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EDC·HCl and Potassium Salts of Oxyma and Oxyma-B as Superior Coupling Cocktails for Peptide Synthesis

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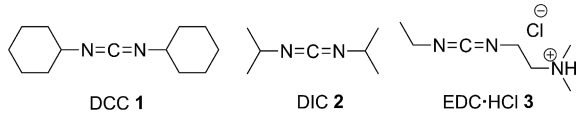
Nowadays, DIC is the most widely used carbodiimide for solid-phase peptide synthesis, while EDC·HCl is mostly used only for solution-phase synthesis. In this paper, we report new coupling cocktails containing EDC·HCl in combination with potassium salts of OxymaPure and Oxyma-B (i.e., K-Oxyma and K-Oxyma-B, respectively). These reagent cocktails gave spectacular purity compared to DIC/classical *N*-hydroxylamine derivatives in the solid-phase peptide synthesis of the Aib-enkephaline (Aib = 2-aminoisobutyric acid) pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2), a hindered peptide. Furthermore, we found that the EDC·HCl/K-Oxyma combination can be used with DMF, THF, or MeCN as the solvent. The optimized cocktail gave less racemization than benzotriazole derivatives, but slightly more than OxymaPure and Oxyma-B during stepwise solution-phase peptide synthesis.

Introduction

Carbodiimide-mediated coupling methods are one of the most traditional approaches for peptide-bond formation.[1–5] *N*,*N*′-Dicyclohexylcarbodiimide (DCC; **1**) has been used as coupling reagent since 1955.[6] *N*,*N*′-Diisopropylcarbodiimide (DIC; **2**) replaced DCC (**1**) in solid-phase peptide synthesis because its urea is more soluble in organic solvents, and therefore it is easily removed by several washings of the resin with dichloromethane.[7] Also, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl; **3**) replaced **1** in solution-phase peptide synthesis because its urea is highly soluble in aqueous solutions, and so is easily removed by an aqueous work-up (Figure 1).[5,8] From a green chemistry point of view, EDC·HCl is greener than DCC and DIC according to the GlaxoSmithKline reagent-selection guide, since its by-product is water-soluble, while DIC is flammable and toxic by inhalation.[7]



DCC **1** DIC **2** EDC·HCl **3**

Figure 1. Structures of commonly used carbodiimides.

To reduce the level of racemization and enhance the coupling yields, carbodiimides are usually used in combination with additives. These serve to reduce the reactivity of the active species formed in the reaction, and thus they inhibit side-reactions such as the formation of *N*-acylureas and oxazolones.[5] Phenol or *N*-hydroxylamine derivatives are commonly used as additives.[9] 1-Hydroxybenzotriazole (HOBt; **4**) was reported in the 1970s, and has been used for decades in most coupling reactions in combination with carbodiimides.[10] Later, Carpino reported that 7-aza-1-hydroxybenzotriazole (HOAt; **5**) is more efficient than HOBt as it decreases racemization while also enhancing the coupling yields, especially with hindered amines.[11–13] Recently, our group reevaluated ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure; **6**) as an additive for peptide synthesis. OxymaPure was shown to be superior to HOBt in all cases, and in many cases it gave the same per-

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formance as HOAt.[14,15] Furthermore, we have also described K-Oxyma (**7**) as a potassium salt of **6**, which proved to be a good alternative, especially when acid-sensitive solid supports, such as CTC resins, were used.[16] More recently, our group reported 5-(hydroxyimino)-1,3-dimethylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (Oxyma-B; **8**) as a new additive for peptide synthesis.[17] Oxyma-B was shown to be superior to additives **4**, **5**, and **6** in its suppression of racemization (Figure 2). In this paper, we describe the use of EDC·HCl (**3**) as a greener carbodiimide in combination with the potassium salts of oximes such as **7** and a new member of the Oxyma-B family, K-Oxyma-B (**9**).

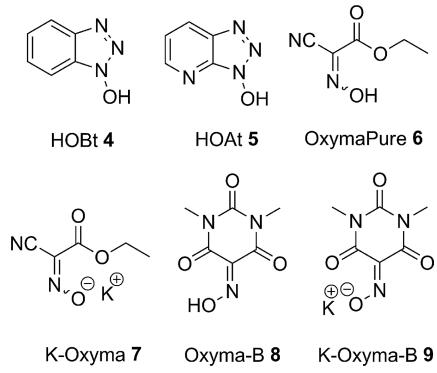
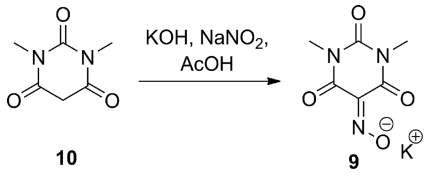


Figure 2. Structures of the additives used in this study.

