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Fast conventional Fmoc solid-phase peptide synthesis: a comparative study of different activators

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The ability to speed up conventional Fmoc solid-phase peptide synthesis (SPPS) has many advantages including increased productivity. One way to speed up conventional Fmoc SPPS is the choice of activator. Recently, several new activators have been introduced into the market, and they were evaluated along with some older activators for their ability to synthesize a range of peptides with shorter and longer reaction times. It was found that HDMC, PyClock, COMU, HCTU, and HATU worked well at shorter reaction times (2 × 1 min), but PyOxim and TFFH only worked well at longer reaction times. The performance of PyBOP at shorter reaction times was poor only for more difficult sequences. These results are important for selecting an appropriate activator for fast SPPS applications. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Fast SPPS; Activators; COMU; HDMC; PyOxim; HCTU; PyClock; HATU

Introduction

In recent years, there has been an increasing interest in fast Fmoc solid-phase peptide synthesis. This is not a new concept, however. In 1997, Alewood *et al*. reported coupling times of 1–2 min using Boc chemistry and the activator HATU [1]. However, at the time, similar fast coupling studies using Fmoc chemistry had not been attempted. To date, HATU is still considered the most efficient activator available on the market today. However, its high cost prevents its routine use. In 2008, a study by Hood, *et al*. [2] showed that the activator HCTU was highly efficient and could be used to synthesize a variety of peptides using Fmoc chemistry and coupling times of 5 min or less. Since then, several new activators have been introduced to the market, including HDMC [3], PyClock [4], COMU [5], and PyOxim [6]. The purpose of this study was to compare the efficiency and speed of these new activators to various other available activators by synthesizing several peptides under the same conditions at shorter and longer coupling times. The effect of the activators and coupling times on peptide purity and yield was compared.

Method

Materials

All resins, solvents, and reagents were supplied by Protein Technologies, Inc. (Tucson, AZ, USA).

Synthesis

The peptides were synthesized on a Symphony peptide synthesizer (Protein Technologies, Inc. Tucson, AZ, USA) at the 25 μmol scale using Fmoc-Gly-Wang resin (0.41 mmol/g) for ACP, and Rink Amide MBHA resin (0.33 mmol/g) for the remaining peptides. The peptides were synthesized using the same fast conditions used in Hood *et al*. [1] (Deprotection: 20% piperidine/DMF for 2 × 30 s; Coupling: 1:1:4 0.1 M AA/0.1 M Activator/0.4 M NMM in DMF (5x excess) for 2 × 1 min; Washes: DMF for 2 × 30 s following deprotection and second coupling steps, DMF for 1 × 30 s between coupling steps; Cleavage: 95/2/2/1 TFA/anisole/water/EDT for 2 h) and again with 2 × 20 min coupling times. All amino acids were dissolved in DMF except for valine that was dissolved in DMSO. This synthesis was repeated using the activators HCTU (≥99% purity), HATU (≥99% purity), HDMC (≥99% purity), COMU (≥98% purity), PyBOP (≥97% purity), PyClock (≥98% purity), PyOxim (≥99% purity), and TFFH (≥99% purity).

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Abbreviations used: *ACP, acyl carrier protein; COMU, morpholinium, 4-[[[(1-cyano-2-ethoxy-2-oxoethylidene)amino] oxayl] (dimethylamino)methylene]-hexafluorophosphate (1-) (1:1); DMF, dimethylformamide; DMSO, dimethylsulfoxide; EDT, ethanedithiol; Fmoc, 9-fluorenylmethyloxycarbonyl; GHRP, growth hormone releasing peptide; HATU, N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-N-*methylmethanaminium hexafluorophosphate *N-oxide; HCTU, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HDMC, 1H-benzotriazolium, 5-chloro-1-[(dimethylamino)-4-morpholinylmethylene]-,3-oxide hexafluorophosphate(1-) (1:1); HPLC, high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; NMM, N-methylmorpholine; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; PyClock, 6-chloro-benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyOxim, [ethyl cyano(hydroxyimino)-acetato-O2) tri-1-pyrrolidinylphosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TFFH, tetramethylfluoroformamidinium hexafluorophosphate.*

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Analysis

Crude peptides were analyzed on a Varian HPLC, equipped with a C18, 300 Å, 5 μm, 250 × 4.6 mm column (Varian Microsorb-MV), run for 60 min with a flow of 1 mL/min and using a gradient of 5–95% B, where Buffer A was 0.1% TFA in water, and Buffer B was 0.1% TFA in acetonitrile. Detection was at 214 nm.

