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COMU: scope and limitations of the latest innovation in peptide acyl transfer reagents

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The methodology for peptide bond formation is undergoing a continuous evolution where the main actors are being renewed. In recent years, coupling reagents based on the Oxyma scaffold, such as the uronium salt COMU, has been a groundbreaking contribution to the field. The advantages of COMU over classic benzotriazole-based reagents (HATU, HBTU, HCTU, TBTU) were proven in terms of solubility and coupling efficiency in bulky junctions in our groups and others. However, some aspects of the use of COMU need to be revised and improved, such as the stability of commercial samples in organic solvents, which hampers the compatibility with long synthesis in automated synthesizers. In this review, an overview of the main features and suggestions to improve the use of COMU are presented, along with a discussion on the best conditions for its use in microwave-assisted peptide robots. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: coupling reagents; COMU; microwave SPPS; stability; automated synthesizers

Introduction

The art of assembling peptides calls for a continuous refinement of applied synthetic tools to achieve an optimal outcome [1]. The organic chemistry within peptide synthesis is indeed highly refined. It covers not only peptide bond formation and removal of urethane-type protecting groups under various conditions, but also linker chemistry and side chain protecting groups. The successful elongation of a peptide chain relies on quantitative conversion of the distinct resin-bound intermediates into their products – an achievement that is difficult to meet even in moderate sterically encumbered sequences. Moreover, an inadequate acid activation may lead to unexpected and detrimental resin-bound by-products [2]. Hence, the choice of the reagent committed to assemble both the acid and the amine components is pivotal [3].

Thus, acid halides and azides are commonly dismissed in the peptide context, on the basis of their extreme reactivity or hazardous profile. Preformed esters are considered for special applications only mainly because of their higher price [2–4]. In the past years, innovative research has contributed new acylation methods, such as the umpolung or isonitrile techniques; however, their practical use remains unproven [5,6]. To date, *in situ* active ester strategies (either through use of carbodiimides or stand-alone coupling reagents) have prevailed in peptide bond formation [2,3]. The introduction of various *N*-hydroxylamine templates as additives in the 1970s decade improved the use of carbodiimides by suppressing or minimizing several side reactions [2,3]. Undoubtedly, 1-hydroxybenzotriazole (HOBt **1**) and its subsequent analogs (HOAt **2**, and 6-Cl-HOBt **3**) surpassed the impact of any other scaffold (Figure 1) [2,3]. In the following years, the benzotriazole core structure was also implicit in the design of a myriad of aminium salts, such as HBTU (**4**), HATU (**5**), HCTU (**6**), TBTU (**7**), TATU (**8**), and TCTU (**9**), standing as powerful alternatives to carbodiimides (Figure 1) [2,3]. In recent times, analogs bearing a dimethylmorpholino-moiety in the carbocation skeleton 1-((dimethylimino)morpholinomethyl)3-H-benzo-[1,2,3] triazolo-1-ium-3-olate hexafluorophosphate (HDMB **10**), 1-((dimethylimino)morpholinomethyl)3-H-[1,2,3]triazolo [4,5-b] pyridine-1–3-olate hexafluorophosphate (HDMA **11**), and 1-((dimethylimino)morpholinomethyl)3-H-6-chlorobenzo [1,2,3] triazolo-1-ium-3-olate hexafluorophosphate (HDMC **12**) flourished, increasing the overall performance of the previously introduced reagents [3,7]. Among non-benzotriazolic reagents, oxime-based O-[(Cyano(ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate HOTU (**13**) and O-[(Cyano (ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium Tetrafluoroborate TOTU (**14**) were two of the most salient coupling reagents, affording low epimerization and remarkable acylation ability (Figure 1) [3,8].

COMU as Efficient Coupling Reagent

Nonetheless, the supremacy of benzotriazoles in peptide chemistry was called into question in 2005, after a safety study concluded that

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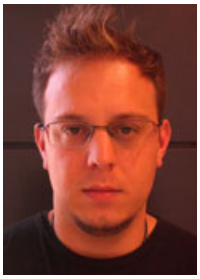
Biographies



