# Use of Onium Salt-Based Coupling Reagents in Peptide Synthesis[1]

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Peptide coupling methods derived from onium salts based on 1-hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HOAt) are becoming incorporated in synthetic strategies more frequently than the classical carbodiimide methods. We have correlated the reactivity of various onium salts derived from HOXt (X = A, B), with the structure of the reagents in question. Thus, we confirmed that the aza derivatives are more reactive than the parent benzotriazole derivatives in both activation and coupling. In addition, the activation step is determined by the structure of the carbon skeleton. Thus, pyrrolidino derivatives appear to be reagents of choice relative to the piperidino analogues or those derived from trialkylamines. Furthermore although phosphonium salts are slightly less reactive than the corresponding aminium/uronium salts, the former should be used for the activation of hindered species, since the latter may lead to the formation of guanidino derivatives.

## Introduction

The success of peptide synthesis is based upon the proper management of temporary and permanent protecting groups and the efficiency of the coupling reagents chosen to elongate the peptide chain.[3] During the past few years, there has been an evolution in the development of new activation methods and their application to both solution and solid-phase methodology.[4] The formerly predominant carbodiimide and active ester techniques have been replaced with onium salts based upon 1-hydroxybenzotriazole (HOBt, **1**)[5] and 7-aza-1-hydroxybenzotriazole (HOAt, **2**).[6] HOAt-based reagents have been shown to be more efficient than the corresponding HOBt analogues in terms of coupling yield and reduced loss of configuration at the C-terminal carboxy acid residue.[6,7] To correlate the reactivity of different onium salts (**3**, **4**) based upon HOXt where X = A, B, model studies were designed to investigate the stability of these reagents in solution and determine the ease of carboxylic acid activation as well as the nature and extent of any undesired side reactions. Furthermore, since practical use of these reagents may require storage in solution for prolonged times, the present study provides guidelines for optimum handling of this class of compounds. The nature of the different reagents, phosphonium (**4**) vs aminium/uronium (**3**),[8] *N*-alkyl substituents (R), and OAt vs OBt influences are studied (Figure 1).

(1) Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in: *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: ACP, acyl carrier protein; AOP, (7-azabenzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; Deg, diethylglycine; DIEA, *N*,*N*-diisopropylethylamine; DMAP, *N*,*N*-dimethyl-4-aminopyridine; DMF, *N*,*N*-dimethylformamide; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethyloxycarbonyl; HAMDU, *O*-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-dimethyleneuronium hexafluorophosphate; HAMTU, *O*-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-trimethyleneuronium hexafluorophosphate; HAPipU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate; HAPyU, 1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4, 5-*b*]pyridin-1-ylmethylene)pyrrolidinium hexafluorophosphate *N*-oxide; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBMDU, *O*-(benzotriazol-1-yl)-1,3-dimethyl-1,3-dimethyleneuronium hexafluorophosphate; HBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBPipU, *O*-(benzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate; HDTU, *O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)1,1,3,3-tetramethyluronium hexafluorophosphate; HMPA, hexamethylphosphoric triamide; HOAt, 7-aza-1-hydroxybenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine]; HOBt, 1-hydroxybenzotriazole; HODhbt, 1-oxo-2-hydroxydihydrobenzotriazine; HPLC, high performance liquid chromatography; MeOH, methanol; PAL, 5-[[(4-amino)methyl]-3,5-dimethoxyphenoxy]valeric acid; PEG−PS, poly(ethylene glycol)−polystyrene (graft resin support); PPTS, pyridinium *p*-toluensulfonate; PS, copoly(styrene-1%-divinylbenzene) support; PyAOP, 7-aza-benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris-(pyrrolidino)phosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; Su, succinimidyl; TFA, trifluoroacetic acid; TMP, 2,4,6-trimethylpyridine (collidine). Amino acid symbols denote L-configuration unless indicated otherwise.

(2) (a) University of Barcelona. (b) University of Massachusetts. (c) PerSeptive Biosystems.