Results and Discussion

First, we prepared the potassium salt of Oxyma-B (i.e., K-Oxyma-B, **9**) as an example of a potassium salt additive. K-Oxyma-B (**9**) was prepared by the reaction of 1,3-dimethylbarbituric acid (**10**) with KOH, $NaNO_2$, and AcOH in water for 2 h at 0 °C to give the desired product as a yellow-orange solid in 75% yield (Scheme 1). K-Oxyma-B (**9**) showed good solubility in DMF, the most commonly used solvent for peptide synthesis. It showed only partial solubility in MeCN, and was even less soluble in CH_2Cl_2 and THF.



Scheme 1. Synthesis of K-Oxyma-B (**9**).

The key parameters used to evaluate peptide coupling reagents are coupling efficiency and racemization. To evaluate the coupling efficiency, Aib-enkephaline (Aib = 2-aminoisobutyric acid) pentapeptide (**11**) was selected as a model for solid-phase assembly of a hindered peptide by standard Fmoc (fluorenylmethoxycarbonyl) solid-phase peptide synthesis.[14,17,18] The nonincorporation of one Aib residue, giving des-Aib, is the most important side-reaction in this process, and it is caused by a slow reaction due to the sterically hindered nature of the two coupling components. This benchmark peptide clearly shows the differences in efficiency of different coupling reagents. Fmoc-SPPS is the most commonly used approach for peptide synthesis for research purposes, and even for large-scale production processes.[19] Pentapeptide **11** was manually assembled stepwise on an Fmoc-RinkAmide-AM-PS-resin using 1 h coupling times (except for the Aib–Aib linkage, where a 1 h double coupling was used), using an excess (3 equiv.) of the Fmoc-amino acid, the additive, and the carbodiimide.

As shown in Table 1, DIC was more efficient in the couplings than EDC·HCl when classical *N*-hydroxylamine derivatives were used (Table 1, entries 1, 3, 5, and 9, vs. entries 2, 4, 6, and 10). However, EDC·HCl was more efficient in the couplings than DIC when potassium salts were used (Table 1, entries 8 and 12, vs. entries 7 and 11). Furthermore, the crude purities obtained with EDC·HCl/K-Oxyma or K-Oxyma-B were more than double those obtained with EDC·HCl/OxymaPure or Oxyma-B (Table 1, entries 8 and 12, vs. entries 6 and 10). In fact, the cocktails of EDC·HCl/potassium salts gave spectacular results compared to HOAt and OxymaPure, which are considered to be the best additives known in this regard.

Table 1. Percentage purity of the pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) during solid-phase assembly, in the presence of the tetrapeptide side-product des-Aib (H-Tyr-Aib-Phe-Leu-NH_2).[a]

Entry	Coupling reagents	Pentapeptide [%]	des-Aib [%][b]
1	DIC/HOBt	8.4	83.1
2	EDC·HCl/HOBt	7.2	79.3
3	DIC/HOAt	37.5	60.2
4	EDC·HCl/HOAt	26.5	58.9
5	DIC/OxymaPure	42.8	50.4
6	EDC·HCl/OxymaPure	41.9	49.0
7	DIC/K-Oxyma	71.0	6.7
8	EDC·HCl/K-Oxyma (**7**)	85.0	1.7
9	DIC/Oxyma-B	26.4	61.1
10	EDC·HCl/Oxyma-B	11.1	71.7
11	DIC/K-Oxyma-B	20.7	74.0
12	EDC·HCl/K-Oxyma-B	61.3	27.1

[a] Fmoc-RinkAmide-AM-PS resin, DMF as a solvent, and 1 h coupling times were generally used, except for the Aib–Aib coupling (1 h double coupling). [b] Deletion tetrapeptide (des-Aib) was identified by peak overlap in HPLC with an authentic sample obtained on the solid phase. The crude H-Tyr-Aib-Aib-Phe-Leu-NH_2 was analysed by reverse-phase HPLC using a linear gradient of 20–40% CH_3CN (0.1% TFA)/H_2O (0.1% TFA) over 15 min (TFA= trifluoroacetic acid), detection at 220 nm, and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column. t_R = 6.68 (pentapeptide), 6.78 (des-Aib) min.

