Protocol

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COMU: A third generation of uronium-type coupling reagents

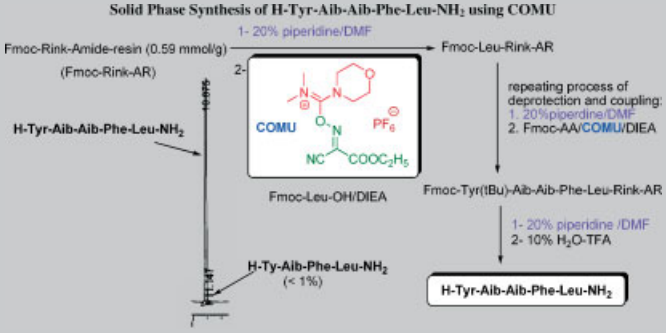
Ayman El-Faham[a,b,c]* and Fernando Albericio[a,d,e]*

COMU is a third generation of uronium-type coupling reagent based on ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) as well as a morpholino carbon skeleton. The presence of the morpholino group has a marked influence on the solubility, stability and reactivity of the reagent. COMU performed extremely well in the presence of only 1 equiv. of base, thereby confirming the effect of the hydrogen bond acceptor in the reaction. The by-products of COMU are water soluble and easily removed, making it an excellent choice of coupling reagent for solution-phase peptide synthesis. Finally, COMU shows a less hazardous safety profile than benzotriazole-based reagents, such as HATU and HBTU, which in addition exhibit unpredictable autocatalytic decompositions and therefore a higher risk of explosion. Furthermore, in contrast to benzotriazole-based reagents, COMU is significantly less likely to cause allergic reaction. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: coupling reagent; uronium salt; Oxyma; peptide synthesis; solid-phase methodology

REACTION SCHEME

Solid Phase Synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2 using COMU

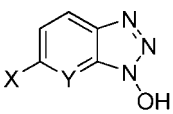


GENERAL OPTIMISED PROCEDURE

Fmoc-Rink-Amide-AR (100 mg, 0.59 mmol/g) was deprotected by 20% piperidine (1mL) in DMF for 10 min., washed with DMF (2x1 mL), DCM (2x1 mL) and then DMF (2x1 mL). Fmoc-Leu-OH (3 equiv.), **COMU** (3 equiv.) and DIEA (6 equiv.) were mixed in DMF (0.5 mL) and activated for 1-2 min and then added to the resin. The reaction mixture was then stirred slowly for 1 min and allowed to couple for 30min (1-h. double coupling only for Aib-Aib). The resin was washed with DMF and then deprotected by 20 % piperidine in DMF for 7 min. It was then washed again with DMF, DCM, and DMF, and then coupled with the next amino acid. Coupling and deprotection steps were repeated to obtain the pentapeptide. The crude product was analyzed by HPLC, with the following conditions: linear gradient of 20 to 80 % CH_3CN/0.1 % TFA in H_2O/0.1 % TFA over 30 min, Symmetry Waters C_{18} column (4.6 x 150 mm, 4 µm), detection at 220 nm, flow rate 1.0 ml/min, t_R penta = 10.87min (99.74%), t_R des-Aib = 11.15 min (0.26%).

Scope and Comments

Peptide synthesis is based on the proper combination of protecting groups and a suitable choice of coupling method [1–3]. Almost all peptide bonds formed are currently carried out in the presence of HOBt (**1**) or its derivatives (HOAt, **2**; 6-Cl-HOBt, **3**) [4–10].



1, X=H, Y=CH, HOBt
2, X=H, Y =N, HOAt
3, X=Cl, Y=CH, 6-Cl-HOBt

* *Correspondence to: Ayman El-Faham and Fernando Albericio, Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain. E-mail: aymanel_faham@hotmail.com; albericio@irbbarcelona.org*

a *Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain*

b *Alexandria University, Faculty of Science, Department of Chemistry, P.O. Box 246 Ibrahimia, 21321 Alexandria, Egypt*

c *King Saud University, Department of Chemistry, College of Science, P.O. Box 2455, 1451-Riyadh, Kingdom of Saudi Arabia*

d *CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain*

e *Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1-11, 08028-Barcelona, Spain*