Results & Discussion

$^{65-74}$ACP

The 65–74 fragment of the acyl carrier peptide ($^{65-74}$ACP: VQAAIDYING-OH) is a more difficult sequence frequently used to test new protocols or synthesis techniques [7,8]. The final valine was coupled using a 1:1 mixture of DMF/DMSO to overcome aggregation [2,7]. For the majority of activators, crude purities ranged between 70.27% and 83.63%, regardless of coupling times, with the best results being obtained from HATU at shorter coupling times, and HCTU at longer coupling times (Table 1, Figure 1). These results are similar to the previously obtained results of 79% using COMU, manual synthesis, and 2 min coupling times [5]. TFFH produced crude purities under 25% regardless of reaction time, whereas at shorter reaction times, PyBOP and PyOxim produced lower purities of 48.11% and 19.13%, respectively, due in part to incomplete coupling of the isoleucines.

G-LHRH

G-LHRH (H-GHWSYGLRPG-NH_2) is a modified version of the luteinizing hormone releasing hormone (LHRH) in which the *N*-terminal pyro-glutamic acid is replaced with a glycine [9]. This relatively easy to synthesize peptide produced crude purities ranging from 78.52% to 90.84% for the majority of activators regardless of reaction time, with the best results being obtained from COMU at shorter coupling times and HCTU at longer coupling times (Table 2, Figure 2). TFFH produced crude purities below 63% regardless of reaction time, whereas at shorter reaction times, PyOxim produced a crude purity of 40.10%, due in part to incomplete coupling of histidine and arginine.

GHRP-6

GHRP (H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) actively releases growth hormone *in vivo* in humans and animals [10]. It is a hexapeptide that contains two D-amino acids (D-Trp and D-Phe). As seen before, most of the activators produced crude purities between 86.89% and 94.02% regardless of coupling time, except PyOxim and TFFH (Table 3, Figure 3). The best results were obtained from COMU at shorter coupling times and HCTU at longer coupling times. PyOxim decreased in purity by 36.31% to only 54.08% purity at shorter coupling times. TFFH produced lower purity results compared with the other activators with 72.03% at longer coupling times and 52.45% at shorter coupling times.

Table 1. Des-Ile (1) (%) and Des-Ile (2) (%) for both 2 x 1 min and 2 x 20 min columns

Activator	2 × 1 min				2 × 20 min			
	ACP (%)	Des-Val (%)	Des-Ile (1)	Des-Ile (2)	ACP (%)	Des-Val (%)	Des-Ile (1)	Des-Ile (2)
HCTU	74.68	9.06	1.72	1.77	83.63	7.76	N/A	N/A
HATU	78.08	3.53	1.84	2.40	80.27	4.52	0.26	0.38
HDMC	70.27	3.22	1.78	1.48	75.77	3.96	0.19	0.09
COMU	71.07	6.32	3.03	2.29	78.10	6.49	0.36	0.27
PyBOP	48.11	1.67	11.44	12.59	74.96	2.49	0.29	0.29
PyClock	73.84	4.29	2.73	3.05	80.04	4.49	0.31	0.27
PyOxim	19.13	3.83	11.50	11.05	74.69	5.43	0.90	0.96
TFFH	3.16	1.03	2.20	1.09	24.47	2.51	4.10	6.19

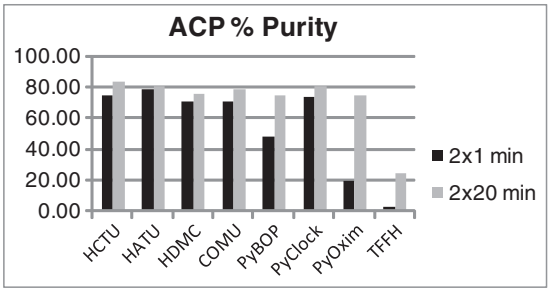

Figure 1. Crude % purity data for $^{65-74}$ACP synthesized with multiple activators using coupling times of either 2 x 1 minute or 2 x 20 minutes.