Recently, we reported that THF or MeCN can be a good alternative to DMF in terms of increasing the coupling efficiency when used in combination with ChemMatrix resins and DIC-mediated coupling methods.[20,21] Thus, we evaluated EDC·HCl/potassium salts using a ChemMatrix resin and THF or MeCN as a solvent, which are considered more environmentally friendly than DMF. As shown in Table 2, the use of THF/MeCN resulted in lower coupling efficiencies than those obtained with DMF. However, in the

case of K-Oxyma, the product was obtained in spectacular purity in all cases (almost 90%), and therefore we can conclude that the EDC·HCl/K-Oxyma cocktail can be used in combination with ChemMatrix resin and THF or MeCN.

Table 2. Percentage purity of the pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) during solid-phase assembly, in the presence of the tetrapeptide side-product des-Aib (H-Tyr-Aib-Phe-Leu-NH_2)[a] using ChemMatrix resin and different solvents.

Entry	Coupling reagent	Solvent	Pentapeptide [%]	des-Aib [%][b]
1	EDC·HCl/K-Oxyma	DMF	93.3	1.1
2		THF	88.7	11.3
3		MeCN	89.9	3.5
4	EDC·HCl/K-Oxyma-B	DMF	44.8	55.1
5		THF	15.4	73.5
6		MeCN	31.9	51.8

[a] Fmoc-RinkAmide-AM-ChemMatrix resin and 1 h coupling times were generally used, except for the Aib–Aib coupling (1 h double coupling). [b] See footnote [b] in Table 1.

We also evaluated the extent to which optical configuration was retained during peptide assembly using this new cocktail. To address the racemization, we chose previously studied peptides as coupling models, namely Z-Phg-Pro-NH_2 (**12**) for (1 + 1) stepwise coupling, and Z-Phe-Val-Pro-NH_2 (**13**) for (2 + 1) segment coupling.[14,17,18,22]

In the first model system, the α-phenyl moiety in phenylglycine ensures a high sensitivity to racemization.[23] The EDC·HCl/potassium salt cocktails gave lower racemization levels than DIC/classical benzotriazoles (Table 3, entries 5 and 8, vs. entries 1 and 2). However, these cocktails gave slightly more racemization than DIC/potassium salts (Table 1, entries 5 and 8, vs. entries 4 and 7) and also than DIC/OxymaPure or DIC/Oxyma-B (Table 3, entries 5 and 8, vs. entries 3 and 6).

Table 3. Yield and racemization during the formation of Z-Phg-Pro-NH_2 (**12**) (solution-phase synthesis).[a]

Entry	Coupling reagents	Yield [%][b]	DL [%][c]
1	DIC/HOBt	94.4	9.9
2	DIC/HOAt	91.8	3.7
3	DIC/OxymaPure	94.4	0.9
4	DIC/K-Oxyma	96.1	1.5
5	EDC·HCl/K-Oxyma	93.2	1.6
6	DIC/Oxyma-B	91.0	1.0
7	DIC/K-Oxyma-B	89.3	1.2
8	EDC·HCl/K-Oxyma-B	97.4	1.6

[a] Couplings were carried out in DMF at room temperature without preactivation. [b] Conversion yield of the product (LL + DL) was calculated by HPLC. Retention times of Z-Phg-OH and Z-Phg-Pro-NH_2 were identified by injection of a pure sample. [c] Retention times for each epimer were identified after coinjection with pure LL and DL samples on reverse-phase HPLC using a linear gradient of 25–50% CH_3CN (0.1% TFA)/H_2O (0.1% TFA) over 15 min, detection at 220 nm, and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column. t_R(LL) = 6.4 min, t_R (DL) = 6.8 min.

The second model system (2 + 1) is known to give more racemization than the previous stepwise coupling model because oxazolone formation is promoted during the activation of dipeptide as a result of the electron-donating effect of the *N*-aminoacyl substitutent.[24,25] The potassium salts generally gave more racemization than the *N*-hydroxylamine derivatives (Table 4), but K-Oxyma gave less racemization when used in combination with EDC·HCl than it did when used with DIC (Table 4, entry 5 vs. entry 4).

