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Effect of Residual Water and Microwave Heating on the Half-Life of the Reagents and Reactive Intermediates in Peptide Synthesis

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Abstract: Precise microwave heating has changed the way many small molecules are being synthesized and, currently, the field of solid-phase peptide synthesis is undergoing dramatic changes owing to the use of microwave heating. To fully reap the benefits of precise microwave heating for the formation of amide bonds in peptide synthesis, it is important to understand the kinetics of formation and break-down of activated esters and their N-acylation of the nascent peptide chain at elevated temperatures. Herein, we present systematic studies of, first, the rate of formation of activated esters by NMR spectroscopy and, second, their N-acylation during peptide synthesis. A study of the amount of residual water in the solvents revealed a significant effect on electrophilic reagents and intermediates. This observation was expanded into a general study of microwave heating in peptide synthesis.

Keywords: microwave chemistry · NMR spectroscopy · peptides · reactive intermediates · water

Introduction

The organic chemistry behind solid-phase peptide synthesis (SPPS) is highly refined and often very efficient. Amide-bond formation in SPPS requires, first, a suitable electrophile that reacts with the carboxylic acid of the "incoming" amino acid to form a reactive activated-ester intermediate.[1,2] Then, this activated ester will react with the N-terminal amine of the nascent peptide. However, besides the main reaction, a number of competing inter- or intramolecular side-reactions can occur. Despite the evident successes of SPPS, this technology is still limited by the length of the sequences that can be synthesized and by the problems in assembling so-called "difficult sequences". Precise microwave heating has emerged as a new, powerful tool in SPPS and has been crucial for the synthesis of otherwise difficult peptides and long sequences.[3] However, raising the coupling temperature from ambient to, for example, 75 °C may also promote side-reactions, especially related to the activated ester, as well as the potential for epimerization or intramolecular side-reactions. To utilize elevated temperatures in SPPS, there is a need to understand its effect on the N-acylation reaction versus the side-reactions, which requires an understanding of the half-lives of the key intermediates. This vital information has so far been missing, but herein, we describe the rate of formation of activated esters from several electrophiles at different temperatures, as well as their half-lives.

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Carbodiimides, such as DIC, are commonly used electrophiles in combination with nucleophiles, such as 1*H*-benzotriazole and its analogues.[2] DIC-mediated coupling reactions require the presence of an additive, such as HOBt, to suppress undesired side-reactions, such as racemization and the formation of *N*-acylisoureas. However, the potentially explosive properties of 1*H*-benzotriazole-based additives, that is, HOBt and HOAt, have limited their accessibility owing to transport restrictions.[4] The most-common in situ coupling reagents in SPPS are based on 1*H*-benzotriazoles, that is, HBTU and HATU, which both contain the electrophile and the nucleophile auxiliary.[1] HATU is the most-efficient coupling reagent of the two; however, HBTU is considerably less expensive and often sufficiently effective.

Over the last decade, there has been an increased focus on developing new and safe coupling reagents for SPPS that are not based on the benzotriazole skeleton. However, only a few of these reagents have been shown to equal the efficiency of HATU. The immonium-based coupling reagent, COMU, which contains an ethyl-2-cyano-2-(hydroxyimino)acetate (Oxyma) skeleton, was recently reported to be an efficient coupling reagent for amide-bond formation and is now also commercially available.[5–7] COMU has been reported to provide superior coupling efficiency compared to HBTU and comparable coupling efficiency to HATU during standard automated SPPS, as well as in automated microwave-assisted SPPS, without an increased level of racemization.[6,8,9] Furthermore, the non-explosive additive Oxyma has also recently been reported to be a viable alternative to the 1*H*-benzotriazole-based additives (HOBt and HOAt) in carbodiimide-mediated coupling reactions, with epimerization levels that are comparable to those obtained with HOAt.[7]

An ensemble of different aminium- and immonium-type coupling reagents that combine two different electrophilic