Abbreviations used: *Aib, α-aminoisobutyric acid; COMU, 1-[(1-(cyano-2-ethoxy-2-oxo-ethylideneaminooxy)-dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate; DIEA, N,Ndiisopropylethylamine; HATU, N-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-b] pyridin-1-yl-methylene)-N-methylmethanaminium hexafluorophosphate N-oxide; NMP, N-methylpyrrolidone; TMP, 2,4,6-trimethylpyridine or collidine. Source: Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature [IUPAC-IUB Commission on Biochemical Nomenclature Symbols for Amino-Acid Derivatives and Peptides. Recommendations (1971). J. Biol. Chem. 1972;* **247:** *977–983.*

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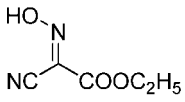
Recent reports have confirmed the explosive properties of HOBt derivatives [11]. In a previous study [12], we showed that Oxyma (**4**) is an excellent replacement for HOBt and its analogs. Here we devised a third generation of uronium salt, COMU (**5**), which involves the combination of a morpholonium-based immonium moiety as proton acceptor [13], and Oxyma (**4**) as leaving group to provide a superior and safe coupling reagent for amide formation.



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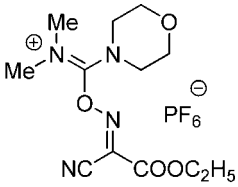

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The oxygen in the imminium structure increased the stability of the coupling reagent compared with the tetramethyl derivatives. Furthermore, Oxyma derivatives have higher stability than the benzotriazole derivatives, HATU and HBTU. These observations are of practical relevance for both solid-phase and solution strategies.

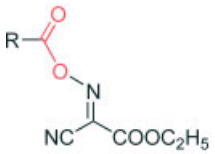
Although a typical protocol for the use of these reagents involves 2 equiv. of base, usually DIEA, the presence of the morpholinium moiety also allows good results with COMU when just 1 equiv. of DIEA is used [14]. Alternatively, the less basic TMP (2 or even 1 equiv.) can be used instead of DIEA and provides good yields and reduces racemization [14].

A further characteristic of COMU is that the course of reaction can be followed as a result of change of color, depending on the type of base used. Once the reaction is complete, the solution becomes colorless to yellow, again depending on the type of base used (Figure 1).

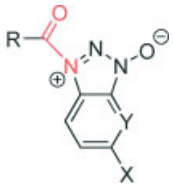
To check the effectiveness of COMU, the demanding leu-enkephalin derivative H-Tyr-Aib-Aib-Phe-Leu-NH_2 [14] was chosen as an example (see General Optimal Procedure). Synthesis with COMU (**5**) led to only 0.26% of des-Aib when 2 equiv. of DIEA was used, while HDMA and HDMB gave 1% and 10% of des-Aib, respectively. In contrast, HATU and HBTU gave 17% and 53% of des-Aib, respectively. These results are consistent with what is discussed earlier.

Furthermore, COMU is compatible with microwave-assisted peptide synthesizers [15]. Consistent with previous reports, COMU displayed higher efficiency than HATU/HBTU in the demanding synthesis of the Aib derivative of the leu-enkephalin pentapeptide and produced no Oxyma-based by-products. Thus, the combination of microwave irradiation and COMU resulted in a similar performance to that observed by manual synthesis in considerably shorter time.

In conclusion, we have introduced a third generation of *O*-form uronium-type coupling reagent that differs in immonium moiety and also in the leaving group. The presence of the morpholino group has a marked influence on the polarity of the carbon skeleton, which affects the solubility, stability and the reactivity of the reagent. Remarkably, Oxyma derivatives often gave similar or better results than the aza derivatives and performed extremely well in the presence of only 1 equiv. of base. This observation confirms the effect of the oxygen as a hydrogen bond acceptor in the reaction. Although the acidity of Oxyma derivatives is similar to that HOBt and higher than HOAt, the excellent performance of the former can be explained by the fact that the *O*-form is the only form present during the coupling. This feature differs to that of benzotriazole derivatives, in which the the *N*-form is predominant.



