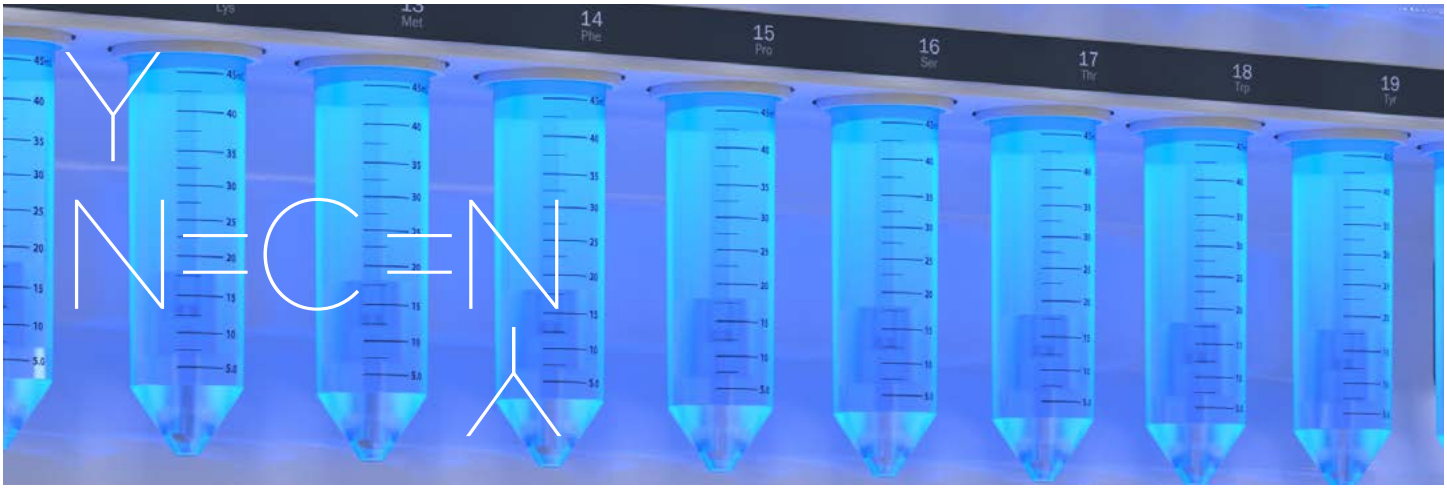
CarboMAX™ – Enhanced Peptide Coupling at Elevated Temperature



Summary

CarboMAX is a new patented coupling methodology for peptide synthesis at elevated temperature. It offers improvements over existing carbodiimide methodology by providing faster activation and corresponding reduction in epimerization. Additionally, it offers increased stabilization of acid sensitive linkages at high temperature which is particularly beneficial for phosphopeptides, glycopeptides, and synthesis of peptides on hyper-acid sensitive linkers.

Introduction

It has been recently shown that rapid and efficient peptide couplings can be performed by *in-situ* carbodiimide activation at 90°C using a high efficiency solid phase peptide synthesis process (*HE*-SPPS).[1] *HE*-SPPS eliminates large amounts of strong base present with onium salt based methods such as HCTU and HATU which lead to significant epimerization (particularly cysteine) and arginine deletions from δ-lactam formation. CarboMAX coupling methodology offers further improvements to carbodiimide based methodology through two major improvements:

(i) Higher crude purities and reduced epimerization levels:
CarboMAX provides a more rapid formation of the slow forming O-acylisourea intermediate necessary for activation by carbodiimides. This is achieved by using a higher ratio of carbodiimide relative to the amino acid (2 equivalents).[2] Increasing the rate of O-acylisourea formation leads to a higher amount of activated amino acid present earlier in the coupling step. Faster coupling rates are then achieved while correspondingly reducing epimerization. In many cases, noticeably higher crude purities are achieved with CarboMAX.

(ii) Avoiding undesirable cleavage of acid sensitive functionalities/linkers:
Activators (Oxyma Pure) used with carbodiimide chemistry, are acidic and can prematurely remove acid sensitive groups at higher temperatures. With CarboMAX methodology, small amounts of base (≤ 0.4 equivalents of DIEA relative to the activated amino acid) stabilize acid sensitive linkages.[3] The presence of this base is low enough to not interfere with the activation process while also maintaining minimal epimerization.