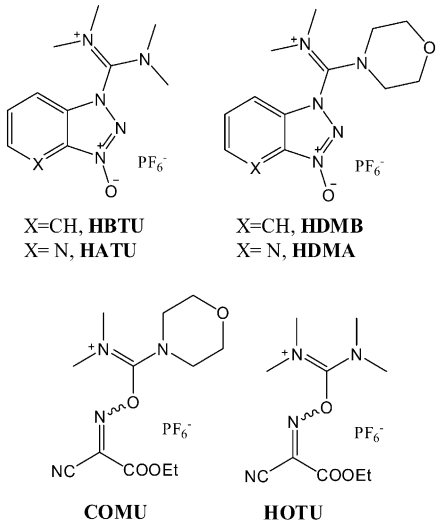


Figure 1. Six coupling reagents that combine two different iminium moieties and three different leaving groups (HOBt, HOAt, and Oxyma).

moieties and three different leaving groups have previously been published (Figure 1).[5–10] HDMB, HDMA, and HOTU[1] are not currently commercially available and, consequently, these were not tested in this study. It has previously been reported that the morpholinium-based iminium moiety affords coupling reagents with superior coupling efficiency and higher solubility. Moreover, they were reported to have a higher hydrolytic stability in both open- and closed vials.[5,11] However, these conclusions are compromised by the results presented herein in which NMR spectroscopic analysis shows a very low hydrolytic stability of COMU in DMF compared to HBTU and HATU. The stability of HBTU and HATU, amongst others, has also been investigated previously;[10–12] these reports showed that, in particular, HBTU is relatively stable in solution and, therefore, very suitable for automated synthesis. The stability of coupling reagents in solution is one of the key considerations when shifting from manual to automated SPPS. Because SPPS was originally developed mainly for reactions at ambient temperature, with only occasional cooling or heating in special circumstances, it is important to understand the stability of the activated esters at different temperatures.

Herein, we present a comprehensive study of the rate of formation of activated esters in SPPS and their half-lives, both at ambient and elevated temperatures. Moreover, the role of residual water in the solvents was studied. This study was complemented with studies of the hydrolytic stability of COMU, HATU, and HBTU in solution. Finally, the stability of these reagents was correlated with their efficiency in the synthesis of difficult peptide sequences by SPPS with microwave heating.

Results and Discussion

During automated peptide synthesis, stock solutions of the coupling reagents in DMF or NMP are often used for several days without being replaced. First, we analyzed the hydrolytic stability of HBTU, HATU, and COMU in [D_7]DMF by ^{1}H NMR spectroscopy at room temperature (Figure 2 and Table 1). COMU was almost completely hydrolyzed after

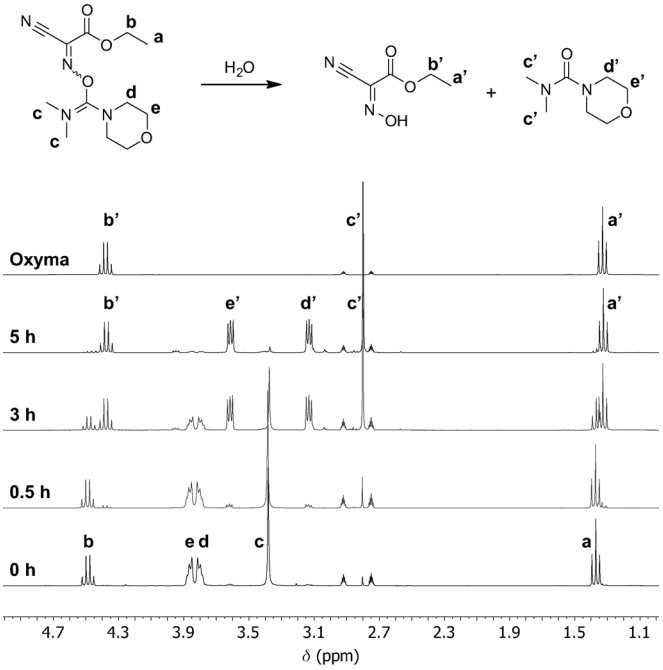


Figure 2. Stability of COMU in an open vial, studied by ^{1}H NMR spectroscopy.

Table 1. Hydrolytic stability of the coupling reagents in [D_7]DMF[a] in open vials.

Coupling reagent[b]	Half-life ($t_{1/2}$)
HBTU	5.1 days
HATU	3.2 days
COMU	3.0 h

[a] Fresh [D_7]DMF from a closed-glass ampulla had a water content of 100 ppm, as estimated by the Carl Fischer test. [b] Concentration: 0.13 M.

only 5 h in DMF (starting water content: 100 ppm H_2O) in an open vial; however, in a closed vial, just 15% had decomposed after 23 h (data not shown). In comparison, less than 5% of HBTU and HATU in open vials had been hydrolyzed after 24 h. These results show that benzotriazole-based coupling reagents have much better storage stability in solution in the open reagent flasks that are used in some synthesizers. As expected, HBTU is also more stable in closed reagent flasks, which are used in some automated synthesizers, compared to COMU.