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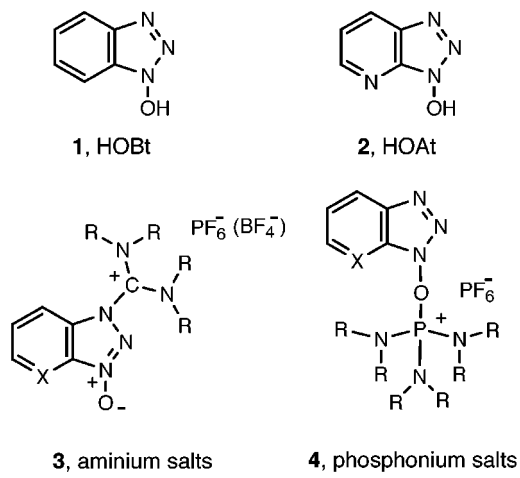


**Figure 1.** Structures of additives HOBt and HOAt. General structures of aminium/uronium and phosphonium salts.

## Results and Discussions

The first onium salts introduced for solid-phase peptide assembly, BOP (**12**)[9] and HBTU (**5**),[10] were based on HOBt and contained dimethylamino moieties. Due to the carcinogenecity and respiratory toxicity associated with HMPA formed when BOP is used in coupling reactions, researchers set limits in the use of the latter reagent.[11] Instead, the safer pyrrolidino derivative (PyBOP, **14**)[12] was developed. Subsequently, the piperidino derivative (HBPipU, **8**, A = B)[13] and the dihydroimidazole derivative (HBMDU, **9**)[14] of HBTU have also been successfully used in SPPS. These reagents as well as the uronium salt derived from HODhbt,[15] HDTU (**16**),[10b,16] are objects of the present study (Figure 2).

**Stability of Xt Derivatives.** The data in Table 1 show that aza derivatives are as expected less stable than the benzotriazole analogues (1.1 vs 1.2; 1.8 vs 1.9, 1.10 vs 1.11), and the phosphonium derivatives are less stable than the aminium/uronium analogues (1.8 vs 1.1, 1.9 vs 1.2, 1.10 vs 1.3). Furthermore, the nature of the carbon skeleton structure is of marked importance in connection with the stability of a compound. Both dihydroimidazole derivatives (1.5 and 1.6) as well as HDTU (1.12) are very unstable, while the salts derived from dimethylamine are the most stable, and the pyrrolidino derivatives are of intermediate stability. These results should be considered mainly for those syntheses carried out in automatic synthesizers where the coupling reagents are located in open vessels.

**Table 1. Hydrolytic Stability of Xt Derivatives in DMF[a]**

| | | stability (%) open vial | | | stability (%) closed vial |
|---|---|---|---|---|---|
| entry | reagent | 5 h | 20 h | 42 h | 48 h |
| 1.1 | HATU | >99 | 95 | 78 | >99 |
| 1.2 | HBTU | 100 | 98 | 88 | 100 |
| 1.3 | HAPyU | 96 | 79 | 22 | 93 |
| 1.4 | HAPipU | 98 | 95 | 83 | 97 |
| 1.5 | HAMDU | 0[b] | | | 0 |
| 1.6 | HBMDU | 0[b] | | | |
| 1.7 | HAMTU | 71 | 31 | 0 | 52 |
| 1.8 | AOP | 91 | 23 | 1 | 78 |
| 1.9 | BOP | 96 | 53 | 6 | 98 |
| 1.10 | PyAOP | 29 | 0 | | 13 |
| 1.11 | PyBOP | 39 | 0 | | 57 |
| 1.12 | HDTU | 0 | | | |

[a] Stability studies were conducted via HPLC analysis of aliquots removed from stock solutions (0.05 M) of the various coupling reagents in DMF at indicated times. Yields were calculated according to the integration of the peak area at 220 nm of the signal associated with the aminium/phosphonium salt with respect to the corresponding HXBt (see Experimental Section for more details). [b] After 30 min, only 1% of HAMDU and 27% of HBMDU are present.

