An Improved Coupling Method for Peptide Synthesis at Elevated Temperature

Jonathan M. Collins, Sandeep K. Singh, Keith A. Porter
CEM Corporation, Matthews, NC 28106, USA

Introduction

The most commonly used and studied activation method for peptide synthesis is based on the use of carbodiimides. Carbodiimides, whose use dates back to 1955 for peptide synthesis[1], contain a slightly basic nitrogen atom which will react with the carboxylic acid of an amino acid derivative to form a highly reactive o-acylisourea compound. However, the use of carbodiimide based activation methods can lead to incomplete coupling due to both a relatively slow activation process and a more acidic environment for subsequent acylation. This led to the more recent development of onium salt-based coupling methods which require use of a base, but feature a more rapid activation and acylation rate[2]. While more rapid at room temperature, onium salts have the undesirable requirement of at least one equivalent of base to generate the carboxylate anion which rapidly reacts with the electrophilic onium salt activator.

An improved coupling method for SPPS is presented which overcomes the limitations of coupling with both standard carbodiimide and onium salt based methods at elevated temperatures. This method is a modified carbodiimide activation strategy which features the use of a base. It was found that a strong base added at less than 1-equivalent compared to the amino acid, carbodiimide, and activator additive could be present during the entire activation and coupling process while both enhancing the overall coupling reaction and avoiding potential side reactions.

Methods

All peptides were synthesized sequentially using a Liberty Blue™ HT12 under *HE*-SPPS[3] conditions at 0.1 mmol scale using 5-fold excess of reagents. The peptides were cleaved with TFA/TIS/H_2O/DODT (92.5/2.5/2.5/2.5) in 30 min at 38 °C using an Accent™ microwave cleavage system. The resin was filtered and the peptide crashed out upon addition of ice cold ether. The peptides were collected and analyzed on a Waters UPLC ACQUITY H-Class with 3100 Single Quad MS using acetonitrile/water with 0.1 % TFA as the solvent system on C18 Column (1.7mm, 2.1 x 100mm).

Results and Discussion

To evaluate the effect of base (DIEA) present during a carbodiimide with additive (DIC/Oxyma) coupling method, the difficult coupling of Fmoc-Aib-OH onto Aib-IDYING was selected. The couplings were performed at 75, 90, or 100 °C for various times in the presence of either 0, 0.1, or 1.0 equivalents of DIEA. It was found that the presence of 0.1 equivalents of DIEA was preferred over 0 or 1 equivalents at each time point and temperature tested (Table 1).

Limiting the amount of base present is ideal for controlling side reactions during coupling such as epimerization of cysteine and δ-lactam formation of arginine. Analysis of a difficult cysteine coupling, even at 100°C (VYWTSPFMKLIHEQ**C**NRADG-NH_2) revealed no significant increase in D-Cys formation compared to room temperature conditions. In contrast, use of larger amounts of base >1 equivalent caused significant epimerization (Table 2).

Use of this coupling method was then further evaluated on the difficult $^{1\text{-}29}$Glucagon peptide (HSQGTFTSDYSKYLDSRRAQDFVQWLMNT). It was found that the presence of 0.1 equivalents of DIEA during the coupling reaction provided complete stability for the Trityl linker and prevented premature cleavage even at 90 °C (Table 3). Additionally, the use of a Thr(tBu)-Trityl-SpheriTide® resin at 0.85 mmol/g allowed for a higher purity to be obtained (62%) compared to a Fmoc-Thr(tBu)-Wang-PS resin (47%).

Finally, use of this coupling method was evaluated on the difficult AS-48 75-mer protein (VVEAGGWVTTIVSILTAVGSGGLSLLAAAGRESIKAYLKKEIKKKGKRAVIAWMAKEFGIPAAVAGTVLNHVKKK-NH_2). This protein was synthesized using single coupling and DIC/Oxyma/DIEA (1:1:0.1) with 5-equivalents of amino acid for 2 min at 100 °C. The protein was synthesized in > 80% crude purity in only 6 h 42 min.