To evaluate the compatibility of HBTU, HATU, and COMU with automated microwave-assisted SPPS, a model peptide was synthesized by using stock solutions of HBTU, HATU, and COMU. The Jung and Redemann (JR) H-WFTTLISTIM-NH_2 10-mer peptide was chosen as a test sequence.[13,14]

This sequence has previously been used as a test sequence within our group, where it was synthesized in crude purities of 40–70%, depending on the coupling reagent and the conditions.[9,15,16] The JR sequence is a particularly difficult sequence to synthesize and, therefore, optimized conditions are crucial. The synthesis was performed on a Biotage Syro*Wave*™ instrument, which is an automated *x*–*y* robotic peptide synthesizer with microwave heating (with open coupling reagent flasks, although inert gas can be applied) and vortexing of the reactor inside the microwave cavity. The overall synthesis time was 4 h and 36 min, which was within the time-frame of the stability of the coupling reagents (HBTU and HATU). The use of fresh stock solutions of the three different coupling reagents gave, on average, peptide purities for the JR sequence of 46% with HBTU, 56% with COMU, and 60% with HATU. This result clearly showed an advantage of HATU and COMU over HBTU, with HATU providing a slight advantage over COMU in this particular case. In this series of test experiments (Table 2), the DMF that was used for solubilizing the amino acid derivatives had a water content of approximately 400–700 ppm unless otherwise stated.

When the stock solutions of the coupling reagents were left for 24 h in an open vial, the peptides that were synthesized with HBTU solutions gave comparable peptide purity levels to those prepared with a fresh coupling solution. A similar experiment with HATU showed a decrease in peptide purity from approximately 60 to 52% when using a fresh stock solution of HATU compared to a 24 h old solution (Table 2). The decrease in purity was, to a certain extent, accounted for by the formation of an increased amount of deletion peptides. This result appears to correlate with the stability/lability of the electrophilic coupling reagents in the stock solutions, as shown by ^{1}H NMR spectroscopy (Table 1). Attempts to synthesize the JR peptide with a >24 h old COMU solution gave neither the desired peptide nor any deletion sequences (Figure 3 and Table 2). In our hands, not even a relatively fresh 4 h old COMU solution could provide the desired peptide or any deletion peptides. Again, this result was reflected in the ^{1}H NMR studies, which showed very little intact COMU after 5 h (8%; Figure 2). These findings appear to be somewhat inconsistent with previously reported data on the hydrolytic stability of COMU.[5,17] Therefore, we determined the water content of different DMF solutions (Table 3) and found that our standard DMF had a water content of approximately 450 ppm immediately after opening the bottle, 700 ppm 24 h after opening (stored in a closed vial), and 5000–18000 ppm when opened regularly over the course of a week. This increased water content affects the half-life of the reagent solution negatively and decreases the coupling efficiency.

Table 2. Synthesis of the JR sequence by using standard 0.44 M solutions of the coupling reagents in DMF (open vial).

Entry	Coupling reagent	Time in solution [h][a]	HPLC [purity][b][%]			
			JR[e]	DesThr[e]	DesPhe[e]	DesTrp[e]
1	HBTU/HOBt	0	46	5	10	10
2	HBTU/HOAt	0	45	8	9	9
3	HBTU	0	46	6	9	9
4	HBTU	24	46	7	10	9
5	HBTU	96	30	5	15	16
6	HATU/HOAt	0	60	3	6	7
7	HATU	0	60	2	7	7
8	HATU	24	52	3	7	10
9	HATU	48	0	trace	trace	trace
10	COMU/Oxyma	0	56	5	8	8
11	COMU/HOAt	0	56	5	9	7
12	COMU	0	56	3	8	7
13	COMU	4	0	0	0	0
14	COMU	24	0	0	0	0
15	COMU[d]	4	52	4	9	7
16	COMU[d]	24	45	4	7	7
17	COMU[d]	96	0	trace	trace	trace
18	COMU (anh. DMF)[c]	0	55	4	9	8
19	COMU (anh. DMF)[c]	4	39	7	10	10
20	COMU (anh. DMF)[c]	24	0	0	0	0
21	DIC/Oxyma	0	58	4	8	5
22	DIC/Oxyma	96	54	5	9	8
23	DIC/Oxyma (without DIEA)	0	57	5	8	7
24	DIC/HOAt (without DIEA)	0	34	10	18	3