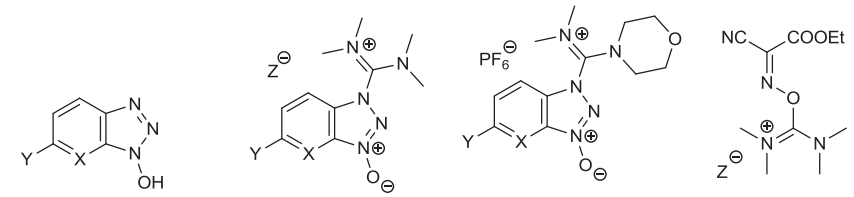
Dr Ramon Subirós-Funosas was born in Barcelona, Spain, in 1983. Before receiving his BSc in Chemistry in 2007, he moved for 1 year to GlaxoSmithKline, S.L., Stevenage, UK, in 2005, where he joined the Medicinal Chemistry Department as an Industrial Placement Student, working on Synthetic Organic Chemistry. In 2007, he joined the group of Prof. Fernando Albericio in the University of Barcelona, Barcelona, Spain, receiving his PhD in Organic Chemistry in 2011, developing a new family of coupling reagents based on ethyl 2-cyano-2-hidroxiiminoacetate (Oxyma) as additive to carbodiimides. In 2010, he visited the group of Phillip E. Dawson at The Scripps Research Institute, La Jolla, CA, USA, for a 4 month internship, learning ligation techniques for the assembly of proteins. His major research interests include novel applications of *N*-hydroxylamines and methodology of native chemical ligation.

Lidia Nieto-Rodriguez was born in 1983 in Barcelona, Spain. She received her BSc in Chemistry with specialisation in Organic Chemistry in 2010 at the University of Barcelona. Shortly thereafter, she stayed as an undergraduate student in Professor Riera's group, performing research on chiral phosphines for asymmetric chemistry at the IRB Barcelona. For 2 years, she worked in multi-gram peptide synthesis as a Junior Research Assistant in Professor Albericio's group at the Science Park of Barcelona. She is studying Master of Science in Chemistry in the Free University of Berlin.

Professor Knud J. Jensen holds degrees in Organic Chemistry and Philosophy from the University of Copenhagen. He obtained a PhD degree in Synthetic Bioorganic Chemistry with Professor Morten Meldal in 1992, after which he did postdoctoral research with Professor George Barany, University of Minnesota. He became Assistant Professor at the Technical University of Denmark in 1997 and Associate Professor at KVL in Copenhagen in 2001; in 2007, he became part of the University of Copenhagen. In the same year, he was promoted to Full Professor in Nanobioscience. He is now a Full Professor at the Department of Chemistry, University of Copenhagen. He is the co-author of >100 peer-reviewed publications, as well as numerous book chapters and proceedings. In 2010, he was the co-chair of the 31st European Peptide Symposium, which was held in Copenhagen. His research covers a broad range of topics at the interface between synthetic chemistry, biology, biophysics, medicinal chemistry, and nanobioscience. A starting point is often provided by the development of new synthetic methodology, including reagents for solid-phase peptide synthesis and for chemoselective chemistry on carbohydrates, peptides, and proteins. Other recent examples from his research include the use of abiotic ligands to control self-assembly of peptides and proteins, and to control their quarternary structure. He has developed the concept of carbohydrates as templates in *de novo* design of proteins. This formed the background for an entry into peptide medicinal chemistry. He has an interest in the chemistry and chemical biology of glycans as well as in glyconanotechnology. He was awarded the Zervas Award of the European Peptide Society in 2012.

Professor Fernando Albericio was born in Barcelona, Spain, in 1953. He obtained his PhD in Chemistry from the University of Barcelona in 1980. After postdoctoral work at Tufts University, at the Université d'Aix-Marseille, and at the University of Minnesota (1981-1984), he joined back the University of Barcelona as an Associate Professor. In 1992-1994, he was appointed Director of Peptide Research at Milligen/Biosearch, Boston, USA, and then rejoined the University of Barcelona in 1995, when he was promoted to Professor. Currently, he is holding a triple appointment as Professor at the University of Barcelona, Group Leader at the Institute for Research in Biomedicine, and Honorary Research Professor at the University of KwaZulu-Natal (Durban, South Africa). From 2005 to 2011, he has been Executive Director in the Barcelona Science Park. His major research interests cover practically all aspects of peptide synthesis and combinatorial chemistry methodology, as well as synthesis of peptides and small molecules with therapeutic activities. He is currently Editor of the International Journal of Peptide Research and Therapeutics. He received the Leonidas Zervas award from the European Peptide Society in 1994 and the Research Chair from the Autonomous Government of Catalonia in 2004. Recently, Professor Albericio has honored with a Doctorate Honoris Causa by the Universidad de Buenos Aires (Argentina) and the Vincent du Vigneaud Award (American Peptide Society).

