

Fast conventional Fmoc solid-phase peptide synthesis with HCTU

CHRISTINA A. HOOD, GERMAN FUENTES, HIRENDRA PATEL, KAREN PAGE, MAHENDRA MENAKURU and JAE H. PARK*

Department of Chemistry, Protein Technologies Inc., Tucson, Arizona, 4675 South Coach Drive, Tucson, Arizona, 85714, USA

Received 28 June 2007; Revised 18 July 2007; Accepted 23 July 2007

Abstract: 1*H*-Benzotriazolium 1-[bis(dimethyl-amino)methylene]-5-chloro-hexafluorophosphate (1-),3-oxide (HCTU) is a nontoxic, nonirritating and noncorrosive coupling reagent. Seven biologically active peptides (GHRP-6, ^{65–74}ACP, oxytocin, G-LHRH, C-peptide, hAmylin_{1–37}, and β -amyloid_{1–42}) were synthesized with reaction times reduced to deprotection times of 3 min or less and coupling times of 5 min or less using HCTU as the coupling reagent. Expensive coupling reagents or special techniques were not used. Total peptide synthesis times were dramatically reduced by as much as 42.5 h (1.8 days) without reducing the crude peptide purities. It was shown that HCTU can be used as an affordable, efficient coupling reagent for fast Fmoc solid-phase peptide synthesis. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: HCTU; fast Fmoc SPPS; human amylin (1–37); oxytocin; β -amyloid

INTRODUCTION

The ability to assemble peptides faster is highly desirable and has several benefits, including identifying difficult couplings in target sequences as well as increasing the production of small complex peptides, protein mutants and peptide segments used in ligation methods for the construction of small proteins [1].

Fmoc conventional solid-phase peptide synthesis (SPPS) is a very practical method used worldwide for the synthesis of peptides in pharmaceutical companies, active pharmaceutical ingredient (API) manufacturers, university research laboratories and custom peptide manufacturers. Most conventional methods today use extended reaction times to achieve high-purity crude peptides at high yields. Typically, Fmoc protecting groups are removed for 10–30 min with 20% piperidine in dimethylformamide (DMF), and Fmoc-protected amino acids are coupled for 20 min to over an hour to the free amines attached to the solid support, which may result in cycle times of up to 2 h, depending on the choice of chemistry and the synthetic protocol followed.

Over the years, methods to accelerate SPPS have been proposed, which mostly focus on the coupling step. In a kinetic study of the coupling reaction by Hetnarski and Merrifield, it was found that diffusion was not the rate-limiting step for the coupling reaction [1]. Rather, as Miranda and Alewood report, the rate of amino acid acylation is heavily dependent on the properties of the coupling reagents [2]. There have been a few studies on fast conventional SPPS using different coupling

reagents. In 1990, one manufacturer of automated synthesizers illustrated FastMoc chemistry using HBTU with fast coupling times of 10–30 min [3–5]. In 1993, the coupling agent *O*-(7-azabenzotriazole-1-yl)-*N,N,N'*-tetramethyluronium hexafluorophosphate (HATU) was introduced [6], and 4 years later, Kent and Alewood *et al.* reported on an accelerated peptide synthesis with coupling times of 1–2 min using Boc chemistry and HATU [7]. While HATU has also been used as a coupling reagent with Fmoc chemistry [8,9], to our knowledge coupling times less than 15 min were not attempted.