O-Form



N-Form

Experimental Procedure

General procedure for preparation of COMU [16,17]

Dimethylcarbamoyl chloride (0.6 mol) was added dropwise to a stirring mixture of DCM (400 ml) and 4 N NaOH (250 ml) and morpholine (0.5 mol) at 0 °C. When the addition was completed, the mixture was stirred for 3 h at r.t. The organic layer was collected and the aqueous layer was washed with DCM (100 ml). The combined DCM solution was washed with a saturated solution of NaCl (2 × 100 ml). Finally, the organic solvent was dried over anhydrous $MgSO_4$ and filtered. The solvent was then removed under reduced pressure to give an oily residue. The product was distilled and collected at bp 127–129 °C as a colorless oil with a 93% yield.

(***N,N*-Dimethyl-4-morpholinecarboxamide)** ^{1}H NMR ($CDCl_3$): δ 2.84 (s, 6H, 2 CH_3), 3.22–3.20 (m, 4H, $2CH_2$), 3.68–3.70 (m, 4H, $2CH_2$) ppm. ^{13}C NMR ($CDCl_3$): δ 38.6, 47.5, 66.9, 165.0 ppm. (The NMR of the crude product was 99.8% pure, so we can use it directly for the next step without further purification.)

Oxalyl chloride (100 mmol) in DCM (100 ml) was added dropwise to a solution of urea (***N,N*-Dimethyl-4-morpholinecarboxamide)**

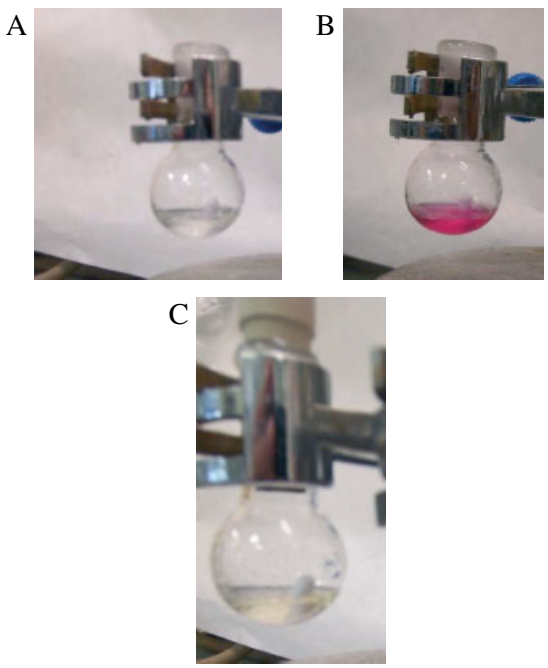


Figure 1. (a) Time 0; (b) after 2 min of the addition of COMU and TMP; (c) after 1 h of the addition of COMU and TMP.

(100 mmol) in dry DCM (200 ml) at r.t. over 5 min. The reaction mixture was stirred under reflux for 3 h. and the solvent was removed under vacuum. The residue was washed with anhydrous ether (2 × 100 ml) and then bubbled with N_2 to remove the excess of ether. The residue was highly hygroscopic, and was therefore dissolved directly in DCM, and a saturated aqueous potassium hexafluorophosphate (100 mmol in 50 ml H_2O) solution was then added at r.t. with vigorous stirring for 10–15 min. The organic layer was collected, washed once with water (100 ml), dried over anhydrous $MgSO_4$ and filtered. The solvent was removed under reduced pressure to give a white solid which recrystallized from DCM-ether or acetonitrile-ether to give white crystals in a yield of 89.6%, m.p. 94–95 °C; ^{1}H NMR (CD_3COCD_3): δ 3.39 (s, 6H; $2CH_3$), 3.75 (t, 4H; $2CH_2$), 3.86 ppm (t, 4H; $2CH_2$); ^{13}C NMR (CD_3COCD_3): δ 44.36, 52.82, 65.99, 162.79 ppm).