the parent triazole-containing *N*-hydroxylamine and its analogs merited labeling as Class 1 explosive, which put restrictions on their transport [9]. Only 4 years later, one of our groups reevaluated ethyl 2-cyano-2-(hydroxyimino)acetate (renamed as Oxyma **15**, Figure 2), which had first been reported a few decades ago [10]. Although cyanooximes are well appreciated in organometallic chemistry, Oxyma (**15**) had not been used in peptide synthesis, beyond the related uronium salts HOTU (**13**) and TOTU (**14**) [3,8,10]. The recent exhaustive reconsideration of Oxyma as an additive to carbodiimides revealed an improved suppression of stereomutation and coupling yields than HOBt (**1**) and often comparable with HOAt (**2**). In addition, Oxyma offers a much safer decomposition behavior than benzotriazoles [10]. Moreover, the success of Oxyma (**15**) prompted the introduction of several derivatives for peptide bond formation, which were equal or superior to benzotriazolic counterparts [11,12]. 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate (COMU **16**, Figure 2) can be rapidly accessed from dimethylcarbamoyl chloride and Oxyma (**15**) in a few steps. It is the most powerful representative of the Oxyma-based family of coupling reagents and is now commercially available from numerous suppliers [13]. The fast



X=CH; Y=H; HOBt (**1**)
X=N ; Y=H; HOAt (**2**)
X=CH; Y=Cl; 6-Cl-HOBt (**3**)

X=CH; Y=H; Z=PF_6; HBTU (**4**)
X=N ; Y=H; Z=PF_6; HATU (**5**)
X=CH; Y=Cl; Z=PF_6; HCTU (**6**)
X=CH; Y=H; Z=BF_4; TBTU (**7**)
X=N ; Y=H; Z=BF_4; TATU (**8**)
X=CH; Y=Cl; Z=BF_4; TCTU (**9**)

X=CH; Y=H; HDMB (**10**)
X=N ; Y=H; HDMA (**11**)
X=CH; Y=Cl; HDMC (**12**)

Z=PF_6; HOTU (**13**)
Z=BF_4; TOTU (**14**)

Figure 1. Structure of the main additives and aminium/uronium salts currently employed in peptide chemistry.

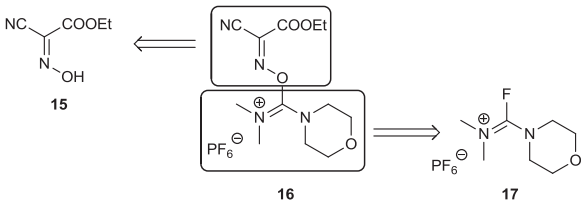


Figure 2. Structural design of COMU from Oxyma and the corresponding fluoroformadinium salt.

and extensive recognition of COMU (**16**) in a few years has led to several systematic studies by other groups in the peptide and organic synthetic community. In this review, we provide a critical evaluation of the performance of COMU (**16**), on the basis of available literature reports.

The design of COMU (**16**) brings together the excellent leaving group ability of Oxyma (**15**) and the markedly reactive dimethylmorpholino core contained in *N*-(chloro)morpholino) methylene)-*N*-methylmethanaminium hexafluorophosphate (DMCH **17**) (Figure 2) [7,10,13]. The advantageous properties of the morpholine moiety observed in benzotriazole aminium salts were also noted in the comparison between HOTU (**13**) and COMU (**16**) [7,13]. Thus, the inclusion of the dimethylmorpholino skeleton in COMU (**16**) resulted in 50% higher solubility in DMF than HOTU (**13**) [13]. In addition, COMU (**16**) also allowed the preparation of approximately four times more concentrated solutions than benzotriazolic HBTU (**4**) and HATU (**5**) in DMF, with direct implications in coupling efficiency [13]. The remarkable solubility properties of COMU in organic solvents used in peptide synthesis were confirmed by White and co-workers, showing also a significant improvement over HDMC (**12**), due to the contribution of Oxyma (**15**) [14]. Furthermore, solution-phase acylations with COMU (**16**) are facilitated by the great water solubility of its by-products, such as the corresponding urea, deriving in cleaner workup removal in contrast to benzotriazole-based reagents [13,15,16]. An additional distinctive feature of COMU (**16**) is that its coupling reactions can be monitored by a change of color of the solution, which depends on the base used, thus indicating the end point [13,15].