Materials and Methods

Reagents

All standard Fmoc amino acids were obtained from CEM Corporation (Matthews, NC) and contained the following side chain protecting groups: Arg(Pbf), Asn(Trt), Asp(OMpe), Cys(Trt), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc) and Tyr(tBu). Phosphoamino acids Fmoc-Ser(PO(OBzl)OH)-OH and Fmoc-Thr(PO(OBzl)OH)-OH, Oxyma Pure and Rink Amide ProTide™ LL resin were also obtained from CEM Corporation (Matthews, NC). Fmoc-Thr(β-D-GlcNAc(Ac)$_3$)-OH and Fmoc-Lys(palmitoyl-Glu-OtBu)-OH were obtained from Bachem (Switzerland). *N,N*-Diisopropylcarbodiimide (DIC), *N,N*-Diisopropylethylamine (DIEA), Piperidine, Trifluoroacetic acid (TFA), 3,6-Dioxa-1,8-octanedithiol (DODT), Triisopropylsilane (TIS) and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), and anhydrous Diethyl ether (Et_2O) were obtained from VWR (West Chester, PA). HPLC-grade water (H_2O) and HPLC-grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Peptide Synthesis

Peptides were prepared at 0.05 or 0.1 mmol scale using a CEM Liberty Blue™ automated microwave peptide synthesizer on 0.18 mmol/g Rink Amide ProTide LL resin (CEM Part # R002) unless mentioned otherwise. Deprotection was performed with 20% piperidine in DMF. Coupling reactions were performed using 5-equivalents of amino acid with Fmoc-AA-OH/DIC/Oxyma Pure in DMF. Cleavage was performed for 30 min at elevated temperature using the CEM Razor high-throughput peptide cleavage system with TFA/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated in cold Et_2O and lyophilized overnight.

Peptide Analysis

Peptides were analyzed on a Waters Acquity UPLC system with PDA detector equipped with an Acquity UPLC BEH C8 column (1.7 mm and 2.1 x 100 mm). The UPLC system was connected to a Waters 3100 Single Quad MS for structural determination. Peak analysis was achieved on Waters MassLynx software. Separations were performed with a gradient elution of 0.05% TFA in (i) H_2O and (ii) MeCN.

Results

A significant increase in peptide purity was achieved by applying CarboMAX methodology (2 equivalents of DIC relative to the amino acid) instead of the traditional 1 equivalent normally used.

This was demonstrated on the synthesis of 8 well-known peptides synthesized using standard carbodiimide chemistry versus CarboMAX methodology (**Table 1; & Figures 1 – 6**). Importantly, no evidence of undesirable capping was observed from the use of excess carbodiimide.

Table 1: Improvement in peptide purity by application of CarboMAX coupling methodology

Peptide	Sequence	Crude Purity (Standard)	Crude Purity (CarboMAX)
Thymosin	SDAAVDTSSEITTKDLKEKKEVVEEAEN	63%	75%
GRP	VPLPAGGGTVLTKMYPRGNHWAVGHLM	62%	74%
Bivalirudin	fPRPGGGGNGDFEEIPEEYL	80%	82%
$^{1\text{-}34}$PTH	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF	67%	85%
$^{35\text{-}55}$MOG	MEVGWYRSPFSRVVHLYRNGK	77%	91%
Magainin 1	GIGKFLHSAGKFGKAFVGEIMKS	71%	79%
Dynorphin A	YGGFLRRIRPKLKWDNQ	74%	82%
Liraglutide*	HAEGTFTSDVSSYLEGQAAK(γ-Glu-palmitoyl) EFIAWLVRGRG-OH	74%	88%

*Synthesized with ~ 0.32 mmol/g Fmoc-Gly-Wang PS resin

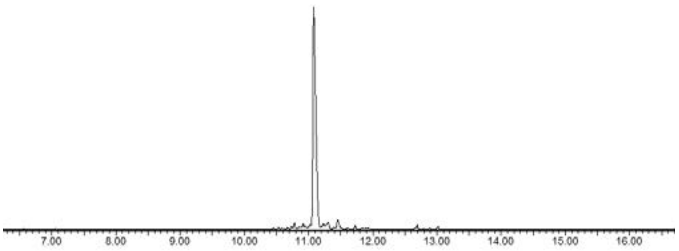


Figure 1: UPLC-MS Analysis of crude Liraglutide (CarboMAX)

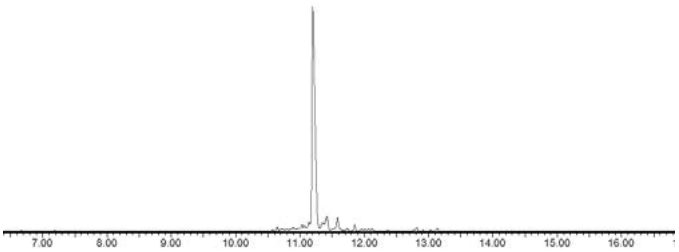


Figure 2: UPLC-MS Analysis of crude Liraglutide (Standard)