Table 1. Coupling Fmoc-Aib-OH onto Aib-IDYING-NH_2

Entry	Coupling Temp (°C)	Coupling Time (min)	Base (Equivalent)	% Purity (UPLC-MS)
1	75	20	None	92
2	90	2	None	30
3	90	2	DIEA - (0.1)	53
4	90	2	DIEA - (1.0)	24
5	90	4	None	65
6	90	4	DIEA - (0.1)	68
7	90	4	DIEA - (1.0)	62
8	90	6	None	73
9	90	6	DIEA - (0.1)	76
10	90	6	DIEA - (1.0)	72
11	100	6	DIEA - (0.1)	89
12	100	10	None	86
13	100	10	DIEA - (0.1)	93
14	100	10	DIEA - (1.0)	85

Table 2. Epimerization of Fmoc-Cys(Trt)-OH during various coupling conditions

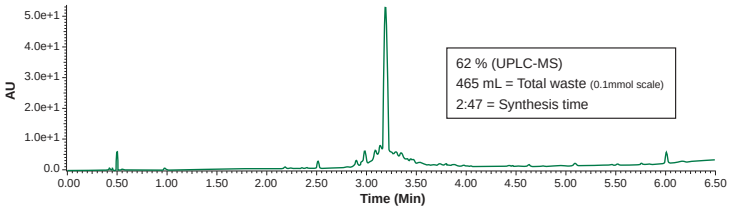
	Reagent	Coupling Temp (°C)	Coupling Time (min)	% -D
1	HBTU/DIEA	RT	30	1.34-Cys
2	HBTU/DIEA	75	5	**4.33**-Cys
3	HBTU/DIEA	90	2	**6.60**-Cys
4	HBTU/DIEA	100	2	**8.52**-Cys
5	DIC/Oxyma	90	2	0.69-Cys
6	DIC/Oxyma/DIEA (1/1/0.1)	90	2	1.46-Cys
7	DIC/Oxyma/DIEA (1/1/0.1)	100	2	0.85-Cys
8	DIC/Oxyma/DIEA (1/1/**1.0**)	90	2	**3.91**-Cys

Table 3. Optimized Synthesis of $^{1\text{-}29}$Glucagon with Trityl-SpheriTide®

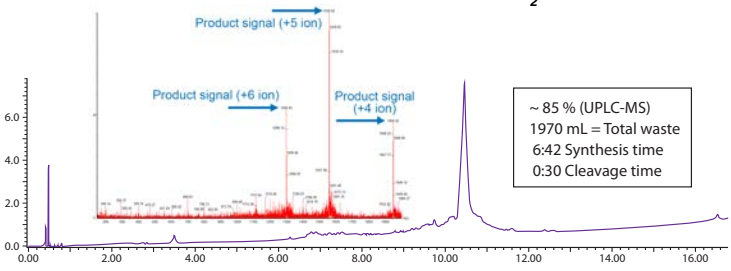
Entry	Resin	Coupling Method	% Purity (UPLC-MS)	Crude Yield (%)
1	H-Thr(tBu)-Trt-SpheriTide® (0.85 mmol/g)	**A**	60	**69**
2	**H-Thr(tBu)-Trt-SpheriTide® (0.85 mmol/g)**	**B**	**62**	**93**
3	Fmoc-Thr(tBu)-Wang PS (0.65 mmol/g)	B	47	87
4	H-Thr(tBu)-2Cl-Trt PS (0.67 mmol/g)	B	56	**29**

A = Fmoc-AA/DIC/Oxyma (1:1:1) – 90°C
B= Fmoc-AA/DIC/Oxyma/DIEA (1:1:1:0.1) - 90°C

$^{1\text{-}29}$Glucagon *HSQGTFTSDYSKYLDSRRAQDFVQWLMNT*



AS-48 (75-mer) *VVEAGGWVTTIVSILTAVGSGGLSLLAAAGRESIKAYLK-KEIKKKGKRAVIAWMAKEFGIPAAVAGTVLNHVKKK-NH_2*



Conclusion

This new coupling method based on 0.1 equivalents of DIEA for DIC/Oxyma based activation appears ideal for SPPS at elevated temperatures.[4] Use of this method not only improved peptide purities over standard carbodiimide and onium salt based methods, but also uniquely avoided cysteine epimerization, minimized δ-lactam formation of activated arginine, and provided stability of the hyper-acid sensitive Trityl linker at 90°C. Use of this coupling chemistry with *HE*-SPPS chemistry demonstrates significant value for peptide synthesis.

References

[1] Sheehan, J.C.; Hess, G.P. *JACS*, **1955**, *77*, 1067-1068.
[2] Gawne, G.; Kenner, G.W.; Sheppard, R.C. *JACS*, **1969**, *91*, 5669-5671.
[3] Collins, J. M.; Porter, K. A.; Singh, S. K.; Vanier, G. S. *Org. Lett.*, **2014**, *16*, 940 – 943
[4] Patent Pending