Above all qualities of COMU (**16**) as peptide coupling reagent is its pronounced reactivity, which in many cases supersedes that of the benzotriazole-based analogs [13]. Apart from the effect by the Oxyma (**15**) and the morpholino moieties, X-ray and ^{13}C-NMR studies proved that COMU (**16**) exists as uronium salt (i.e., *O*-form). Thus, it belongs to a more reactive subfamily than the aminium salts (*N*-form) such as HBTU (**4**), HATU (**5**), HCTU (**6**), TBTU (**7**), TATU (**8**), TCTU (**9**), HDMB (**10**), HDMA (**11**), and HDMC (**12**) [13,17]. This structural feature may be partially responsible for the markedly high acylation rate both in solution and solid-phase synthesis. Experimental observations indicate that the advantage of COMU (**16**) and Oxyma-containing reagents over other reagents increases with the sterical hindrance at the coupling junction. This could be due to the flexibility of Oxyma (**15**) in contrast to the rigidity of the benzotriazolic moiety [13]. A crystal clear example is provided during assembly of Fmoc-Val-Val-NH_2 in solution (Table 1) [13]. Thus, whereas COMU (**16**) accomplishes quantitative conversion in 1 h (Entry 3), HATU (**5**) renders 94% (Entry 1) of the dipeptide after 2 h [13]. In addition, COMU (**16**) also offers slightly faster peptide bond formation than HDMA (**11**, Entry 5) and HOTU (**13**, Entry 6). Most remarkably, COMU (**16**) can be efficiently used in presence of only 1 equiv. of base, as a result of the hydrogen-acceptor nature of the morpholine ring [7,13]. Hence, COMU (**16**) achieves higher conversion under these conditions in 30 min than

Table 1. Extent of coupling over time during formation of Fmoc-Val-Val-NH_2 using various coupling reagents and amounts of base in DMF[a]

Entry	Coupling reagent	Base (equiv.)	Coupling Time (min)				
			10	20	30	60	120
1	HATU (**5**)	DIEA (2)	87.6	90.5	92.5	93.0	94.0
2		DIEA (1)	76.0	80.0	82.0	82.0	83.0
3	COMU (**16**)	DIEA (2)	96.0	98.0	98.5	100.0	100.0
4		DIEA (1)	86.0	90.1	94.5	96.0	98.0
5	HDMA (**11**)	DIEA (2)	95.0	96.4	98.0	99.0	99.0
6	HOTU (**13**)	DIEA (2)	89.0	91.0	93.0	94.0	96.0

[a]Table shows percentages of target dipeptide, as detected by HPLC. See reference [13] for more experimental and analytical details.

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HATU (**5**) in 2 h using 2 equiv. of base (Entries 1 vs. 3) [13]. A similar tendency on the relative coupling extent was observed in the formation of Z-Aib-Val-OMe in solution and of acyl carrier protein (65–74) and Aib-enkephaline peptides in solid-phase [13]. Hence, COMU (**16**) has become a preferred peptide coupling reagent in many research groups.

The possibility of using COMU (**16**) without excess base is an attractive methodology to reduce the degree of stereomutation in peptide couplings, which is a dramatic side reaction with severe implications in peptide purity and activity [3,18]. The optical purity of peptides synthesized by COMU (**16**) is much higher than HOBt-analogs in fragment couplings and even superior to HATU (**5**) in stepwise models [3]. Thus, using 2 equiv. of DIEA in the assembly of Z-Phg-Pro-NH_2, COMU (**16**) gives only rise to 0.12% of DL epimer, whereas HBTU (**4**) and HATU (**5**) render 8.2 and 3.1%, respectively [3]. In [2 + 1] fragment couplings, COMU (**16**) offers the lowest percentage of epimerization (30.5 and 90% yield) when 1 equiv. of 2,4,6-trimethylpyridine is employed [3]. The outstanding ability of COMU (**16**) to reduce loss of configuration has been confirmed recently by other authors [15]. The combination of COMU (**16**) and Oxyma (**15**) is an alternative to maintain acceptable epimerization levels, which can also be used to decrease the risk of guanidylation. Moreover, COMU (**16**) and Oxyma (**15**) mixtures are reported to display higher acylation capacity than HDMA (**11**)/HOAt (**2**) and HCTU (**6**)/HOAt (**2**) [19,20].