[a] If nothing else is stated, the solutions were kept in an open vial in standard DMF (water content approx. 400–700 ppm). [b] These are the average peptide purities obtained from two different syntheses with two independent cleavages/releases from the solid support. [c] Anhydrous DMF, water content 40–60 ppm. [d] Closed vial. [e] JR = JR sequence; DesThr = H-WFTLISTIM-NH_2, DesPhe = H-WTTLISTIM-NH_2, DesTrp = H-FTTLISTIM-NH_2.

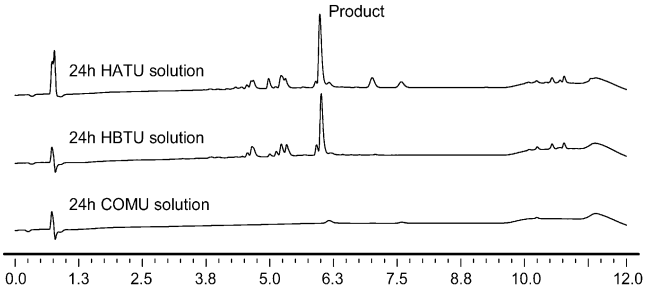


Figure 3. HPLC chromatograms of the different peptides (JR sequence, Table 2).

Table 3. Water content (Carl Fischer) of DMF in bottles.

DMF	Fresh [ppm]	24 h after first opening [ppm]	Regularly opened during a week [ppm]
standard DMF	450	700	5000–18000
anhydrous DMF	50	60	70
[D_7]DMF (glass ampules)	100–130	n.a.[a]	n.a.[a]

[a] n.a. = not available.

An "anhydrous" solution of DMF with a water content of 40–60 ppm was also tested in the synthesis of the JR sequence. Interestingly, under these conditions, a 4 h old stock solution of COMU in "anhydrous" DMF gave a peptide purity of 39% compared to zero for a similar synthesis that was performed with our standard DMF. Nevertheless, a stock solution of COMU in anhydrous DMF that was stored for 24 h in an open vial had decomposed, owing to an increase in the water content. This result was supported by the attempted synthesis of the JR sequence, which, in all cases with 24 h old solutions of COMU in open vials, gave neither product nor deletion products. It was evident from these experiments that the specific water content in the DMF that was used to dissolve COMU had a great impact on the hydrolytic stability of COMU. Even with relatively anhydrous DMF, the activity of the COMU solution decreased quickly in an open vial. Experiments in which the stock solution of COMU had been stored in a closed vial prior to the synthesis showed a higher stability with respect to hydrolysis. However, the hydrolytic stability depended on the initial water content of the DMF solution. SPPS is often, especially manually, performed in open reactors and with open reagent containers, as well as wash solutions.

Next, the decrease in the coupling efficiency of a COMU solution was studied by investigating its use in solid-phase peptide synthesis. Leu-enkephalin (H-YGGFL-NH_2), which is an endogenous ligand for the opioid receptor, was chosen owing to the ease with which it can be synthesized, with a coupling time as short as 5 min at RT, thus giving an overall synthesis time of 84 min on the Syro*Wave*™. The experiments were performed by using a stock solution of COMU in standard DMF (400–700 ppm) in both open- and closed vials (Table 4). Two time-course experiments were performed in which the syntheses were repeated every 2 h with the same stock solution of COMU. The results of this experiment supported the previously obtained results that the coupling efficiency of the COMU solution is maintained longer in a closed vial compared to an open vial, although the purity of the crude peptides synthesized still decreased over time. In an open vial system, a decrease from 96 to 62% peptide purity was observed over the course of 6 h, whereas the same decrease (96 to 65%) was observed over the course of 24 h in a closed-vial system.

Table 4. Synthesis of H-YGGFL-NH_2 by using COMU in DMF.