Despite its efficiency, HATU has not enjoyed widespread general use owing to its high cost (The price for 25 g of coupling reagents as of June 2007 from Novabiochem. HATU: \$340, HBTU: \$55, BOP: \$60, TBTU: \$60, HCTU: \$80, PyBOP: \$112 and ByBroP: \$248.). Typically, it is reserved only for use in difficult coupling reactions. In 2002, the 6-Cl-HOBt-based coupling reagent 1*H*-benzotriazolium 1-[bis(dimethyl-amino)methylene]-5-chloro-hexa-fluorophosphate (1-), 3-oxide (HCTU) was introduced, and found to be nontoxic, stable in DMF and close in efficiency to HATU, but commercially available at the cost of HBTU [10–13]. Internal studies conducted at Protein Technologies, Inc. also investigated the coupling efficiency of HCTU. The 65–74 fragment of the Acyl Carrier Protein (^{65–74}ACP), (H-VQAAIDYING-OH) was synthesized with various coupling reagents such as HBTU, HATU, HCTU, PyBOP and TBTU. The results showed that HCTU was as effective as HATU, and HCTU gave higher purity peptides than HBTU, PyBOP and TBTU (data not shown). A phosphorylated peptide (H-CRRKGpSQKVS-NH₂) was also synthesized with HBTU and HCTU to compare

*Correspondence to: Jae H. Park, Department of Chemistry, Protein Technologies Inc., Tucson, Arizona, 4675 South Coach Drive, Tucson, Arizona, 85714, USA; e-mail: info@peptideinstruments.com

coupling efficiency. The phosphorylated peptide coupled with HCTU gave a better crude purity than the phosphorylated peptide coupled with HBTU (data not shown).

However, to the best of our knowledge, no study to date has determined the minimum coupling time of HCTU with Fmoc chemistry. In this study, we selected seven biologically active peptides with a broad range of properties including long and short peptides, as well as peptides containing D-amino acids or pseudoproline dipeptides that had been synthesized in our lab using HCTU and long, conservative synthesis times. We then resynthesized these seven peptides using HCTU and short reaction times, and compared the crude purities to evaluate the utility of HCTU as an affordable, highly efficient coupling reagent for fast SPPS.

MATERIALS AND METHODS

Reagents

All protected natural amino acids, HCTU, 20% piperidine in DMF and 0.4 M *N*-methylmorpholine (NMM) in DMF were provided by Protein Technologies, Inc. (Tucson, AZ). Fmoc-Rink amide HMBA resin was purchased from Peptides International (Louisville, KY). Fmoc-Ala-Thr- $\Psi^{Me,Me}$ pro-OH, Fmoc-Ser-Ser- $\Psi^{Me,Me}$ pro-OH and Fmoc-Leu-Ser- $\Psi^{Me,Me}$ pro-OH were provided by Novabiochem (San Diego, CA), while Fmoc-D-Trp-OH, Fmoc-D-Phe-OH and Fmoc-Ala-Wang PS resin were purchased from Novabiochem (San Diego, CA). Fmoc-Ala-Wang Chemmatrix resin was purchased from Matrix Innovation (Montreal, Canada). Solvents were purchased from VWR (West Chester, PA) and used without further purification. Anisole, ethanedithiol (EDT) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Sigma-Aldrich (St. Louis, MO), and trifluoroacetic acid (TFA) was purchased from Spectrum (Gardena, CA). Fmoc-Gly-Wang resin was purchased from Polymer Laboratories (Amherst, MA). HOCT was purchased from Lee BioSolutions, Inc. (St Louis, MO). 1,1,1,3,3,3-Hexafluoroisopropan-2-ol (HFIP) was purchased from TCI America (Portland, OR).

Peptide Synthesis

$^{65-74}$ ACP, G-LHRH, hAmylin₁₋₃₇, oxytocin and GHRP-6 were synthesized on a *Prelude* peptide synthesizer at 40- μ mol scale using fivefold excess of Fmoc-amino acids (200 mM) relative to the Fmoc-Rink amide MBHA resin (0.47 mmol/g, for G-LHRH, hAmylin₁₋₃₇, oxytocin and GHRP-6) and Fmoc-Gly-Wang PS resin (0.41 mmol/g for $^{65-74}$ ACP). Deprotection was performed using 20% piperidine/DMF. Coupling was performed using 1:1:2 amino acid/HCTU/NMM in DMF. DMF top washes (0.5 min) were performed between deprotection and coupling steps. Amino acids with D-configuration and pseudoproline dipeptides were delivered by the *Prelude's* *Single-Shot* feature, which gives a zero dead volume.