Table 2. Crude % purity data for G-LHRH synthesized with multiple activators using coupling times of either 2 × 1 min or 2 × 20 min. % purity data for major deletion peaks are also shown

Activator	2 × 1 min			2 × 20 min		
	G-LHRH (%)	Des-His (%)	Des-Arg (%)	G-LHRH (%)	Des-His (%)	Des-Arg (%)
HCTU	87.84	1.37	1.89	90.84	0.53	0.45
HATU	86.01	2.26	1.42	85.80	2.28	1.42
HDMC	84.50	2.28	2.22	87.02	2.54	0.29
COMU	90.24	0.96	0.44	88.36	0.66	1.15
PyBOP	78.52	4.38	4.13	89.28	1.46	0.24
PyClock	81.82	3.77	2.63	87.40	1.86	0.24
PyOxim	40.10	8.17	4.74	84.12	2.89	1.03
TFFH	39.07	2.58	3.65	62.90	2.12	15.76

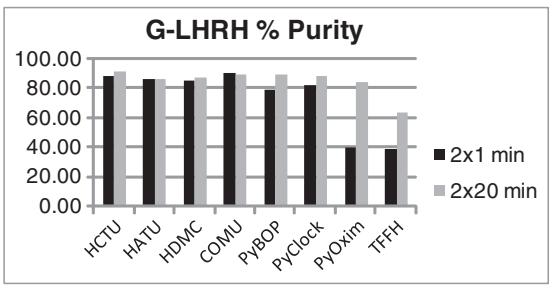

Figure 2. Crude % purity data for G-LHRH synthesized with multiple activators using coupling times of either 2 x 1 minute or 2 x 20 minutes.

9Pbw0

Protaetiamycine is a naturally occurring 43-mer antimicrobial peptide obtained from the larvae of the *Protaetia brevitarsis* beetle. A 9-mer analog, 9Pbw0 (H-RLWLAIGRG-NH_2), was synthesized by Shin *et al.* that showed good antifungal activity against *Candida albicans* [11]. In this case, most of the activators produced crude purities between 72.60% and 86.64% regardless

Table 3. Crude % purity data for GHRP-6 synthesized with multiple activators using coupling times of either 2 × 1 min or 2 × 20 min. % purity data for major deletion peaks are also shown

Activator	2 × 1 min			2 × 20 min		
	GHRP-6 (%)	Des-Ala (%)	Des-His (%)	GHRP-6 (%)	Des-Ala (%)	Des-His (%)
HCTU	91.14	0.96	0.44	92.01	0.96	0.19
HATU	89.96	1.43	0.34	91.09	0.61	0.25
HDMC	90.05	1.34	0.35	90.74	0.37	0.14
COMU	92.11	0.87	0.38	94.02	0.10	0.11
PyBOP	86.89	3.21	0.99	92.48	0.26	0.22
PyClock	89.08	1.18	0.58	90.83	0.36	0.51
PyOxim	54.08	13.10	7.76	90.39	0.68	0.22
TFFH	52.45	3.83	9.90	72.03	0.87	1.97

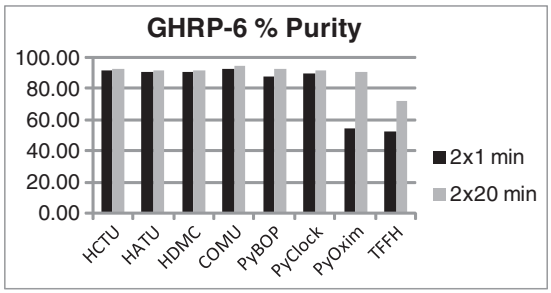

Figure 3. Crude % purity data for GHRP-6 synthesized with multiple activators using coupling times of either 2 x 1 minute or 2 x 20 minutes.

of coupling time, with the best results being obtained from COMU for both coupling times (Table 4, Figure 4). At shorter coupling times, only PyOxim and TFFH had lower purities of 32.22% and 45.98%, respectively.

Linear Oxytocin

Oxytocin (H-CYIQNCPLG-NH_2) is a peptide hormone that causes uterine contractions and milk ejection [12]. It is a peptide of nine amino acids long and contains a disulfide bridge between Cys-1 and Cys-6. For the majority of the activators, the purities of the linear version of this peptide varied between 54.76 and 77.68% regardless of coupling time, with the best results being obtained from COMU for both coupling times (Table 5, Figure 5). TFFH had purities below 15% regardless of coupling times, and at shorter coupling times, PyOxim had a purity of 22.33%.