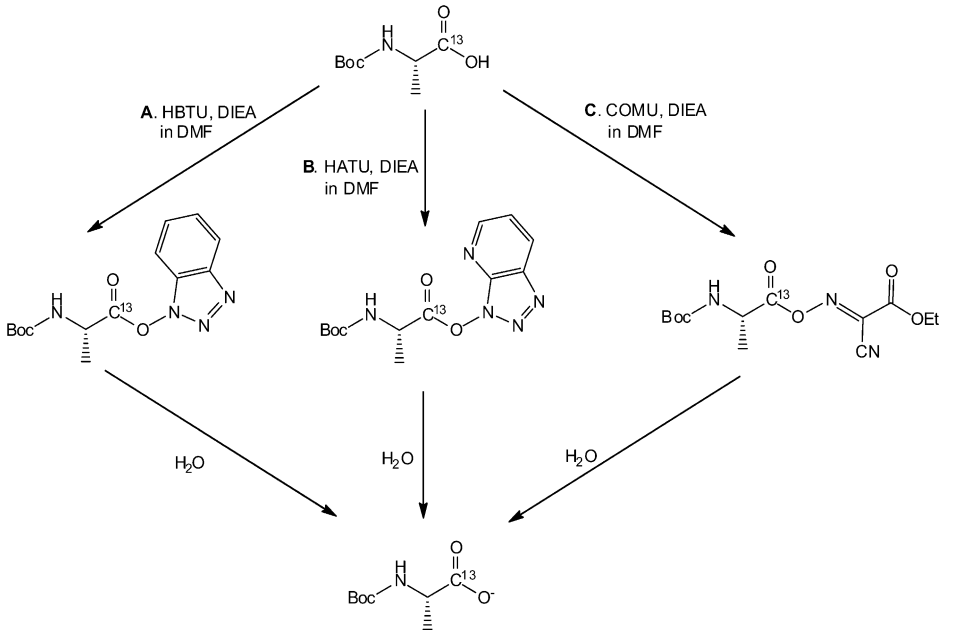
Entry	Time in solution [h]	HPLC purity [%] Open vial	Closed vial
1	0	96	96
2	2	94	91
3	4	78	91
5	6	62	91
6	24	0	65
7	48	n.a.[a]	0

[a] n.a. = not available.

When HBTU and HATU (which contain one molecule of HOBt and HOAt, respectively) were originally evaluated for use in SPPS, the use of additional HOBt or HOAt was recommended. However, in recent years, this idea has been questioned and, in some cases, these additives have been omitted without any reported decrease in coupling efficiency.[18] We decided to assess the effect of these additives on our test system (Table 2).

HBTU, HATU, and COMU were studied with their corresponding additives and no obvious differences in peptide purities were observed for the synthesis of the difficult JR peptide sequence. Furthermore, the combination of HBTU with HOAt, which could generate both OBt- and OAt esters, was tested with no observed benefit in the peptide purity of the obtained JR sequence. In the synthesis of a longer sequence, this result may be different owing to a higher demand on coupling efficiency. Also, the quantified yield has not been determined. Furthermore, we wanted to investigate how comparable the DIC-mediated coupling reactions were to HBTU, HATU, and COMU. Generally, the use of an additive in carbodiimide-mediated coupling reactions is recommended owing to an decrease in coupling times, the suppression of racemization, and an inhibition of the dehydration of the carboxamide side-chains of Asn and Gln.[19,20] The acceleration in coupling rates are caused by the formation of the corresponding OBt, OAt, or Oxyma esters when DIC is mixed in an equimolar amount with HOBt, HOAt, or Oxyma, respectively. Interestingly, we found that DIC-Oxyma-mediated couplings gave overall comparable peptide purities to COMU and slightly lower than HATU (Table 3). We performed the synthesis with and without the addition of *N*,*N*-diisopropylethylamine (DIEA) but found no differences in the coupling efficiency. We compared these results to the DIC-HOAt-mediated coupling reactions and found a significant advantage in the use of Oxyma as an additive instead of HOAt in our test system. Furthermore, no significant decrease in peptide purity was observed after Oxyma and DIC had been stored (separately) as solutions in DMF in an open vial for up to 5 days. This result was opposed to that with COMU, which, as mentioned above, was very unstable in DMF (open vial 4–5 h) and HATU, which could be stored for approximately 24–48 h. In terms of current prices, HBTU is the least expensive, followed by the combination of DIC and Oxyma, whereas HATU is the most expensive.[21]