The C-peptide was synthesized on a *Symphony* peptide synthesizer at 25- μ mol scale using a six-fold excess of Fmoc-amino acid (112 mM) relative to the Fmoc-Gly-Wang resin (0.41 mmol/g). Deprotection was performed using 20%

piperidine in DMF, and coupling was performed using 1:0.9:2 amino acid/HCTU/DIPEA in DMF. DMF washes (0.5 min) were performed between deprotection and coupling steps.

β -Amyloid₁₋₄₂ was synthesized on a *Symphony* peptide synthesizer at 25- μ mol scale using a ten-fold excess of Fmoc-amino acids (200 mM) relative to the Fmoc-Ala-Wang-PS resin (0.25 mmol/g). Deprotection was performed using DBU:Piperidine:DMF (2:20:78). Coupling was performed using 1:1:4 amino acid/HCTU/NMM in DMF. DMF washes (0.5 min) were performed between deprotection and coupling steps.

Cleavage

Cleavage was performed with 95:2:2:1 TFA/water/anisole/EDT ($^{65-74}$ ACP, G-LHRH and GHRP-6), 95:2.5:2.5 TFA/anisole/EDT (hAmylin₁₋₃₇), 92.5:2.5:2.5:2.5 TFA/EDT/water/TIS (oxytocin), 94:1:2.5:2.5 TFA/TIS/water/EDT (C-peptide) and 94:2:2:1:1 TFA/water/anisole/EDT/TIS (β -amyloid₁₋₄₂).

Peptide Analysis

Crude peptides were analyzed on a Varian ProStar HPLC on a Microsorb-MW 300-5 C18 column, 250 \times 4.6 mm. The gradients were (a) 5-65% over 60 min for $^{65-74}$ ACP, G-LHRH and GHRP-6; (b) 5-95% over 60 min for hAmylin₁₋₃₇, oxytocin and C-peptide; and (c) 5-95% over 60 min at 60°C with a Varian Pro Star column oven, model 510 for β -amyloid₁₋₄₂. The solvent was aqueous MeCN with 0.1% TFA run at a flow rate of 1 ml/min. Detection was at 214 nm. Mass measurements were carried out on a Bruker Reflex III MALDI-TOF instrument using a matrix of sinapinic acid and reflectron acquisition in the positive mode.

RESULTS AND DISCUSSION

The seven peptides were chosen from peptides synthesized for instrument quality control purposes, customer applications or chemistry applications using HCTU as the coupling reagent. As a result, four of the seven peptides had been synthesized with PTI's standard test peptide protocol with 2.5 min \times 2 deprotection times and 10 min \times 2 acylation times. The remaining three peptides had used longer reaction times based on their application. We were able to reduce the reaction times for the shorter peptides (ten residues or less) to 1 min for deprotection and 2 min for coupling with no loss in crude peptide purity. In some cases, the crude purity actually improved at the reduced times. This is most likely due to the fact that short reaction times do not allow kinetically slower side reactions a chance to compete [2]. The reaction times for the longer peptides (over 30 residues) were reduced to 2-3 minutes for deprotection and 5 min for coupling, resulting in crude peptides of equal or better purity. Cycle times were reduced from 37-131 to 14-19 min. Specific results for each peptide are detailed below.

Peptides Containing D-Amino Acids

GHRP-6 (H-HwAWfK-NH₂) is a hexapeptide containing two amino acids with D-configuration (D-Trp and D-Phe) that has been shown to actively release growth hormone *in vivo* in humans and animals [14]. Initially, GHRP-6 was synthesized with 15 min × 2 deprotection times and 45 min × 2 acylation times to showcase the *Single-Shot* feature on the *Prelude*. GHRP-6 was then resynthesized with 0.5 min × 2 deprotection times and 1 min × 2 acylation times. This reduced the total synthesis time from 13.1 to 1.4 h without reducing crude peptide purity (Figure 1(a) and Table 1). In fact, a post-peak impurity in the longer synthesis was reduced with the shorter reaction times. The two peptides were analyzed on different HPLC systems, resulting in slightly different retention times. However, their identities were validated by mass spectrometry.

