addition of 1.2*M* N,N-diisopropylethylamine (DIEA) in DMF. In the case of the DIC solution, only reagent was added and base addition was omitted. In each addition, the reagents were added to four columns of the plate simultaneously, starting with positions A1–A4. Delivery of all reagents took 95 s. The plate was oscillated five times and rested for 50 s. (During oscillation, the plate was rotated at a speed at which the liquid does not overflow the wall of the well and solid support moves toward the outer side of the well. When the rotation was stopped, liquid returned to horizontal position and beads distributed at the well bottom, thus mixing the well content.) This procedure was repeated 30 times. Plate was centrifuged and addition of amino acids and reagents were repeated. After another 30 cycles of oscillation and resting, the reagents were removed by centrifugation, and washing and deprotection was repeated to prepare the plate for the next cycle of synthesis.

At the end of the synthesis, the plate was dried in vacuo and 150 μL of mixture K^{16} [trifluoroacetic acid (TFA)/thioanisol/water/phenol/EDT: 82.5:5:5:5:2.5 v/v] was added. The plate was capped and shaken on the plate shaker for 3 h. The suspension was transferred by a multichannel pipettor to filter plate (Orochem Technologies, Lombard, IL, USA). The eluate in the deep well plate (VWR) was precipitated by ether (600 μL); after standing in the refrigerator for 3 h, the pellet was formed by centrifugation, the supernatant removed by a surface suction device,[3,18] and the pellet was resuspended in ether (600 μL) and centrifuged again. The process of supernatant removal and resuspension was repeated three times. The product was dried in a Speedvac (ThermoSavant, Waltham, MA, USA), dissolved in 200 μL of H_2O or

50% dimethylsulfoxide (DMSO)–50% H_2O, and samples of 20 μL were added to 180 μL of water. Twenty microliters were injected onto an HPLC column (Waters, Milford, MA, USA, μBondapak, C18, 10 μ particle, 125 Å pore, 3.9 $\times$ 150 mm, gradient 0.05% TFA in H_2O to 70% acetonitrile, 0.05% TFA in 15 min, flow rate 1.5 mL/min, detection by UV at 217 nm). LC-MS was performed at HT-Labs (San Diego, CA, USA) using the same gradient.

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