Table 4. Yield and racemization during the formation of Z-Phe-Val-Pro-NH_2 (**13**) (solution-phase synthesis).[a]

Entry	Coupling reagents	Yield [%][b]	LDL [%][c]
1	DIC/HOBt	96.7	12.9
2	DIC/HOAt	97.7	5.6
3	DIC/OxymaPure	92.4	7.2
4	DIC/K-Oxyma	92.3	25.3
5	EDC·HCl/K-Oxyma	94.7	17.2
6	DIC/Oxyma-B	91.1	4.9
7	DIC/K-Oxyma-B	93.8	8.8
8	EDC·HCl/K-Oxyma-B	87.0	9.2

[a] Couplings were carried out in DMF at room temperature without preactivation. [b] Conversion yield of the product (LLL + LDL) was calculated from HPLC. Retention times of Z-Phe-Val-OH and Z-Phe-Val-Pro-NH_2 were identified by injection of a pure sample. [c] Retention times for each epimer were identified after coinjection with pure LLL and LDL samples on reverse-phase HPLC using a linear gradient of 30–60% CH_3CN (0.1% TFA)/H_2O (0.1% TFA) over 15 min, detection at 220 nm, and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column. t_R(LLL) = 5.8 min, t_R(LDL) = 6.9 min.

Conclusions

Based on these results, combinations of EDC·HCl and potassium salts of oximes are new and unique coupling cocktails that could be used in particular for the formation of hindered peptides such as Aib-enkephaline pentapeptide (**11**). For instance, EDC·HCl/K-Oxyma gave this product with double the purity obtained with DIC/Oxyma, and it also gave a better coupling efficiency than DIC/K-Oxyma. Furthermore, this unique cocktail gave an acceptable racemization level during stepwise coupling. The compatibility of EDC·HCl with THF/MeCN opens the possibility of using a more environmentally friendly system than the system most commonly used: EDC·HCl instead of DIC, and THF or MeCN instead of DMF.

Experimental Section

General Remarks: The solvents used were HPLC reagent grade. Melting points were determined with a Buchi B-540 apparatus (Buchi Labortechnick GmbH, Essen, Germany). NMR spectra (^1H and ^{13}C) were recorded with a Bruker AVANCE III 400 MHz spectrometer (Rheinstetten, Germany). Chemical shift values are expressed in ppm downfield from tetramethylsilane, which was used as an internal standard. IR spectra were recorded with a Bruker-ALPHA spectrophotometer. Reactions were monitored and the purities of products were checked by TLC on silica-gel-coated aluminum sheets (Type 60 GF254, Merck Millipore, Bedford, MA, USA). Spots were detected by exposure to a UV lamp at λ = 254 nm for a few seconds. Analytical HPLC was carried out with an Agilent 1100 system (Kyoto, Japan), and Chemstation software

was used for data processing. LCMS was carried out with a Shimadzu 2020 UFLC-MS (Kyoto, Japan) instrument using a YMCTriartC18 (5 μm, 4.6 × 150 mm) column, and data processing was carried out using the LabSolution software. Buffer A: H_2O (with 0.1% formic acid); and buffer B: CH_3CN (with 0.1% formic acid). High-resolution mass spectrometric data was obtained using a Bruker micrOTOF-Q II instrument (Bremen, Germany) operating at room temperature and a sample concentration of approximately 1 ppm.

Synthesis of K-Oxyma-B (9; Potassium Salt of Oxyma-B): 1,3-Dimethylbarbituric acid (15.6 g, 0.1 mol) was dissolved in water (60 mL) containing KOH (8.4 g, 0.15 mol). Methanol (10 mL) was added to the resulting clear solution, then sodium nitrite (7.6 g, 0.11 mol) was added, and the resulting mixture was stirred for 10 min. The mixture was cooled to 0 °C, then acetic acid (18 g, 0.3 mol) was added dropwise, and the mixture was kept at that temperature for 2 h. The resulting precipitate was collected by filtration, washed with a mixture of water and methanol (1:1) and, after that, with water. The product was recrystallized from a mixture of water and methanol (2:1) to give K-Oxyma-B (**9**) (16.7 g, 75.0%) as yellow-orange crystals, m.p. 228 °C (decomposition). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 3.05 (s, 3 H, CH_3), 3.21 (s, 3 H, CH_3) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 26.8, 27.7, 141.2, 151.2, 151.4, 161.3 ppm. HRMS (ESI): calcd. for $C_6H_6N_3O_4^-$ [M]$^-$ 184.0358; found 184.0367.