The 65–74 Fragment of the Acyl Carrier Protein

^{65–74}ACP is a well-known, difficult sequence used to test new synthesis protocols and used at Protein Technologies, Inc. for quality control purposes. ACP was first synthesized using our standard test peptide protocol in 5.6 h, and then it was synthesized with reduced times in 2.1 h (Table 1). HPLC analysis of the crude peptides shows a significant pre-peak due to incomplete coupling of the valine (Figure 1(b)). However, we have shown that this pre-peak can be nearly eliminated by coupling the valine in 1 : 1 DMF : DMSO for 5 min × 2 [15].

Oxytocin

Oxytocin (H-CYIQNCPLG-NH₂) is a component of the prohormone. It is located at its *N*-terminal end and contains a Cys-1 to Cys-6 disulfide bridge. Linear oxytocin was initially synthesized with deprotection times of 3 min followed by 20 min and acylation times of 45 min × 2, resulting in a total synthesis time of 18.8 h (Table 1). This time was reduced to 2.1 h, again with absolutely no loss in crude peptide purity (Figure 1(c)). As Figure 1(c) illustrates, HCTU did not generate racemization of the cysteine residues, even with long coupling times.

Modified Luteinizing Hormone Releasing Hormone

G-LHRH (H-GHWSYGLRPG-NH₂) is another test peptide used for quality control of the *Symphony* and *Prelude* peptide synthesizers. G-LHRH was synthesized in 6.2 h using our standard test peptide protocol, then in 2.3 h with reduced times (Table 1). The crude purity was unchanged (Figures 1(d)).

C-Peptide

Chain A of the human proinsulin C-peptide [16] (H-EAEDLQVGGQVELGGGPGAGSLQPLALEGSLG-OH) was initially synthesized using PTI's standard test peptide protocol resulting in a total synthesis time of 18.5 h (Table 1)(C-terminal Q is replaced with G). The reaction times were then reduced to 1.5 min × 2 and 2 min × 2 for deprotection and acylation, respectively, halving

Table 1 Summary of deprotection and acylation of seven biologically active peptides in long and short reaction time

	Long reaction time ^a				Short reaction time				Time reduction	
	Deprotection ^b	Acylation ^c	Cycle Time ^d (min)	Synthesis Time ^e (h)	Deprotection ^b	Acylation ^c	Cycle Time ^d (min)	Synthesis Time ^e (h)	Cycle time ^f (min)	Synthesis Time ^g (h)
GHRP-6 (6 Cycle)	15 min × 2	45 min × 2	131	13.1	0.5 min × 2	1 min × 2	14	1.4	117	11.7
^{65–74} ACP (9 Cycle)	2.5 min × 2	10 min × 2	37	5.6	0.5 min × 2	1 min × 2	14	2.1	23	3.5
Oxytocin (9 Cycle)	3 and 20 min	45 min × 2	125	18.8	0.5 min × 2	1 min × 2	14	2.1	111	16.7
G-LHRH (10 Cycle)	2.5 min × 2	10 min × 2	37	6.2	0.5 min × 2	1 min × 2	14	2.3	23	3.9
C-Peptide (30 Cycle)	2.5 min × 2	10 min × 2	37	18.5	1.5 min × 2	2 min × 2	18	9	19	9.5
hAmylin 1–37 (34 Cycle)	3 and 20 min	30 min × 2	94	53.3	1 min × 2	2.5 min × 2	19	10.8	75	42.5
β-Amyloid 1–42 (41 Cycle)	15 min × 1	35 min × 1	70 ^h	47.8	1 min × 2	5 min × 1	17	11.6	53	36.2

^a All seven peptides were synthesized at different times for our instrument quality assurance, an application note or a customer.