**Table 2. Hydrolytic Stability of Xt Derivatives in DMF Stored under $N_2$[a]**

| | | stability (%) | | | |
|---|---|---|---|---|---|
| entry | time (days) | HATU | HAPyU | PyAOP | PyBOP |
| 2.1 | 1 | 100 | 100 | 98 | 100 |
| 2.2 | 3 | 98 | 96 | 91 | 95 |
| 2.3 | 7 | 92 | 82 | | 93 |
| 2.4 | 14 | | 77 | <65 | |
| 2.5 | 21 | 87 | 68 | | |

[a] Stability studies were conducted via HPLC analysis of aliquots removed from stock solutions (0.05 M) of the various coupling reagents in DMF at indicated times. Yields were calculated according to the integration of the peak area at 220 nm of the signal associated with the aminium/phosphonium salt with respect to the corresponding HXBt (see Experimental Section for more details).

As expected, all the coupling reagents were more stable when the DMF solutions were stored under $N_2$ atmosphere (Table 2),[17] conditions used in some of the automatic synthesizers.

To determine the feasibility of the use of HATU after prolonged storage under $N_2$, the model peptide acyl carrier protein (ACP) (65-74)[18] was synthesized using a 0.5 M solution of HATU in DMF which had been stored for 21 days. Analysis of the HPLC data indicated an excellent purity, similar to that obtained with a freshly prepared solution of HATU in DMF (Figure 3).

The stability of these compounds has also been examined in the presence of DIEA (Table 3), because peptide bond formation is usually carried out in the presence of an extra equivalent of base. Analysis of these results confirms that the various coupling reagents rapidily

(8) X-ray analysis has shown that HATU, HBTU, and HAPyU crystallize in the form of the aminium salts; see: (a) Abdelmoty, I.; Albericio, F.; Carpino, L. A.; Foxman, B. M.; Kates, S. A. *Lett. Pept. Sci.* **1994**, *1*, 52−67. (b) Henklein, P.; Costisella, B.; Wray, V.; Domke, T.; Carpino, L. A.; El-Faham, A.; Kates, S. A.; Abdelmoty, A.; Foxman, B. M. In *Peptides 1996. Proceedings of the 24th European Peptide Symposium*; Ramage R., Epton, R., Eds.; Mayflower Worldwide Ltd.: Birmingham, 1997. For those compounds for which X-ray data have not yet been obtained, generic structural representations are shown. For these reagents, traditional nomenclature has arbitrarily been retained.

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(17) Under these conditions, HOAt (0.5 M solution in DMF) is totally stable up to 1 month.

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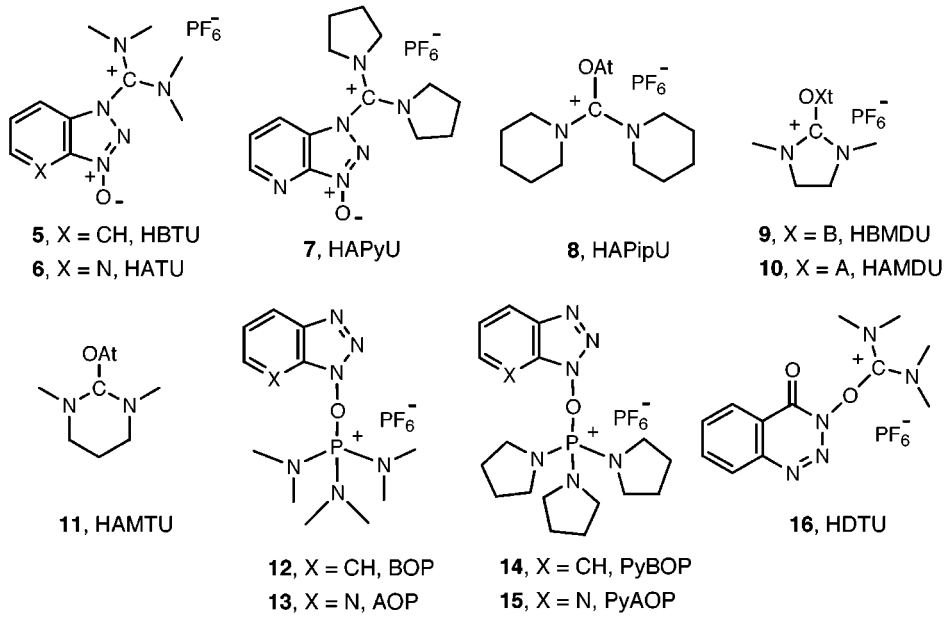