The chlorine salt (20 mmol) was added to a solution of oxime potassium salt (20 mmol) in acetonitrile (50 ml) at 0 °C. The reaction mixture was stirred at this temperature for 30 min and allowed to reach r.t. while stirring for 6 h. The crude product was filtered and washed with acetonitrile. The solvent was concentrated to a small volume (1/4) under reduced pressure. Dry ether was then added to afford the product (**COMU**) as white crystals in a yield of 88.8%, m.p. 159–60 °C.

^{1}H NMR (CD_3COCD_3): δ 1.38 (t, 3H; CH_3), 3.41 (s, 6H; $2CH_3$), 3.82 (t, 4H; $2CH_2$), 3.89 (t, 4H; $2CH_2$), 4.48 ppm (q, 2H; CH2); ^{13}C NMR (CD_3COCD_3): δ 13.48, 40.70, 49.94, 64.59, 66.04, 106.76, 135.03, 156.14, 160.61 ppm.

General procedure for coupling reaction using COMU in solution-phase

COMU (0.25 mmol) was added to a mixture of the *N*-protected amino acid (0.25 mmol), the amino component (0.25 mmol) and base (0.50 mmol or 0.75 mmol in case of ester hydrochloride) in DMF (2 ml) at 0 °C and the reaction mixture was stirred at 0 °C for 1 h and at r.t. for 2–3 h. The mixture was diluted with EtOAc (25 ml) and extracted with 1 N HC1 (2 × 5 ml), 1 N $NaHCO_3$ (2 × 5 ml) and saturated NaCl (2 × 5 ml). The EtOAc was then dried with $MgSO_4$, the solvent was removed, and the crude peptide was directly analyzed by HPLC and NMR.

General procedure for coupling reaction using COMU in solid-phase

N-Protected amino acid (3 equiv.), base (6 equiv.) and COMU (3 equiv.) were pre-activated in DMF (0.3 M) for 1 min and then added to the amino-resin with manual stirring for 2–5 min and allowed to stand at r.t. for 10–30 min (1 h for hindered residues or 1-h double coupling, also). The resin was filtered and washed with DMF.

General Notes

- COMU should be treated like other related stand alone coupling reagents, such as HATU or HBTU.

Solvent

- DMF (Fisher HPLC grade) is aspirated with a stream of N_2 for 15 min and stored over molecular sieves.
- In the microwave mode, NMP is more recommended than DMF, which can provoke formylation of the amino group (this is unrelated to the use of COMU or other coupling method).
- After the coupling has taken place, the resin has a color, which is due to the retention of some Oxyma. This is removed for the piperidine treatment or by further washings with alcohol.

Base

- The use of immonium/onium salts requires careful attention to the tertiary base used.
- For the comparison experiments using distinct coupling reagents and because of discrepancies in racemization levels when using different samples of commercial tertiary amines, DIEA (Aldrich, 99%) and NMM (Aldrich, 99%) are distilled first from ninhydrin and then from CaH_2 (bp 126 °C and 114–116 °C, respectively) and stored over molecular sieves. TMP (Eastman Kodak, 97%) is distilled from CaH_2 (bp 170–172 °C) and stored over molecular sieves. Other bases should be treated similarly.
- For regular synthesis, untreated DIEA, which may contain various amounts of primary or secondary amines (positive ninhydrin test), leads to enhanced racemization (2–3%). In contrast, TMP (Aldrich, 99%), taken directly from the bottle, gives racemization levels comparable to those obtained with material distilled over CaH_2.

Pre-activation time

- The pre-activation time is crucial for the optimization of the yield and the level of racemization. However, in some synthesizers, pre-activation time is dictated by the instrument, while in others and for manual syntheses, it can and should be modulated.
- In solution-phase coupling, pre-activation is not required so the coupling reagent is added last at 0 °C.
- Incorporating HOAt, HOBt or Oxyma in immonium/onium salts, the activation of ordinary amino acids gives the corresponding OAt, OBt, Oxyma esters almost instantly. Thus, in such cases, the pre-activation time should be kept to a minimum, since the activated species can give rise to several side-reactions, including the following:
  - *Racemization*, either by direct formation of the enolate or by formation of oxazolone, which is prone to racemize.
  - *Loss of reactivity* by: (i) formation of the oxazolone, which is also less reactive than the active esters; (ii) hydrolysis of the active intermediates; (iii) by shift of the active ester from the *O*-acyl to the *N*-acyl, which is less reactive. However, this rearrangement has not been detected for COMU.
  - *δ-Lactam formation* during the activation of Arg, which is not incorporated into the growing peptide chain, but results in a lower yield.
  - *Cyano derivatives or α-aminocrotonic acid formation* for Asn/Gln and Thr, respectively. These two side-reactions are less frequent that that described above.