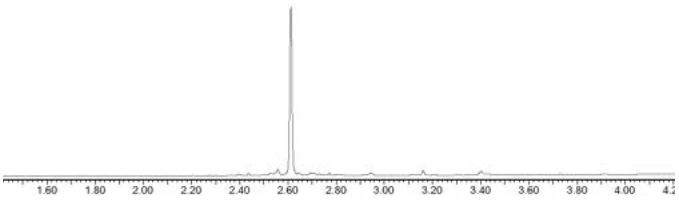


Figure 3: UPLC-MS Analysis of crude $^{35–55}$MOG (CarboMAX)

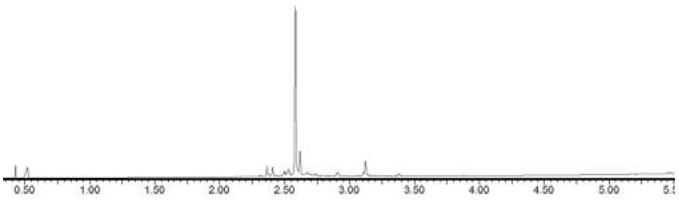


Figure 4: UPLC-MS Analysis of crude $^{35–55}$MOG (Standard)

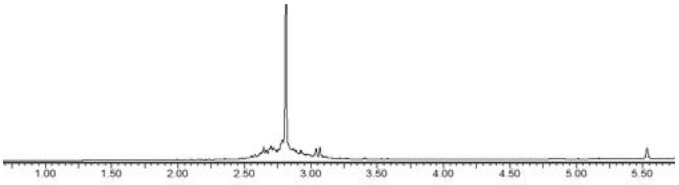


Figure 5: UPLC-MS Analysis of crude $^{1–34}$PTH (CarboMAX)

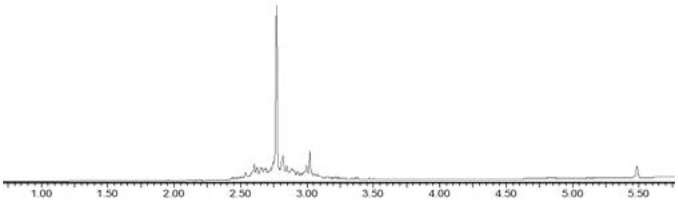


Figure 6: UPLC-MS Analysis of crude $^{1–34}$PTH (Standard)

By increasing the rate of O-acylisourea formation, CarboMAX provides a higher concentration of activated amino acid more quickly. This is beneficial for reducing the lifetime of sensitive ester species before subsequent acylation occurs. To test this, the epimerization level of each amino acid was investigated through hydrolysis, subsequent derivatization, and analysis by gas chromatography (C.A.T. GmbH) on the Liraglutide sequence. As shown in **Table 2**, epimerization levels observed with CarboMAX suggest a trend of reduced epimerization that supports the theory of faster O-acylisourea formation. Thus, CarboMAX coupling offers higher crude purities by both enhancing coupling rates and reducing epimerization over any previous method described for coupling at elevated temperature.

Table 2: Epimerization levels in Liraglutide

%-D Epimer	2 min-90 °C Standard	2 min-90 °C CarboMAX
D-Ala	0.33	0.25
D-Arg	0.29	0.20
D-Asp	0.23	0.31
D-Glu	0.39	0.30
D-Ile L-allo Ile D-allo Ile	< 0.10 < 0.10 < 0.10	< 0.10 < 0.10 < 0.10
D-Leu	0.17	0.13
D-Lys	< 0.10	0.10
D-Phe	0.20	0.16
D-Ser	0.16	0.12
D-Thr L-allo Thr D-allo Thr	< 0.10 < 0.10 < 0.10	< 0.10 < 0.10 < 0.10
D-Trp	0.24	< 0.10
D-Tyr	0.12	0.11
D-Val	< 0.10	< 0.10

An optional addition to CarboMAX methodology is the use of ≤ 0.4 equivalents of base compared to the amino acid. The presence of a catalytic amount of DIEA during the entire activation and coupling process stabilizes acid sensitive linkages (**Table 3**). This was first examined using a hyper-acid sensitive resin linkage. Use of hyper-acid sensitive linkers such as 2-Cl-Trityl, Trityl (Trt), and Cl-TCP is of importance in peptide synthesis as they allow for protected peptide fragment condensation, suppression of diketopiperazine formation, avoidance of DMAP during resin loading, and minimize beta-elimination of C-terminal cysteine residues.