**Figure 2.** Structures of reagents used in this study.

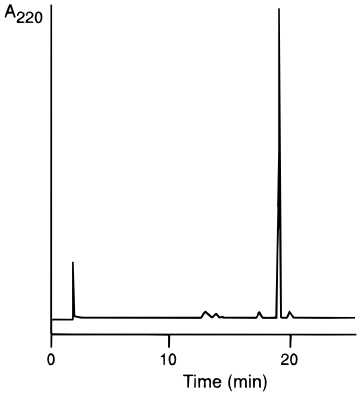



**Figure 3.** HPLC of crude ACP (65-74) directly after extraction with $CHCl_3$ and $H_2O$−HOAc (7:3) of the cleavage reagent [TFA−$H_2O$ (9:1)]. A reversed phase Delta Pack C-18 column was used for the analysis with elution by a linear gradient over 20 min of 0.1% TFA in $CH_3CN$ and 0.1% aqueous TFA from 1:19 to 1:4, flow rate 1.0 mL/min, detection at 220 nm.

**Table 3. Hydrolytic Stability of Xt Derivatives in DMF in the Presence of DIEA (1 equiv)[a]**

| | | stability (%) | |
|---|---|---|---|
| entry | reagent | 2 min | 1 h |
| 3.1 | HATU | 93 | 36 |
| 3.2 | HBTU | 95 | 62 |
| 3.3 | HAPyU | 59 | 9 |
| 3.4 | PyAOP | 78 | 12 |
| 3.5 | PyAOP (2 equiv DIEA) | 77 | 2 |
| 3.6 | PyBOP | 82 | 31 |
| 3.7 | AOP | 96 | 76 |
| 3.8 | BOP | 96 | 82 |

[a] Stability studies were conducted via HPLC analysis of aliquots removed from stock solutions (0.05 M) of the various coupling reagents in DMF at indicated times. Yields were calculated according to the integration of the peak area at 220 nm of the signal associated with the aminium/phosphonium salt with respect to the corresponding HXBt (see Experimental Section for more details).

degraded in the absence of a carboxylic acid function. This observation has practical consequences in solid and solution-phase peptide assembly. Thus, if activation of a carboxylic acid is slow, then the coupling reagent will degrade and no longer function properly in the reaction medium. Under these conditions, aza derivatives are more labile than the benzotriazole derivatives, pyrrolidinio derivatives are more labile than dimethylamino, and aminium salts are less stable than phosphonium salts.

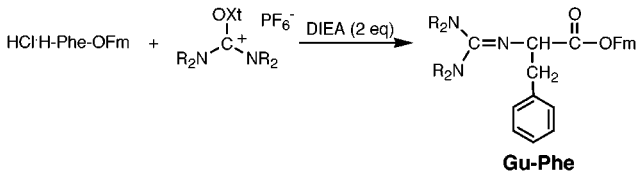
**Table 4. Activation of Fmoc−Deg−OH[1]**

| | | Fmoc−Deg−OXt (%) | | |
|---|---|---|---|---|
| entry | activation method | 2 min | 1 h | 2 h |
| 4.1 | DCC−HOAt | 60 | 83 | 89 |
| 4.2 | HBTU−DIEA | 70 | 95 | 96 |
| 4.3 | HATU−DIEA | 88 | 90 | 90 |
| 4.4 | HATU−TMP | 29 | 59 | 64 |
| 4.5 | HAPyU−DIEA | 89 | 92 | 92 |
| 4.6 | HAMDU−DIEA | 60 | 62 | − |
| 4.7 | HAMDU−TMP | 44 | 63 | 67 |
| 4.8 | HAMDU | 13 | 17 | |
| 4.9 | AOP−DIEA | 77 | 81 | 81 |
| 4.10 | BOP−DIEA | 65 | 89 | 89 |
| 4.11 | PyAOP−DIEA | 89 | 92 | 92 |
| 4.12 | PyBOP−DIEA | 75 | 83 | 83 |
| 4.13 | HDTU−DIEA | 98 | 99 | |

[a] Activation studies were conducted via HPLC analysis of aliquots removed from a DMF stock solution (0.33 M of each reagent) of Fmoc−Deg−OH (1 equiv), coupling reagent (1 equiv), and base (1 equiv) when required, at the indicated times. Yields were calculated according to the integration of the peak area at 220 nm of the signal corresponding to Fmoc−Deg−OXt with respect to Fmoc−Deg−OH (see Experimental Section for more details).