Acknowledgements

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References

1 Lloyd-Williams P, Albericio F, Giralt E. *Chemical Approaches to the Synthesis of Peptides and Proteins*. CRC: Boca Raton (FL, USA), 1997.

2 Goodman M, ed., *Synthesis of Peptides and Peptidomimetics. Houben-Weyl, Methods of Organic Chemistry*, Vol. E22a, Thieme: Stuggart·New York, 2002.
3 Amblard M, Fehrentz J-A, Martinez J, Subra G. Methods and protocols of modern solid phase peptide synthesis. *Mol. Biotech.* 2006; **33**: 239–254.
4 Dourtoglou V, Ziegler JC, Gross B. O-Benzotriazolyl-*N*,*N*-tetramethyluronium hexafluorophosphate: a new and effective reagent for peptide coupling. *Tetrahedron Lett.* 1978; **19**: 1269–1272.
5 Albericio F, Carpino LA. Coupling reagents and activation. In *Methods in Enzymology, Solid-Phase Peptide Synthesis*, Vol. 289, Fields GB (ed.). Academic Press: Orlando, 1997; 104–126.
6 Humphrey JM, Chamberlin AR. Chemical synthesis of natural product peptides: coupling methods for the incorporation of noncoded amino acids into peptides. *Chem. Rev.* 1997; **97**: 2243–2266.
7 Kaminski ZJ. Triazine-based condensing reagents. *Biopolymers* 2000; **55**: 140–165.
8 Albericio F, Chinchilla R, Dodsworth DJ, Najera C. New Trends in Peptide Coupling Reagents. *Org. Prep. Proc. Int.* 2001; **33**: 203–303.
9 Han S-Y, Kim Y-A. Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron* 2004; **60**: 2447–2467.
10 Montalbetti CAGN, Falque V. Amide bond formation and peptide coupling. *Tetrahedron* 2005; **61**: 10827–10852.
11 Wehrstedt KD, Wandrey PA, Heitkamp D. Explosive properties of 1-hydroxybenzotriazoles. *J. Hazard. Mat.* 2005; **A126**: 1–7.
12 Subirós-Funosas R, Prohens R, Barbas R, El-Faham A, Albericio F. Oxyma: an efficient additive for peptide synthesis to replace benzotriazole-based HOBt and HOAt with a lower risk of explosion. *Chem. Eur. J.* 2009; **15**: 9394–9403.
13 El-Faham A, Albericio F. Immonium-type coupling reagents: application in solution and solid phase peptide synthesis. *Organic Lett.* 2007; **9**: 4475–4477.
14 El-Faham A, Subirós-Funosas R, Prohens R, Albericio F. COMU, a safer and more effective replacement for benzotriazole based uronium coupling reagents. *Chem. Eur. J.* 2009; **15**: 9404–9416.
15 Subiros-Funosas R, Acosta GA, El-Faham A, Albericio F. Microwave irradiation and COMU: a superior tool for SPPS. *Tetrahedron Lett.* 2009; **50**: 6200–6202.
16 El-Faham A, Khattab SN, Abdul-Ghani M, Albericio F. Design and synthesis of a new immonium type coupling reagents. *Eur. J. Org. Chem.* 2006; **6**: 1563–1573.
17 El-Faham A. New syntheses of bis(tetramethylene)fluoroformamidinium hexafluorophosphate (BTFFH) and 1,3-dimethyl-2-fluoro-4,5-dihydro-1H-imidazolium hexafluorophosphate (DFIH). Utility in peptide coupling reactions. *Org. Prep. Procd. Int.* 1998; **30**: 477–481.