Table 4. Crude % purity data for 9Pbw0 synthesized with multiple activators using coupling times of either 2 × 1 min or 2 × 20min. % purity data for major deletion peak are also shown

Activator	2 × 1 min		2 × 20 min	
	9Pbw0 (%)	Des-Arg (%)	9Pbw0 (%)	Des-Arg (%)
HCTU	81.12	5.24	83.94	3.11
HATU	83.29	6.44	82.88	5.75
HDMC	83.01	7.48	83.47	6.59
COMU	84.87	3.39	86.64	0.75
PyBOP	80.81	9.33	72.60	17.98
PyClock	76.26	13.17	80.71	5.40
PyOxim	32.22	12.65	75.97	3.43
TFFH	45.98	5.79	72.69	1.68

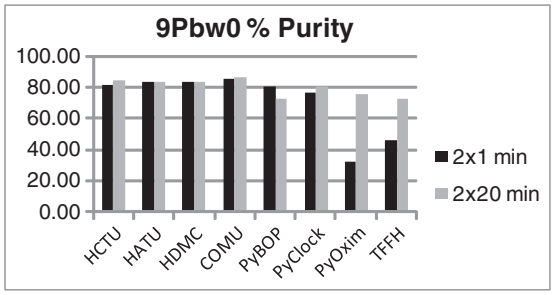

Figure 4. Crude % purity data for 9Pbw0 synthesized with multiple activators using coupling times of either 2 x 1 minute or 2 x 20 minutes.

Table 5. Crude % purity data for linear oxytocin synthesized with multiple activators using coupling times of either 2 × 1 min or 2 × 20 min. % purity data for major deletion peak are also shown

Activator	2 × 1 min		2 × 20 min	
	Oxy (%)	Des-Ile/Leu (%)	Oxy (%)	Des-Ile/Leu (%)
HCTU	70.80	1.01	73.97	N/A
HATU	58.40	1.46	59.86	0.22
HDMC	54.76	1.03	58.68	0.17
COMU	75.74	3.46	77.68	0.16
PyBOP	62.10	10.62	76.40	0.23
PyClock	65.31	3.60	63.85	0.17
PyOxim	22.33	10.10	73.65	1.32
TFFH	4.03	1.24	14.34	1.45

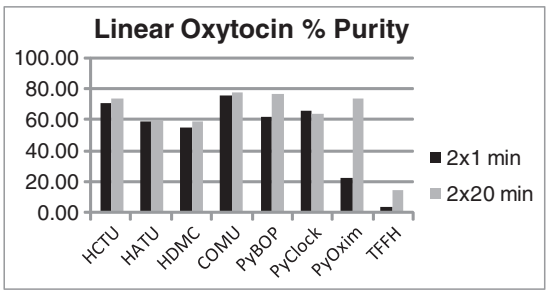

Figure 5. Crude % purity data for linear oxytocin synthesized with multiple activators using coupling times of either 2 x 1 minute or 2 x 20 minutes.

Although there are some variations between the results generated by HCTU, HATU, HDMC, COMU, and PyClock, in general, they produce largely similar results to one another regardless of the coupling time. COMU frequently produced the highest purity peptides at shorter coupling times. This shows that these coupling reagents, COMU in particular, are especially suited for performing fast chemistry protocols. PyBOP and PyOxim produced similar results to the previously mentioned coupling reagents as well but only consistently at longer coupling times. Depending on the sequence, PyBOP produced significantly lower purities at shorter coupling times. However, PyOxim consistently produced significantly lower purities at shorter coupling times, whereas TFFH consistently produced lower purities than the rest of the coupling reagents, especially at shorter reaction times. Based on these data, PyBOP, PyOxim, and TFFH may not be as suitable for fast coupling protocols.

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Occasionally, shorter reaction times produced higher purity products. This may be due to the tendency of highly reactive coupling reagents to result in side reactions during extended couplings [6].