^b Fmoc protecting group was removed with 20% piperidine in DMF except β-amyloid_{1–42} (2 : 20 : 78 DBU/Piperidine/DMF).

^c Acylation was performed with 0.2 M HCTU/0.4 M NMM/DMF except β-amyloid_{1–42} (0.1 M HCTU/0.1 M HOCT/0.4 M NMM/DMF).

^d Determined from *Prelude* with 0.5 min × 6 DMF top washes.

^e Cycle time × number of cycles.

^f Long cycle time – short cycle time.

^g Long synthesis time – short synthesis time.

^h Cycle time includes 5 min capping with 1 : 1 : 3 Acetic anhydride/DIPEA/DMF.

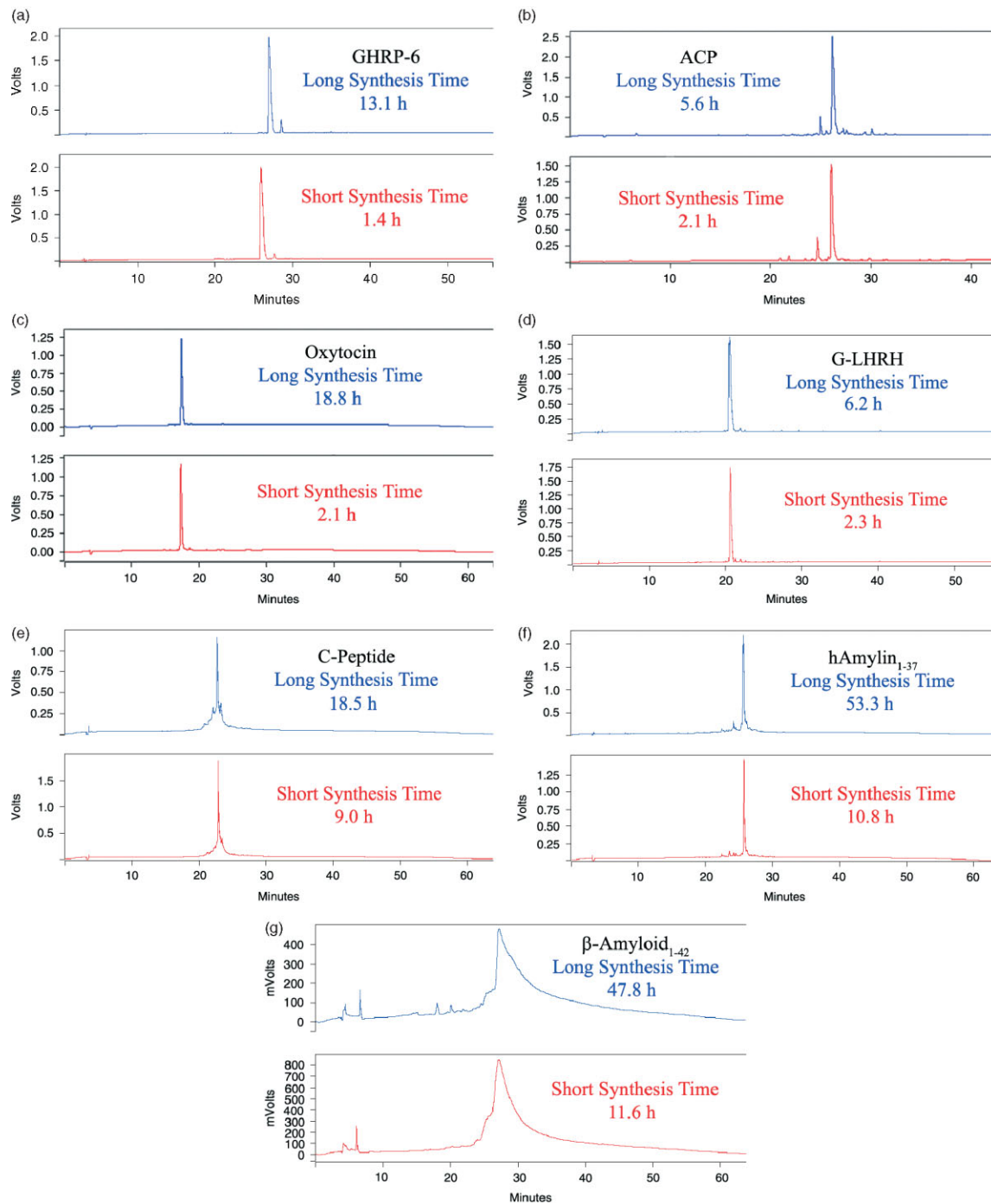


Figure 1 Crude HPLC comparison of (a) GHRP-6, (b) $^{65-74}$ ACP, (c) oxytocin, (d) G-LHRH, (e) C-peptide (f) human amylin (1–37) and (g) human β -amyloid (1–42) in long and short reaction time.

the total synthesis time to 9 h (Table 1). The shorter reaction times produced a higher-purity crude peptide than the longer reaction times (Figure 1(e)).

Human Amylin (1–37)

Human amylin (1–37) (H-KCNTATCATQRLANFLVHSS-NNFGAILSSTNVGSNTY-NH₂) is a major component of the amyloid deposits found in the pancreases of type-II diabetes patients and contains a disulfide

bridge between Cys-2 and Cys-7. Linear hAmylin_{1–37} was initially synthesized with deprotection times of 3 min followed by 20 min, and acylation times of 30 min \times 2, resulting in a total synthesis time of 53.3 h. Pseudoproline dipeptides were incorporated into the sequence during the synthesis. Fmoc-Ala-Thr- $\Psi^{Me,Me}$ pro-OH was coupled in position A⁸T, Fmoc-Ser-Ser- $\Psi^{Me,Me}$ pro-OH was coupled at S¹⁹S and Fmoc-Leu-Ser- $\Psi^{Me,Me}$ pro-OH was coupled at position L²⁷S.

