Low-Cost, Fast, Conventional Peptide Synthesis With HCTU and Automated Peptide Synthesizers

Cut of a concern for purity and yield, many peptide chemists today choose to extend their reaction times excessively in order to obtain as high a purity and yield as possible. Typical reaction times for conventional Fmoc solid-phase peptide synthesis (SPPS) are deprotection times of 10–30 min, and coupling times of 20 min to over an hour, which can result in cycle times of up to 2 hr. As a result, conventional peptide synthesis chemistry is perceived as slow, but this is not necessarily the case.

Historically, the biggest contribution to increasing the speed of peptide synthesis was undoubtedly the invention of solid-phase peptide synthesis by Bruce Merrifield in 1963. By attaching the growing peptide chain to a solid support, his method eliminated time-consuming purification steps and paved the way for automating the process. Since then, advances in automation and chemistry have made it possible to increase the speed at which peptides can be made. Most chemistry methods have focused on the development of more efficient activators for the coupling step. O-benzotriazole-N,N,N,N′-tetramethyluronium hexafluorophosphate (HBTU) was the first of these activators, and was introduced in 1990 for performing FastMoc™ chemistry (Applied Biosystems, Foster City, CA) with coupling times of 10–30 minutes.

In 1993, Carpino introduced the activator O-(7-azabenzotriazole-1-yl)-N,N,N,N′-tetramethyluronium hexafluorophosphate (HATU), which was used four years later by Alewood and Miranda to perform 1–2 min couplings with Boc chemistry. Similar fast methods have not been developed for Fmoc chemistry, and for many laboratories, HATU is too expensive to use for all but the most difficult couplings. In 2002, the activator H-benzotriazolium-1-[(dimethylamino)methyl]ene-5-chloro-hexafluorophosphate (HCTU) was introduced by Luxembourg Laboratories (Rehovot, Israel), and is available at a significantly lower price than HATU.

The authors tested the efficiency of HCTU by synthesizing a phosphorylated peptide (H-CRRKGP5QKVS-NH2) using HBTU and HCTU, and found that HCTU produced the higher-purity peptide (data not shown). They then synthesized the 65–74 fragment of the acyl carrier protein (O-TACP) (H-QIAAIDYING-OH) using HCTU; HATU; HBTU; benzotriazol-1-yl-oxytripyrrolidino phosphonium hexafluoro-phosphate (PyBOP); and O-(benzotriazol-1-yl)-N,N,N,N′-tetramethyluronium tetrafluoroborate (TBTU). They found that HCTU and HATU produced peptides of extremely similar purity, while the remaining activators had additional impurities (data not shown). From this, it was concluded that HCTU was a highly efficient coupling reagent.

The goal was to synthesize peptides as quickly and inexpensively as possible. The authors were able to achieve extremely rapid coupling times using the activator HCTU. This paper demonstrates this on seven peptides with a variety of properties: long, short, hydrophobic, hydrophilic, cyclic, and peptides containing D-amino acids and pseudoproline dipeptides.

Experimental

Peptides were synthesized as described previously either on either a Symphony™ or Prelude™ peptide synthesizer from Protein Technologies, Inc. (Tucson, AZ). The Prelude automated peptide synthesizer was used to save money on expensive monomer additions, because in Single-Shot™ delivery feature delivers the entire contents of an amino acid bottle to a specified reaction vessel without priming or waste. HPLC and mass spectrometry analysis were also carried out as described previously.

Results and discussion

Using HCTU as the coupling reagent, the authors were able to synthesize the shorter peptides (10 residues or less) using 1-min deprotection times and 2-min coupling times. The reaction times for the longer peptides (over 30 residues) were reduced to 2–3 min for deprotection and 5 min for coupling, resulting in cycle times of 14–19 min (Table 1). Specific results for each peptide are detailed below.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Length</th>
<th>Dep. time</th>
<th>Coup. time</th>
<th>Cycle time5</th>
<th>Synth. time6</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHRP-6</td>
<td>6-mer</td>
<td>2 × 30 sec</td>
<td>2 × 1 min</td>
<td>14 min</td>
<td>1.4 hr</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>9-mer</td>
<td>2 × 30 sec</td>
<td>2 × 1 min</td>
<td>14 min</td>
<td>2.1 hr</td>
</tr>
<tr>
<td>455-TACP</td>
<td>10-mer</td>
<td>2 × 30 sec</td>
<td>2 × 1 min</td>
<td>14 min</td>
<td>2.1 hr</td>
</tr>
<tr>
<td>G-LHRH</td>
<td>10-mer</td>
<td>2 × 30 sec</td>
<td>2 × 1 min</td>
<td>14 min</td>
<td>2.3 hr</td>
</tr>
<tr>
<td>C-peptide</td>
<td>31-mer</td>
<td>2 × 1.5 min</td>
<td>2 × 2 min</td>
<td>18 min</td>
<td>9 hr</td>
</tr>
<tr>
<td>hAmylin-47</td>
<td>37-mer</td>
<td>2 × 1 min</td>
<td>2 × 2.5 min</td>
<td>19 min</td>
<td>10.8 hr</td>
</tr>
<tr>
<td>β-Amyloid-42</td>
<td>42-mer</td>
<td>2 × 1 min</td>
<td>1 × 5 min</td>
<td>17 min</td>
<td>11.6 hr</td>
</tr>
</tbody>
</table>

*Deprotection time × number of cycles.