**Activation of Fmoc−Deg−OH.** The formation of a peptide bond between two amino acids involves two steps. The first step is the activation of the carboxyl group of one residue, and the second step is the nucleophilic atack of the amino group of the other amino acid derivative to the activated carboxylic group. To study the activation step of the carboxyl group, Fmoc−diethylglycine (Fmoc−Deg−OH) was chosen for inherent steric hindrance to the carboxyl group. Results in Table 4 are in agreement with the previously discussed observations (Tables 1−3). Thus, aminium and phosphonium salts were more effective than carbodiimide in the presence of HOAt. Examination

**Table 5. Guanidinio Formation**[a]




| | | Gu-Phe | | | |
|---|---|---|---|---|---|
| entry | reagent | 2 min | 30 min | 3 h | 5 h |
| 5.1 | HATU | 78 | 91 | 94 | 94 |
| 5.2 | HAPyU | 65 | 66 | 66 | |
| 5.3 | HAMDU | 62 | 67 | 67 | |
| 5.4 | HBTU | <1 | | 44 | 51 |

[a] Guanidino studies were conducted via HPLC analysis of aliquots removed from a DMF stock solution of HCl·H−Phe−OFm (1 equiv, 0.33 M), coupling reagent (1 equiv, 0.33 M), and DIEA (2 equiv, 0.66 M) at the indicated times. Yields were calculated according to the integration of the peak area at 220 nm of the signal corresponding to Gu−Phe with respect to H−Phe−OFm (see Experimental Section for more details).

of the activation of Fmoc−Deg−OH at 2 min indicates that the aza derivatives are seen to be more reactive than their corresponding benzotriazole analogues (4.2 vs 4.3, 4.9 vs 4.10, 4.11 vs 4.12). Furthermore, formation of the active species competes with hydrolysis of the coupling reagent. Thus, the most reactive onium salt, HAMDU, gives poorer activation when compared with the less reactive compounds HATU and HAPyU, due to the fact that after a few seconds no activation reagent is present in the medium. Collidine (TMP), a weaker base than DIEA, was less effective (4.4 vs 4.3 and 4.7 vs 4.6). Experiment 4.8 was carried out to determine whether the most reactive coupling reagent HAMDU is effective in the absence of base. The results indicate clearly that for significant activation it is necessary that the carboxylate anion be present. Thus, the presence of a base is mandatory. HDTU, as previously shown in Table 1, was an excellent activating reagent.

**Side Reactions: Guanidino Formation.** Aminium salts can react with the *N*-terminal amino component leading to a guanidino derivative, a process that terminates peptide chain elongation.[19] Such side reactions are not prevalent during the solid-phase coupling of single amino acids, since the activation step often occurs prior to the addition to the amino component. In addition, the activation is fast and the aminium salt is rapidily consumed or hydrolyzed before exposure to a resin containing an amino terminus. However, during the much slower activation of hindered amino acids, protected peptide segments, or carboxylic acids involved in cyclization steps, the aminium salt may undergo reaction with the amino component. Table 5 shows the progress over time of this side reaction relative to the particular aminium salts derivatives, taking as example the reaction of the fluorenylmethyl ester of the phenylalanine with various coupling reagents.

Analysis of the results indicate that guanidinium formation occurs in all four cases examined. Again the high reactivity of the aza derivatives induces the reaction to occur more rapidily than in the case of HBTU (**5**). Thus, for the most reactive reagents HAPyU and HAMDU (5.2, 5.3) there was competition between hydrolysis of the reagent and guanidino formation. In both cases, after a few minutes the aminium reagent was hydrolyzed and was not present in the medium. Similar experiments carried out with phosphonium salts (PyAOP and PyBOP) show disapperance of the reagent, but no formation of any new product related to the amino component. In these cases, if after 1 h Fmoc−Deg−OH was added together with fresh coupling reagent, formation of the protected dipeptide (Fmoc−Deg−Phe−OFm) occurred, showing clearly that phosphonium salts are not involved in any reaction analogous to that which occurs in the case of the corresponding aminium salts.