In the peptide field, COMU (**16**) is compatible with a broad range of solid supports, either PS-based or PEG-based, and the assembly of Boc-amino and Alloc-amino acids, where it is preferred over HCTU (**6**) [13,15,19,21–29].

However, the application of COMU (**16**) is not merely limited to peptide manufacture but also extends to general amide and ester synthesis. The outstanding acyl transfer capacity of COMU (**16**) has been successfully applied in recent years in amide bond formation. Indeed, COMU (**16**) is able to modify sensitive scaffolds such as 2′,3′-diisopropropylidene-protected 5′-modified nucleosides with fluorinated, constrained, cyclic, and chiral amines, showing chemoselectivity with other functional groups [30]. COMU (**16**) has also been employed in C-terminal amidation in excellent yields without need of purification [31]. Moreover, COMU (**16**) has been described to display substantially higher acylation rates than HATU (**5**) in solution and solid-phase approaches towards *p*-arylopeptoid and *m*-arylopeptoid and also during screening of green solvents for amide bond formation [25,26,32]. Successful Weinreb amidation of several Boc-amino acids (63–97% yield) and aliphatic acids has been recently achieved [15,33]. In addition, the use of COMU (**16**) in bioconjugation, anilide, and PNA-based construct formation has been described in numerous occasions [27,34–39].

In the last decade, traditional methods of ester bond formation are being replaced by milder strategies, such as peptide coupling reagents. In the last couple of years, COMU (**16**) has been explored in alcohol acylation, particularly challenging due to their poor nucleophilicity and competition of moisture. For example, the use of COMU (**16**) is preferred over HATU (**5**) in the solution-phase *N*-acylation and *O*-acylation of ethanolamines, providing complete acylation in 3 to 6 h (85–90% yield)[16]. Similarly, Cao and colleagues reported the superiority of COMU (**16**) over HBTU (**4**) in fatty acid ester bond formation [40]. In addition, Twibanire and colleagues performed an exhaustive screening of substrates, observing that the performance of COMU (**16**) in comparison with TBTU (**7**) and TATU (**8**) increased with the bulkiness of the alcohol [41]. Thus, whereas the benzotriazole reagents rendered acyl transfer to primary and secondary alcohols (except TBTU **7**, in the case of secondary alcohols) faster than COMU (**16**), the Oxyma-based reagent was the only activator displaying acylation of tertiary alcohols such as tert-butyl alcohol [41].

Furthermore, there is no need for acid preactivation when using COMU (**16**), in contrast to the 30-min preincubation time that benzotriazole analogs demand [41]. With concern to safety measures required when handling the coupling reagent, COMU (**16**) implies considerably less thermal risk than other related activators based on benzotriazoles, such as HDMA (**11**) or HDMB (**10**), apart from not displaying dangerous autocatalytic decompositions [13].