Standard carbodiimide coupling with DIC/Oxyma Pure for 2 min at 90°C resulted in a low yield for synthesis of the ACP peptide on a Trityl resin linkage. However, CarboMAX coupling at 90°C when utilizing 0.1 equivalents of DIEA protected the Trityl linker bond and raised the yield from 38% to 96%.

Table 3: Synthesis of $^{65\text{-}74}$ACP using hyper-acid sensitive Fmoc-Gly-NovaSyn TGT resin

Coupling Temp. (°C)	Coupling Time (min)	Activation	DIEA (equiv.)	Crude Purity (%)	Crude Yield (%)
90	2	DIC/Oxyma Pure	0	90	38
90	2	DIC/Oxyma Pure	0.1	95	96

$^{65\text{-}74}$ACP: VQAAIDYING

In a similar manner, synthesis involving phosphoamino acid derivatives pose significant challenges at high temperature from potential loss of the phospho group due to β-elimination during deprotection and/or acidic cleavage during coupling. Once incorporated into the sequence, a phospho amino acid causes considerable steric hindrance leading to difficult coupling of the consecutive residues. Use of CarboMAX coupling with 0.4 equivalent DIEA resulted in successful synthesis of the phosphopeptide containing 3 phosphoamino acid residues as the major target as shown in **Figure 7a**. In comparison, the synthesis without using DIEA did not show any target peptide (**Figure 7b**).

TpTGNGKPVECpSQPESSFKMpSFKR

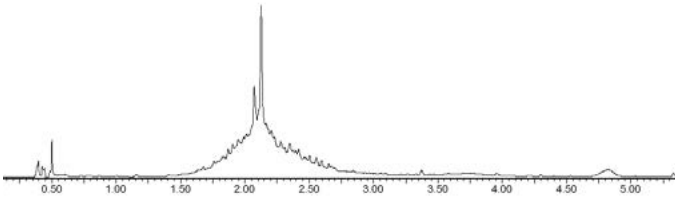


Figure 7a: UPLC-MS Analysis of crude phosphopeptide using 0.4 equivalent DIEA (CarboMAX)

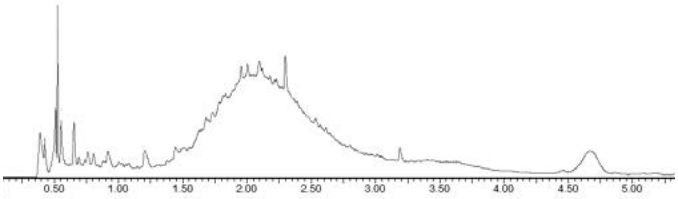


Figure 7b: UPLC-MS Analysis of crude phosphopeptide (Standard)

Additionally, O-linked glycoamino acids contain sensitive linkages that can potentially break during high temperature carbodiimide coupling. Using CarboMAX coupling with 0.4 equivalent DIEA allowed the MUC-1 glycopeptide to be synthesized with 72% crude purity as shown in **Figure 8**. Additionally, CarboMAX method allowed the use of only 2 equivalent excess of glycoamino acid resulting in remarkable savings considering the high-cost of glycosylated amino acids.

PAHGVT[β-D-GlcNAc(Ac)$_3$]SAPDTRPAPGSTAP

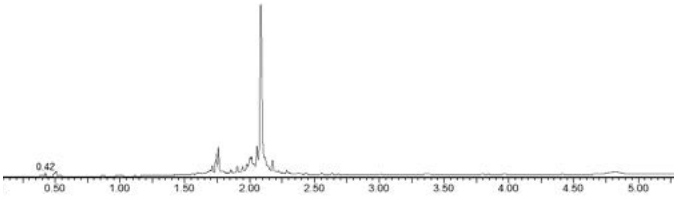


Figure 8: UPLC-MS Analysis of crude MUC-1 glycopeptide using 0.4 equivalent DIEA (CarboMAX)

Conclusion

CarboMAX method offers faster coupling and low epimerization with microwave-assisted SPPS. Expensive or difficult to synthesize amino acid residues can be coupled with only 2 equivalents. Optional use of catalytic amounts of DIEA (0.4 equivalents) prevents undesirable cleavage of acid labile linkages at high temperature.

References

(1) J. Collins, K. Porter, S. Singh and G. Vanier, "High-Efficiency Solid Phase Peptide Synthesis (HE-SPPS)," *Org. Lett.*, vol. 16, pp. 940-943, 2014.

(2) Patent Pending: US15686719; EP17188963.7

(3) Patent Pending: US20160176918; EP3037430; JP2016138090; CN105713066; AU2017204172

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