**Peptide Bond Formation.** Formation of the peptide bond in high yields represents the final confirmation that the activation and coupling steps have functioned well. The synthesis of the protected dipeptide Fmoc−Deg−Phe−OFm, from equimolar amounts of the two amino acids and the coupling reagent in the presence of DIEA (3 equiv) has been taken as a model. Table 6 confirms the results obtained in the previous experiments. Thus, aza derivatives were more effective than the benzotriazole analogues (6.1 vs 6.2, 6.5 vs 6.6, 6.7 vs 6.8) in both activation of the carboxylic acid moiety and the coupling step. The pyrrolidino derivatives [HAPyU (6.3), PyAOP (6.5), PyBOP (6.6)] were slightly superior to the dimethylamino analogues [HATU (6.1), AOP (6.7), BOP (6.8)]. The use of the most reactive onium reagent HAMDU (6.4) is not recommended, because of its clear instability. HDTU (6.9) was the most effective system in terms of activation (after 2 min, all Fmoc−Deg−OH was converted to the active ester), but the corresponding active ester was less reactive than either the OAt or OBt esters. Finally, even under the demanding conditions chosen for this test (hindered carboxylic acid and equimolar amounts of reagents), formation of the guanidino product is not an important side reaction.

## Conclusions

Results presented in this work indicate that all of the coupling reagents discussed, except HAMDU, HBMDU, and HAMTU, can be incorporated efficiently in solid-phase and solution peptide synthesis (Tables 1, 2, 4, and 6). Aza derivatives are more reactive for both steps, activation and coupling (Tables 4 and 6). The structure of the carbon skeleton has a determining role in the efficiency of the reagent for the activation step (Tables 1 and 4). For optimal activation of a hindered amino acid, such as Fmoc−Deg−OH, the presence of DIEA rather than a weaker base such as TMP is recommended (Table 4). All coupling reagents including the most reactive (HAMDU) require the presence of a base for efficient activation (Table 4, 4.8). Phosphonium salts are less stable than the corresponding aminium/uronium derivatives in the absence of base (Table 1), whereas, in the presence of base, the phosphonium species are slightly more stable (Table 3), thus indicating that aminium/uronium salts are somewhat more reactive, as shown in Tables 4 and 6. HDTU is very efficient for activating the carboxylic acid residue (Table 4, 4.13, and Table 6, 6.9), but the resulting active ester is less reactive than the corresponding OAt and OBt esters (Table 6). In addition,

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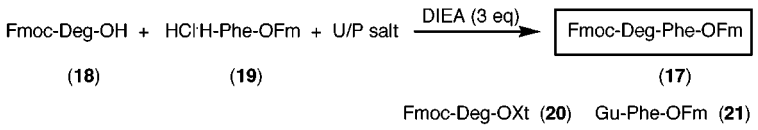
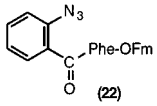
**Table 6. Synthesis of Protected Dipeptide Fmoc-Deg-Phe-OFm[a]**