The relative performance of the phosphonium salts, PyBOP, PyClock, and PyOxim observed in this study could be explained by the acidity of their respective additives: (pK_a of HOBt = 4.60 for PyBOP, pK_a of 6-Cl-HOBt = 3.35 for PyClock, and pK_a of Oxyma = 4.60 for PyOxim). High acidity is attributed to a higher reactivity [6]. In this case, 6-Cl-HOBt has the highest acidity, indicating that PyClock should display the highest reactivity, which it does at fast coupling times. PyBOP and PyOxim both displayed lower reactivity at faster coupling times, although PyOxim consistently produced the lowest purities. The lower steric hindrance of PyOxim should have resulted in higher reactivity compared with both PyBOP and PyClock; however, the data show this is not the case for shorter coupling times, indicating other factors may be at work.

Conclusion

Overall, HCTU, HATU, HDMC, COMU, and PyClock produce similar results at shorter as well as longer coupling times, as seen in the previous study with HCTU [2], although COMU frequently produced the highest purities regardless of coupling time. PyOxim consistently produced significantly lower purities at shorter coupling times, indicating it would probably not be a good choice for fast chemistry protocols, whereas PyBOP produced lower purities at shorter times for more difficult sequences, such as $^{65-74}$ACP but was equally effective at longer and shorter coupling times for easier sequences. TFFH was the poorest performer overall and consistently resulted in the lowest purities regardless of peptide sequence.

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References

1 Alewood P, Alewood D, Miranda L, Love S, Meutermans W, Wilson D. Rapid *in situ* neutralization protocols for Boc and Fmoc solid-phase chemistries. *Methods Enzymol.* 1997; **289**: 14–29.
2 Hood CA, Fuentes G, Patel H, Page K, Menakuru M, Park JH. Fast conventional Fmoc solid-phase peptide synthesis with HCTU. *J. Pept. Sci.* 2008; **14**: 97–101.
3 El-Faham A, Albericio F. Morpholine-based immonium and halogenoamidinium salts as coupling reagents in peptide synthesis. *J. Org. Chem.* 2008; **73**: 2731–2737.
4 Subiros-Funosas R, Moreno JA, Bayo-Puxan N, Abu-Rabeah K, Ewenson A, Atias D, Marks RS, Albericio F. PyClock, the phosphonium salt derived from 6-Cl-HOBt. *Chimica Oggi-Chemistry Today* 2008; **26(**Supplement**)**: 10–12.
5 El-Faham A, Subiros-Funosas R, Prohens R, Albericio F. COMU: a safer and more effective replacement for benzotriazole-based uronium coupling reagents. *Chem. Eur. J.* 2009; **15**: 9404–9416.
6 Subiros-Funosas R, El-Faham A, Albericio F. PyOxP and PyOxB: the oxyma-based novel family of phosphonium salts. *Org. Biomol. Chem.* 2010; **8**: 3665–3673.
7 Hyde C, Johnson T, Sheppard RC. Internal aggregation during solid phase peptide synthesis. Dimethyl sulfoxide as a powerful dissociating solvent. *J. Chem. Soc. Chem. Commun.* 1992; **21**: 1573–1575.
8 a) Johnson T, Quibell M, Owen D, Sheppard RC. A reversible protecting group for the amide bond in peptides. Use in the synthesis of 'difficult sequences'. *J. Chem. Soc. Chem. Commun.* 1993; **4**: 369–372.
b) Howe J, Quibell M, Johnson T. A new generation of reversible backbone-amide protection for the solid phase synthesis of difficult sequences. *Tet. Lett.* 2000; **41**: 3997–4001.
9 Matsuo H, Arimura A, Schally AV. Synthesis of the porcine LH and FSH-releasing hormone by the solid-phase method. *Biochem. Biophys. Res. Commun.* 1971; **45**: 822–7.
10 Muccioli G, Tschop M, Papotti M, Deghenghi R, Heiman M, Ghigo E. Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. *Eur. J. Pharmacol.* 2002; **440**: 235–254.
11 Shin S, Kim J-K, Lee J-Y, Jung K-W, Hwang J-S, Lee J, Lee DG, Kim I, Shin SY, Kim Y. Design of potent 9-mer antimicrobial peptide analogs of protaetiamycine and investigation of mechanism of antimicrobial action. *J. Pept. Sci.* 2009; **15**: 559–568.
12 Du Vigneaud V, Ressler C, Swan JM, Roberts CW, Katsoyannis PG. The synthesis of oxytocin. *J. Am. Chem. Soc.* 1954; **76**: 3115–3118.