Limitations

However, although freshly prepared solutions of commercially available COMU (**16**) are very effective in promoting amide bond formation, this may not be the case for solutions that have been stored for some time. The latter may occur for manual synthesis using stock solutions or on automated synthesizers. Nowadays, a considerable number of routine Fmoc-based or Boc-based peptide syntheses are conducted on automated robots, either conventional or microwave-assisted, in which solutions of coupling reagents are employed and stored for several days. Thus, hydrolytic stability is a critical issue in automated peptide elongation, where the coupling reagent should remain functional for an average of at least 1 day per run. The original report published by our group in 2009 showed that the COMU (**16**) synthesized in-house on a laboratory-scale is able to remain fully stable 1 day, showing only 6% degradation after 2 days in an open vial (Table 2)[13]. In contrast, 48 h after preparation of the sample, 84% of non-morpholine-containing HOTU (**13**) was left in solution, whereas benzotriazole-based HBTU (**4**) and HATU (**5**) were present in 86 and 76%, respectively [13]. Nonetheless, to our astonishment, large-scale prepared COMU (**16**) lacks the extraordinary hydrolytic stability observed when obtained in our hands. Recently, White and co-workers conducted an NMR-based stability assay of various coupling reagents, finding that after 24 h, more than 50% of COMU (**16**) was hydrolyzed, and 1 day later, degradation was almost complete (Table 2) [14]. Conversely to our study, COMU (**16**) was much less stable than other Oxyma-based reagents (TOTU, **14**) and specially, HBTU (**4**) and HATU (**5**), which were barely degraded after 2 days (Table 2) [14]. However, a similar study carried out on a closed vial (mimicking the actual conditions on a peptide synthesizer) displayed that, although still unacceptable, the stability of COMU greatly increased (67% left after 48 h)[14]. In spite of this poor stability, COMU (**16**) provided a higher content of *N*-MeLeu-enkephaline pentapeptide in an ABI433A peptide synthesizer than HBTU (**4**), HCTU (**6**), and HDMC (**12**) (40 vs. 4, 25, and 21%, respectively), although it could not improve the results obtained by HATU (**5**, 77%) [14]. Similar conclusions were obtained in a stability assay performed by one of our groups, where 85% of COMU (**16**) remained after storage for 23 h as a DMF solution in a closed vial while it had completely hydrolyzed after 5 h in an open vial [41]. Also, H-YGGFL-NH_2 was synthesized with short coupling times using stock solution of COMU (**16**) kept in either open or closed vials. As expected, a fresh solution provided the peptide in an HPLC purity of 96%. The synthesis with a 24-h old stock solution stored in an open vial gave no trace of the peptide, whereas a stock solution kept in a closed vial for 24 h provided a 65% HPLC purity of the peptide. A very recent publication from our groups compared COMU (**16**) and HATU (**5**) for the difficult acylation of

Table 2. Reported hydrolytic stability of COMU and other coupling reagents in DMF[a]

Coupling reagent	Solvent conditions (%)	Method of analysis	Stability (%)					Ref
			5 h	8 h	1day	2day	7day	
HATU(**5**)	Regular DMF	HPLC	99	—	95	76	—	[13]
HBTU(**4**)	Regular DMF	HPLC	100	—	98	86	—	[13]
HOTU(**13**)	Regular DMF	HPLC	100	—	95	84	—	[13]
COMU(**16**)[b]	Regular DMF	HPLC	100	—	100	93	—	[13]
HATU(**5**)	DMF-d^7	^{1}H-NMR	—	—	98	97	—	[14]
HBTU(**4**)	DMF-d^7	^{1}H-NMR	—	—	99	99	—	[14]
TOTU(**14**)	DMF-d^7	^{1}H-NMR	—	—	72	42	—	[14]
COMU(**16**)	DMF-d^7	^{1}H-NMR	—	—	47	14	—	[14]
HATU(**5**)[c]	DMF-d^7	^{1}H-NMR	—	—	—	99	99	[14]
HBTU(**4**)[c]	DMF-d^7	^{1}H-NMR	—	—	—	100	98	[14]
TOTU(**14**)[c]	DMF-d^7	^{1}H-NMR	—	—	—	89	85	[14]
COMU(**16**)[c]	DMF-d^7	^{1}H-NMR	—	—	—	67	46	[14]
COMU(**16**)[c]	Regular DMF	HPLC	53	23	0	—	—	[43]
COMU(**16**)[c]	Anhydrous DMF	HPLC	62	48	10	—	—	[43]
COMU(**16**)[c]	Treated DMF[d]	HPLC	78	76	62	—	—	[43]

[a]Unless otherwise stated, the reagent was stored in an open vial.
[b]In-house prepared sample
[c]Experiments conducted in closed vial.
[d]Regular DMF stored under 4 Å molecular sieves and subjected to N_2 aspiration for 2 to 3 h.

N-methylated peptidyl-resins using microwave heating. By using freshly prepared solutions of reagents, COMU (**5**) in general outperformed HATU [43].

Traces of synthetic intermediates in multi-kg scale synthesis, such as the fluoroformamidinium salt or morpholine (in the range 3–5% as detected by ^{1}H-NMR spectroscopy), may be responsible for such distinct behavior, even observed among different batches. Next, we investigated the effect of DMF purity on the stability of a particularly unstable sample (Table 2) [43]. Thus, although anhydrous solvents decreased the degradation rate, the most striking improvement was accomplished when fully removing secondary amines [44]. Hence, a simple treatment consisting in solvent aspiration under N_2 stream for 2 to 3 h, prior to adding molecular sieves, is enough to remove volatile secondary amines coming from DMF and substantially increase COMU (**16**) stability (Table 2) [44].