| | | product distribution (%) | | | | |
|---|---|---|---|---|---|---|
| entry | coupling reagent | **17** | **18** | **19** | **20** | **21**[b] |
| 6.1.1 | HATU, 2 min | 60 | 4 | 18 | 18 | <0.5 |
| 6.1.2 | 1 h 15 min | 94 | 3 | <1 | 2 | <0.5 |
| 6.1.3 | 2 h 30 min | 95 | 3 | 0 | 2 | <0.5 |
| 6.2.1 | HBTU, 2 min | 35 | 11 | 38 | 15 | |
| 6.2.2 | 1 h 15 min | 85 | | 9 | 15 | |
| 6.2.3 | 2 h 30 min | 90 | | 5 | 4 | |
| 6.2.4 | 5 h | 94 | | 3 | 2 | (1) |
| 6.3.1 | HAPyU, 2 min | 65 | 6 | 18 | 11 | <0.5 |
| 6.3.2 | 1 h 15 min | 92 | 5 | 3 | | <0.5 |
| 6.3.3 | 2 h 30 min | 92 | 5 | 3 | | <0.5 |
| 6.4.1 | HAMDU, 2 min | 46 | 23 | 25 | 3 | 3 |
| 6.4.2 | 1 h 15 min | 57 | 20 | 17 | 3 | 3 |
| 6.4.3 | 2 h 30 min | 57 | 20 | 15 | 2 | 3 (5) |
| 6.5.1 | PyAOP, 2 min | 62 | 1 | 26 | 10 | |
| 6.5.2 | 1 h 15 min | 96 | | 3 | | (1) |
| 6.5.3 | 2 h 30 min | 96 | | 3 | | (1) |
| 6.6.1 | PyBOP, 2 min | 31 | 7 | 42 | 20 | |
| 6.6.2 | 1 h 15 min | 89 | 1 | 5 | 4 | (1) |
| 6.6.3 | 2 h 30 min | 90 | 1 | 5 | 3 | (2) |
| 6.6.4 | 5 h | 92 | 1 | 3 | 1 | (3) |
| 6.7.1 | AOP, 2 min | 62 | 4 | 26 | 8 | |
| 6.7.2 | 1 h 15 min | 94 | | 5 | | (1) |
| 6.7.3 | 2 h 30 min | 94 | | 5 | | (1) |
| 6.8.1 | BOP, 2 min | 12 | 16 | 52 | 15 | |
| 6.8.2 | 1 h 15 min | 85 | 1 | 8 | 3 | (3) |
| 6.8.3 | 2 h 30 min | 89 | 1 | 5 | 1 | (4) |
| 6.8.4 | 5 h | 91 | 1 | 3 | | (5) |
| 6.9.1 | HDTU, 2 min | 16 | | 27 | 55 | 1 |
| 6.9.2 | 1 h 15 min | 64 | | 13 | 21 | 1 (1) |
| 6.9.3 | 2 h 30 min | 77 | | 7 | 13 | 1 (2) |
| 6.9.4 | 5 h | 91 | | | | 1 (4, 4) |

[a] Peptide bond formation was studied by HPLC analysis of aliquots removed from a DMF stock solution of Fmoc−Deg−OH (1 equiv, 0.33 M), HCl·H−Phe−OFm (1 equiv, 0.33 M), coupling reagent (1 equiv, 0.33 M), and DIEA (3 equiv, 0.99 M) at the indicated times. Yields were calculated according to the integration of the peak area at 220 nm of the corresponding signals (see Experimental Section for more details). [b] Numbers in parentheses indicate % of other products formed. In the case of HDTU (6.9), a possible side product may correspond to triazine **22**.



(22)

the use of HDTU often leads to the formation of side products. For the use of the various onium reagents described in automatic solid-phase peptide synthesizers, where the coupling reagent is stored in solution under a $N_2$ atmosphere, one can safely continue the synthesis for a time that exceeds that needed to assemble a long peptide (up to 2−3 weeks) (Table 2). In synthesizers where the coupling reagent solutions are stored in an open vessel, solutions freshly prepared every day of the less reactive reagents, such as HATU or HAPipU, in comparison to HAPyU are advised. Formation of guanidino side products is not critical for the stepwise solid-phase synthesis of standard peptides, where preactivation of the amino acid is carried out. However, if in situ activation is carried out or if hindered amino acids, protected peptides, or peptide cyclization is involved, the use of phosphonium salts is recommended. For systems that undergo slow activation, the addition of additional coupling reagent from time to time is advisable, since the coupling derivative may be hydrolyzed after a few minutes. Finally, the use of theoretical methods has provided a reasonable explanation for the experimental results.[20] In the future, a combination of both theoretical and experimental methods should lead to the rational design of more effective reagents.[21]

## Experimental Section

HATU, HAPyU, PyAOP, Fmoc−Phe−OH, and Boc−Phe−OH were obtained from PerSeptive Biosystems (Framingham, MA); HBTU, BOP, and PyBOP were obtained from Calbiochem-Novabiochem (San Diego, CA). Other coupling reagents were prepared from HOBt [Aldrich, (St. Louis, MI)] or HOAt (PerSeptive Biosystems) according to published methods.[6,9,10,12−14] Fmoc−Deg−OH was obtained from the corresponding amino acid [Peptides International (Louisville, KY)] and Fmoc−OSu (Aldrich). HCl·H−Phe−OFm was obtained by esterification of Boc−Phe−OH with Fm−OH (Aldrich) by use of DCC (Aldrich) in the presence of a catalytic amount of DMAP (Aldrich), followed by removal of the $N^{\alpha}$-Boc group by means of 4 N HCl−dioxane (Aldrich). Peptide grade DMF [Scharlau (Barcelona, Spain)] was stored over molecular sieves under a $N_2$ atmosphere.