These results revealed a relatively low hydrolytic stability of COMU in solution compared to the other coupling reagents. We were interested in investigating whether the stability of the corresponding activated esters exhibited a similar pattern and how these activated esters were affected by the increased temperatures used in microwave-assisted SPPS. The stabilities of the activated esters of HBTU, HATU, and COMU were investigated by NMR spectroscopy by using ^{13}C-labeled Boc-Ala-OH (Scheme 1). The ^{13}C-labeled amino acid and the coupling reagent were dissolved in [D_7]DMF and the reaction was initiated by the addition of DIEA. The sample was pre-heated to the specified tempera-



Scheme 1. ^{13}C-labeled Boc-Ala-OH was treated with HBTU, HATU, or COMU (1–1.1 equiv) in [D_7]DMF. All of the Ala carbon atoms were ^{13}C isotopes but the carbonyl carbon atom is emphasized here. The reaction was initiated by the addition of DIEA. The decomposition of the active ester was promoted by the excess water in the DMF.

tures inside the NMR instrument and time-course experiments were performed. All three carbon atoms in Boc-Ala-OH were ^{13}C isotopes; however, only the NMR resonance signals of the carbonyl groups were used for analysis of the different species and intermediates. The ^{13}C resonance signals for the carbonyl groups were all doublets owing to the $^1J(C_\alpha,COO)$ coupling (average values: acid 53 Hz, ester 60 Hz, and carboxylate 75 Hz). A change in the chemical shifts from the carboxylic acid ($\delta = 175.7$ ppm) to the activated esters ($\delta = 172.7$ [OBt], 171.6 [OAt], and 169.8 ppm [Oxyma]) were observed (Figure 4). Upon decomposition of the activated esters into the carboxylate, a shift to $\delta =$ 175.8 ppm was observed.

The decomposition of the active esters of HBTU, HATU, and COMU at three different temperatures was fitted to a pseudo-first-order reaction, assuming a large excess of water. The water content of [D_7]DMF was approximately 100 ppm, as determined by the Carl Fischer test, which was an approximated 60–80 times excess of water relative to the electrophilic coupling reagent. The resonance signals for the ester were integrated and the natural logarithms (ln) of these values were plotted as a function of time. The half-life ($t_{1/2}$) was determined by using the measured rate constants (Table 6).

The current standard temperature for most microwave-assisted peptide coupling reactions is 75 °C. We found that, at 75 °C, the half-life of Boc-Ala-Oxyma was very short. The first data point after the addition of DIEA that was obtained from the NMR spectrum was at 6 min. Already, at this time point, there was no trace of the signal that corresponded to the carbonyl group on the Oxyma-ester. In comparison, the HOBt ester and the HOAt ester had a half-life of approximately 24–28 min at 75 °C. At 40 °C, the half-life of the Oxyma ester was approximately 77 min, although it was 3–3$^1/_2$-times lower than the HOAt- and the HOBt esters. At room temperature, all three activated esters had relative long half-lives from 10 h up to 38 h.

After discovering the instability of COMU in solution, we decided to test the use of DIC-Oxyma and found, as explained above, that DIC-Oxyma was a very efficient coupling reagent and, in addition, very stable in solution. In the DIC-Oxyma-mediated coupling reactions, the Oxyma ester is formed via an *O*-acyl-isourea intermediate, which then reacts

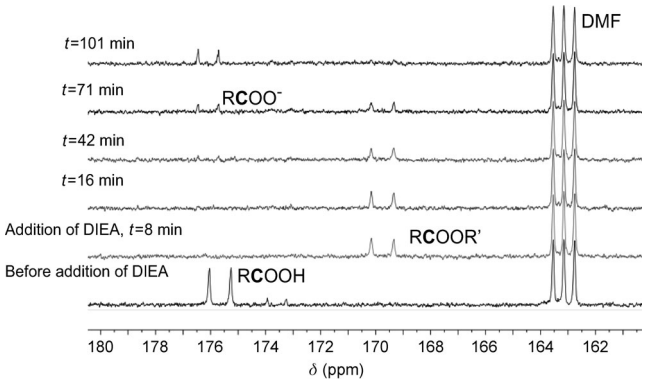


Figure 4. Decomposition of the ^{13}C-labeled Boc-Ala-Oxyma ester at 40 °C. The first data set was obtained after 8 min and then every 3 min for 3 h; only a selection of the spectra are shown.