Several automated peptide synthesizers are combined with microwave irradiation, thereby allowing precise control of temperature to accelerate peptide bond formation and protecting group removal, which results in rapid synthesis of even difficult sequences [44]. One of the prominent representatives is the SyroWave™ synthesis robot (Biotage AB), featuring a vortex mixing within the microwave cavity and the possibility of parallel synthesis at room temperature [45–47]. Besides hydrolytic degradation, COMU (**16**) could theoretically face thermal and chemical stability issues when employed in conjunction with MW-assisted peptide robots. From a thermal point of view, the relatively low decomposition onset of COMU (**16**) leads to the establishment of a 41 °C barrier, temperature that is recommended not to surpass according to calorimetric standards [13]. However, an exhaustive study proved that the risk of thermal runaway ending in explosion of COMU (**16**) is relatively low compared with benzotriazoles [13]. In fact, the Oxyma-based reagent is safely dried for many hours at 60 °C and has been driven to temperature values of 60 to 80 °C in many syntheses described later in the text without incident [24,29,41,42,46–49]. Chemically, working at high temperatures could not affect the reagent's stability (acid activation with COMU **16**, takes place almost instantly) but that of

Table 3. Hydrolytic stability of COMU under closed vial in different solvents and in the presence of various additives[a]

Coupling reagent	Solvent	Stability (%)								
		5 min	30 min	1 h	4 h	8 h	1day	2day	3day	6day
COMU(**16**)	DMF[b]	87	86	86	85	84	77	72	65	55
COMU(**16**) + Oxyma(**15**)		92	89	86	80	77	71	—	—	—
COMU(**16**)	NMP[c]	87	87	87	85	84	83	80	76	75
COMU(**16**)	DMF/toluene 1:1	84	83	80	72	—	—	—	—	—
COMU(**16**)[b]	NMP/ACN[d] 1:1	87	87	86	84	82	78	71	—	—

[a]0.5-M solutions of COMU in each solvent are prepared. A 2-ul aliquot is taken, diluted with 1 ml of ACN and analyzed by HPLC using an XBridge BEH C18 (3.5 μm, 4.6 × 100 mm) column, with a 5 to 60% gradient of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min (detection at 220 nm; flow = 1.0 mL/min)
[b]Regular DMF (peptide synthesis grade, 99.9%) stored under 4 Å molecular sieves and subjected to N_2 aspiration for 2-3 h.
[c]N-methyl-2-pyrrolidone anhydrous 99.5% (Sigma-Aldrich)
[d]ACN, acetonitrile

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the activated species. Thus, one of our groups determined that the half-life of a Boc-Ala-Oxyma ester generated with COMU (**16**) was less than 4 min at 75 °C, whereas at that temperature Boc-Ala-OBt and Boc-Ala-OAt esters had half-lives of 24 and 28 min, respectively [41]. However, in spite of the short half-life at elevated temperature, the remarkably high reactivity of Oxyma esters often provided efficient peptide bond formation under microwave heating. Thus, the efficiency of COMU (**16**) will depend on the kinetics of particular couplings. Moreover, one can speculate that the sterical hindrance of the Fmoc group could increase the half-life of Oxyma esters of Fmoc-amino acids.

Indeed, one of our groups proved that COMU (**16**) can give rise to similar percentages of the Jung–Redemann decapeptide (H-WFTTLISTIM-NH_2) than HATU (**5**) on a SyroWave™ synthesis at 75 °C (56 vs. 60%) [41]. However, because coupling reagent bottles in this case were left exposed to air humidity, a stock solution of COMU (**16**) kept for 4 h prior to peptide assembly did not afford even traces of the decapeptide [41]. An exhaustive investigation on the effects of residual water in DMF proved that although anhydrous batches of the solvent mitigate COMU (**16**) degradation, avoiding contact with air moisture in a closed vial is pivotal [41]. Hence, by using a stock solution of COMU (**16**) stored in DMF for 24 h in a closed vial, similar performance than an HBTU (**4**) fresh sample was obtained (45%) [41]. Nonetheless, by using a fresh sample of COMU (**16**) on an MW-assisted synthesizer, our research and that of other laboratories confirm the superiority of the Oxyma-based reagent over HOBt-based and HOAt-based analogs, with few exceptions [24,29,42,46–49]. In detail, a COMU-mediated approach to Aib-enkephalin pentapeptide using 6-min couplings at 80 °C afforded 92% of the target product, superseding the coupling extent of HBTU (**4**, 23%) and HATU (**5**, 79%)[29]. One of our groups also analyzed the relative efficiency of COMU (**16**) versus HATU (**5**) in Aib-Aib and Ala-*N*-(Me)Ile coupling junctions at 75 °C, in which COMU (**16**) outperformed the HOAt-based uronium salt [42,49]. Similarly, COMU (**16**) gave rise to the highest content of the abovementioned Jung–Redemann sequence with neglectable racemization using a fresh solution in DMF (70 vs. less than 50% for HATU **4**, HBTU **5**, and HCTU **6**) [46,47]. Furthermore, COMU (**16**) was the preferred coupling choice in a microwave-assisted synthesis at 50 °C of RGD-containing peptides [24].