(20) Bofill, J. M.; Albericio, F. Manuscript in preparation.

(21) For an elegant example of the combination of both theoretical and experimental methods in the development of chemical methods for the synthesis of oligodeoxyribonucleotides, see: Wada, T.; Sato, Y.; Honda, F.; Kawahara, S.; Sekine, M. *J. Am. Chem. Soc.* **1997**, *119*, 12710−12721.

HPLC was performed using Spherisob $C_{18}$ reverse-phase columns (0.46 × 25 cm) on either a Waters or Shimadzu instrument, equipped with an automatic injector and with UV detection at 220 nm. MeOH was used for washing the line.[22] Products were eluted at a flow rate of 1 mL/min, using a linear gradient [(1:0) to (0:1) in 20 min] of $H_2O$ and $CH_3CN$ containing 0.045% TFA and 0.036% TFA, respectively.

All stability experiments, except those given in Table 2, were carried out with 0.05 M solutions of the different reagents in DMF. Aliquots removed at different times were evaluated directly by HPLC. Open vials (Table 1) were stored inside the Shimadzu auto injector SIL-6B at 28 °C. Other experiments were carried out at 25 °C. Experiments described in Table 2 were carried out with 0.5 M solutions of the various reagents in DMF stored under $N_2$ in the bottle corresponding to Syringe 2 of a PerSeptive Biosystems 9050 continuous-flow synthesizer.

Solid-phase synthesis of a fragment of the acyl carrier protein, ACP (65-74), was carried out using a 9050 synthesizer on a PAL-PEG-resin using standard protocols with 4 equiv of both Fmoc−amino acid and coupling reagent and 8 equiv of DIEA. Following linear assembly, cleavage and final deprotection was carried out with TFA−$H_2O$ (9:1) at 25 °C for 2 h. The filtrate from the cleavage reaction was collected, $CHCl_3$ was added, the peptide was extracted with $H_2O$−HOAc (7:3), and the aqueous phase was directly evaluated by HPLC.

Activation of Fmoc−Deg−OH (Table 4) was carried out by preparing a 0.33 mmol solution of both Fmoc−Deg−OH and coupling reagent in 1 mL of DMF. Base (0.33 mmol) was added when required (4.2−4.7, 4.9−4.13). Magnetic stirring was performed during the entire process. Aliquots removed at different times were evaluated directly by HPLC.

The study of guanidino formation (Table 5) was carried out by preparing a solution of 0.33 mmol of both HCl·H−Phe−OFm and coupling reagent, and 0.66 mmol of DIEA in 1 mL of DMF. Magnetic stirring was performed during the entire process. Aliquots removed at different times were evaluated directly by HPLC. In the experiments carried out with phosphonium salts, following 1 h of reaction of HCl·H−Phe−OFm and phosphonium salt in the presence of the DIEA, Fmoc−Deg−OH (0.33 mmol) and additional phosphonium salt (0.33 mmol) in 1 mL of DMF were first added, and then DIEA (0.33 mmol). The HPLC analysis of an aliquot removed after 30 min showed the formation of Fmoc-Deg-Phe-OFm.

Peptide bond formation studies (Table 6) were conducted by preparing a solution of 0.33 mmol of Fmoc−Deg−OH, HCl·H−Phe−OFm, and coupling reagent, and 0.99 mmol of DIEA in 1 mL of DMF. Magnetic stirring was performed during the entire process. Aliquots removed at different times were evaluated directly by HPLC.

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(22) Mixtures containing HOAc for washing instrument lines should be avoided, since HOAc can react with the various coupling reagents, generating extra peaks in the HPLC chromatograms.