Deprotection times and coupling times were then reduced to 1.0 min \times 2 and 2.5 min \times 2, respectively, without any effect on the crude peptide purity (Figure 1(f)). In fact, a pre-peak impurity was reduced with the shorter reaction times. In addition, it was found that the pseudoproline dipeptides could be coupled in the same time as the natural amino acids. This reduced the total synthesis time to 10.8 h (Table 1) [17].

β -Amyloid (1–42)

Synthesis of the human β -amyloid (1–42) peptide (H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMV-GGVVIA-OH) by conventional SPPS has been reported to be difficult owing to the high hydrophobicity of the C-terminal segment and on-resin aggregation [18,19]. We initially synthesized β -amyloid_{1–42} with deprotection times of 15 min \times 1, acylation times of 35 min, and capping times of 5 min for a total synthesis time of 47.8 h. β -Amyloid_{1–42} was then synthesized with 1 min \times 2 deprotection 5 min acylation times, and 2.5 min \times 2 capping times giving a total synthesis time of 14 h and produced a crude peptide with similar purity to the long synthesis. It was then found that removing the capping step from the shorter synthesis resulted in the same HPLC profile but with a total synthesis time of only 11.6 h (Figure 1(g)). The HPLC product peaks (β -Amyloid_{1–42} was dissolved in HFIP.) were slightly broadened as seen before with this peptide [20].

CONCLUSION

These results demonstrate that HCTU is a highly efficient coupling reagent that could be used as a low-cost alternative to more expensive, highly efficient reagents such as HATU when fast coupling times (5 min or less) are desired. Fast coupling times promote increased productivity, and in some cases may also result in purer crude peptides by not allowing kinetically slower side reactions to compete.

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