Table 1 Summary of reaction and synthesis times for linear peptides; peptide lengths are also given.

![Figure 1](https://example.com/figure1.png)

**Figure 1** HPLC of crude GHRP-6. This 6-mer peptide was synthesized in 1.4 hr with 2 × 30 sec deprotection times and 2 × 1 min coupling times.

![Figure 2](https://example.com/figure2.png)

**Figure 2** HPLCs of crude a) linear and b) cyclized oxytocin. The 9-mer linear peptide was synthesized in 2.1 hr with 2 × 30 sec deprotection times and 2 × 1 min coupling times. It was then cyclized for 2 × 40 min using thallium (III) trifluoroacetate delivered by the Prelude’s Single-Shot delivery feature prior to cleavage (Figure 2b). The cyclization time was not optimized.
control of the Symphony and Prelude peptide synthesizers. G-LHRH was synthesized in 2.3 hr using deprotection times of 2 × 30 sec and coupling times of 2 × 1 min (Figure 4).

**C-peptide**

Chain A of the human proinsulin C-peptide (H-HEADLVQGVEQGGGPGASGLQ-PLALE GLG-OH) (Q is replaced with G) was synthesized using reaction times of 2 × 2.5 min and 2 × 2 min for deprotection and coupling, respectively, in a total synthesis time of 9 hr (Figure 5).

**Human amylin 1–37**

Human amylin 1–37 (H-KCNTATCATQRLA NFLHSSNFPAGLSTNVSNTYNH) 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. 10 Linear hAmylin 1–37 was synthesized with deprotection times of 2 × 1 min, and acylation times of 2 × 2.5 min, resulting in a total synthesis time of 10.8 hr (Figure 5a). Pseudoproline dipeptides were incorporated into the sequence using the Prelude’s Single-Shot delivery feature. Fmoc-Ala-Thr(Mba) pro-OH was coupled at position A17, Fmoc-Ser-Ser(Mba) pro-OH was coupled at S18, and Fmoc-Leu-Ser(Mba) pro-OH was coupled at position L25. The peptide was then cyclized on the resin in 10 min by treatment with thallium (III) trifluoroacetate delivered by the Prelude’s Single-Shot delivery feature, producing the cyclized peptide in a total synthesis time of 11 hr (Figure 6a).

**β-Amyloid 1–42**

Synthesis of the human β-amyloid 1–42 peptide (H-DAEFRHDSGYEVHHQKLVFFAED-PLALE GLG-OH) by conventional SPPS has been reported to be difficult due to on-resin aggregation and the high hydrophobicity of the C-terminal segment. 11 The authors synthesized β-amyloid 1–42, with deprotection times of 2 × 1 min and acylation times of 1 × 5 min for a total synthesis time of 11.6 hr (Table 1, Figure 7a). The peptide was then purified by analytical HPLC (Figure 7b). The HPLC product peaks were slightly broadened, as seen before with this peptide. 14

**Conclusion**

The authors demonstrated that HCTU is a highly efficient coupling agent by using it to synthesize seven peptides with deprotection times of 3 min or less and coupling times of 5 min or less. Combining fast chemistry with peptide synthesizers like the Prelude or Symphony, which have been optimized for fast fluid deliveries, resulted in cycle times as short as 14 min. Cost-savings were realized using HCTU as a less expensive activator, and minimizing reagent loss with the Prelude’s Single-Shot delivery feature.

**References**


**The 65–74 fragment of the acyl carrier protein**

65–74ACP is a well-known difficult sequence used to test new synthesis protocols and used at Protein Technologies, Inc. for quality control purposes. ACP was synthesized with 2 × 30 sec deprotection times and 2 × 1 min couplings for a total synthesis time of 2.1 hr. HPLC analysis of the crude peptide showed a significant prepeak due to incomplete coupling of the valine (data not shown). However, it was found that this prepeak could be eliminated by coupling the valine in 1:1 dimethylformamide:dimethylsulfoxide (DMF:DMSO) for 2 × 5 min (Figure 3). 8

**G-LHRH**

G-LHRH (H-GHWSYGLRPG-NH2) is a modified version of the luteinizing hormone releasing hormone, and is also used as a test peptide for quality control of the Symphony and Prelude peptide synthesizers. G-LHRH was synthesized in 2.3 hr using deprotection times of 2 × 30 sec and coupling times of 2 × 1 min (Figure 4).

**Figure 3** HPLC of crude 65–74ACP. This 10-mer peptide was synthesized in 2.1 hr with 2 × 30 sec deprotection times and 2 × 1 min coupling times.

**Figure 4** HPLC of crude G-LHRH. This 10-mer peptide was synthesized in 2.3 hr with 2 × 30 sec deprotection times and 2 × 1 min coupling times.

**Figure 5** HPLC of crude C-peptide. This 31-mer peptide was synthesized in 9 hr with 2 × 1.5 min deprotection times and 2 × 1 min coupling times.

**Figure 6** HPLCs of crude a) linear and b) cyclized hAmylin 1–37. The 37-mer linear peptide was synthesized in 10.8 hr with 2 × 1 min deprotection times and 2 × 2.5 min coupling times. It was then cyclized in 10 min for a total synthesis time of 11 hr.

**Figure 7** HPLCs of a) crude and b) purified human β-amyloid 1–42. This 42-mer peptide was synthesized in 11.6 hr with 2 × 1 min deprotection times and 1 × 5 min coupling times.

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