Solid-Phase Synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH$_2$: The synthesis was carried out in a plastic syringe, which was attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The Fmoc-RinkAmide-AM-PS resin (0.6 mmol g^{-1}, 100 mg) or Fmoc-RinkAmide-AM-ChemMatrix resin (0.52 mmol g^{-1}, 50 mg) was washed with DMF, CH_2Cl_2, and DMF (2 × 10 mL each), and was then treated with piperidine (20% in DMF; 10 mL) for 10 min. The resin was then washed with DMF, CH_2Cl_2, and DMF (2 × 10 mL each), and then it was acylated with a solution of Fmoc-Leu-OH (3 equiv.), the corresponding additive (3 equiv.), and the corresponding carbodiimide (3 equiv.) in the minimum amount of solvent (DMF, THF, or MeCN). After the peptide coupling, the resin was washed with DMF, and was then deblocked by treatment with piperidine (20% in DMF) for 7 min. The resin was washed with DMF, CH_2Cl_2, and DMF (2 × 10 mL each). Then coupling with the next amino acid and deblocking were carried out as described above, and repeated to obtain the pentapeptide. The peptide was cleaved from the resin by treatment with TFA/H_2O (9:1) at room temperature for 2 h. The TFA was removed under a stream of nitrogen, and the crude peptide was purified by washing with cold Et_2O (3 × 10 mL), and then lyophilized. The ratio of the penta- and tetrapeptides was determined by HPLC analysis using a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, with a linear gradient of 20–40% of CH_3CN (0.1% TFA)/H_2O (0.1% TFA) over 15 min, flow rate: 1.0 mL min^{-1}, detection at 220 nm. The t_R values for the pentapepide and des-Aib were 6.68 and 6.78 min, respectively. LC–MS showed the expected mass for the pentapeptide at *m*/*z* = 611.0, and also for des-Aib at *m*/*z* = 526.

General Method for the Racemization Experiments: Z-Phg-OH or Z-Phe-Val-OH (0.125 mmol), H-Pro-NH_2 (0.125 mmol), and the corresponding additive (0.125 mmol) were dissolved in DMF (2 mL), and the solution was cooled in an ice bath, and treated with carbodiimide (DIC or EDC·HCl, 0.125 mmol). The mixture was stirred at 0 °C for 1 h, and then at room temperature overnight. Then, an aliquot (10 μL) of the solution was taken, and was diluted to 1 mL with a mixture of CH_3CN/H_2O (1:2), and 5 μL of this solution was injected into a reverse-phase HPLC apparatus.

Z-Phg-Pro-NH$_2$: A linear gradient of 25–50% CH_3CN (0.1% TFA)/H_2O (0.1% TFA) over 15 min was used, with a flow rate of 1.0 mL min^{-1}, detection at 220 nm, and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column. t_R(LL) = 6.4 min, t_R(DL) = 6.8 min, t_R(Z-Phg-OH) = 8.8 min.

Z-Phe-Val-Pro-NH$_2$: A linear gradient of 30–60% CH_3CN (0.1% TFA)/H_2O (0.1% TFA) over 15 min was used, with a flow rate of 1.0 mL min^{-1}, detection at 220 nm, and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column. t_R(LLL) = 5.8 min, t_R(LDL) = 6.9 min, t_R(Z-Phe-Val-OH) = 7.7 min.

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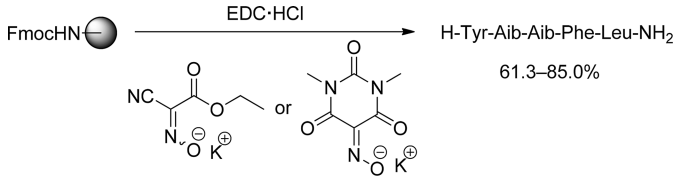
Peptide Coupling



We have shown that combinations of rather simple and green reagents, such as EDC·HCl and the potassium salt of OxymaPure or Oxyma-B, give unique results in terms of yield and low levels of racemization. We envisage a broad application of these reagents in solid-phase and solution synthesis, and especially in the preparation of peptide-based nanomaterials.

Keywords: Peptides / Synthetic methods / Solid-phase synthesis / Peptide coupling / Steric hindrance