A drawback of COMU (**16**), like for other aminium/uronium salts, is that it is not suited for markedly slow couplings, such as cyclizations, because the *N*-terminal guanidylated peptide is obtained as a major product [50]. With regard to the efficiency of COMU (**16**) under fast acylation protocols, contradictory studies have been published [28, 51]. On one hand, the Alewood group found no differences in coupling efficiency between COMU (**16**), HBTU (**4**), and HCTU (**6**) in Boc-SPPS using the *in situ* neutralization method and 1-min couplings, regardless of the type of resin used (although COMU **16** performed better in PEG-resins than PS-ones) [28]. On the other hand, Chantell and colleagues compared various coupling reagents on an Fmoc-SPPS-based synthesizer under fast protocols (2×1 and 2×20-min couplings), where COMU (**16**) was more efficient than HATU (**5**), HCTU (**6**), and HDMC (**12**) in four out of five peptide models tested [51]. An explanation could be that Boc-amino acids are less bulky than Fmoc-ones, and consequently, the sterical factor which is favorable to Oxyma-based reagents in Fmoc-SPPS, is not decisive in Boc-based approaches.

In the past months, advances on the large-scale production process of COMU (**16**) have resulted in a significant increase on the hydrolytical stability of a sample that was tested in our laboratory. By using amine-free DMF, obtained after a simple process, the content of COMU (**16**) 8 h after sample preparation rose from 76 to 84% in comparison to an old batch, and from 62 to 77% after 1 day, a much more acceptable degradation rate to carry out automated synthesis (Table 3). Even after 3 days of preparing the COMU (**16**) solution, 65% of unmodified reagent was left in the bottle. In addition, the effect of additives and other solvents was also investigated. The 1:1 presence of Oxyma (**15**) seemed to increase COMU (**16**) stability shortly after sample preparation, but after 1 h, the mixture did not improve previous results (Table 3) [10]. However, the use of N-methyl-2-pyrrolidone (NMP) as solvent clearly translated into a slower degradation rate in comparison with DMF. Hence, after 1 day, more than 80% of COMU remained unmodified, whereas after 6 days, there was 75% of the reagent left (Table 3). The addition of other solvents to DMF and NMP, such as toluene or acetonitrile, did not provide further improvement.

Conclusions

The implementation of Oxyma-based coupling reagents, and specially its most powerful analog COMU (**16**), represented a groundbreaking innovation in methodology of peptide bond formation. The enhanced solubility of COMU (**16**) in organic solvents, together with the facile removal of its by-products during aqueous workup, possibility of color-monitoring and combination with 1 equiv. of base results in an attractive coupling choice in solution. Moreover, the outstanding epimerization suppression and coupling efficiency of COMU (**16**) has proven superior to the previous standard in the field, HATU (**5**), in numerous syntheses. In addition, COMU (**16**) is well suited for peptide synthesis with microwave heating. Unfortunately, the hydrolytical stability of commercial samples of COMU (**16**) limits its application in automated robots featuring open reagent vials to short synthesis. Nonetheless, COMU (**16**) can be efficiently combined with peptide synthesizers as long as the reagent is stored in closed vials and certain tips are followed, such as simply processing regular DMF to remove secondary amines or choosing NMP as solvent. Under these conditions, the content of functional COMU (**16**) in solution guarantees successful syntheses even if 1-day stored solutions are employed, although fresh samples are recommended. Thus, to become the ultimate acylating reagent in peptide synthesis, the purity of commercial COMU (**16**) must reach that of the lab-scale produced material.

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