with Oxyma to form the active Oxyma-ester. However, NMR studies that were performed on ^{13}C-labeled Boc-Ala-OH at 40 °C with Oxyma and DIC in [D_7]DMF showed that the formation of the active Oxyma ester was slower than similar experiments with COMU. The *O*-acyl-isourea intermediate was not observed by NMR spectroscopy, probably owing to its transient nature. When 2 equiv of DIC were used, the formation of the Oxyma-ester was complete after 1$^1/_2$–2 h, compared to almost-instantaneous formation when using COMU (and DIEA). The half-life of the Oxyma ester apparently also increased to approximately 4 h compared to 77 min for the experiment that was performed with COMU (Table 5). This increase could be due to the presence of excess DIC, which will reform the ester after hydrolysis into

Table 6. Percentage coupling (75 °C, 20 min) of Fmoc-Aib-OH onto resin-bound H-Aib-Ile-Asp(O*t*Bu)-Tyr(O*t*Bu)-Ile-Asn(Trt)-Gly.

Fmoc-Aib-OH	HATU [%]	COMU [%]	DIC-Oxyma [%]
1.1 equiv	34	43	48
4.0 equiv	72	87	90

the carboxylate. An exact half-life could not be obtained because the Oxyma-ester was never fully formed before it started to decompose. However, the DIC-Oxyma-mediated coupling is active in a similar time-frame as the HBTU- and HATU mediated couplings.

These NMR studies inspired a series of experiments in which a peptide was synthesized under different reagent conditions to study whether DIC-Oxyma could be a more-efficient coupling reagent for standard SPPS, as well as in microwave-assisted SPPS. We tested the performance of COMU and HATU versus DIC-Oxyma in a "difficult" coupling process. The incorporation of two sequential Aib residues into a peptide sequence is well-recognized as a difficult coupling process and COMU has previously been shown to be a potent reagent for such a coupling.[8] Herein, we measured the incorporation of Fmoc-Aib-OH onto H-Aib-Ile-Asp(O*t*Bu)-Tyr(O*t*Bu)-Ile-Asn(Trt)-Gly-resin by using 20 min microwave-assisted coupling reactions at 75 °C with either 1.1 or 4 equiv of amino acid and coupling reagent (Table 6). The degree of conversion by using HATU versus

Table 5. Half-life ($t_{½}$) for the degradation of the activated esters in [D_7]DMF.

Activated ester	75 °C	40 °C	RT
Boc-Ala-OBt	28 min(±2 min)	4.5 h(±15 min)	38.3 h(±30 min)
Boc-Ala-OAt	24 min(±2 min)	4.5 h(±15 min)	26.7 h(±30 min)
Boc-Ala-oxyma	<4 min	77 min(±4 min)	10 h(±30 min)

COMU was in agreement with previous data reported for another peptide sequence.[8] However, the use of DIC-Oxyma showed higher peptide purities than coupling with COMU and considerably higher than coupling with HATU. DIC-Oxyma activates the amino acids at a slower rate, and thus the duration in which the un-reacted Oxyma and DIC is in solution without being activated is longer compared to COMU. The incorporation is higher when using DIC-Oxyma because the reagents are more stable in solution compared to COMU, but also, the activated esters of DIC-Oxyma have longer apparent half-lives; hence an increased incorporation of Aib was observed when using DIC-Oxyma. These data are in agreement with our finding that COMU is less stable in solution and that the activated Oxyma ester that is formed during coupling has a very short half-life. This result is in contrast to the high stability of DIC and Oxyma in solution and the slower rate by which the activated Oxyma-ester is formed.

Conclusions

Herein, we have addressed several key questions related to peptide synthesis: 1) the effect of residual water on the stability of different electrophiles that are used for the formation of active esters, 2) the rates of formation and subsequent hydrolysis of these activated esters, and 3) the effect of temperature on (1) and (2), as well as on the outcome of solid-phase peptide synthesis. All of the coupling reagents and activated amino acid esters in SPPS are electrophiles and are prone to reaction with water. However, the amount and role of water in the commonly used solvents for SPPS appears to have been underappreciated, with concentrations in the range 50–18,000 ppm. We investigated and compared the stability of HBTU, HATU, and COMU in DMF. We found that there were some inconsistencies with other literature reports with regard to the hydrolytic stability of COMU in solution. Our findings point to the varying water content of different DMF solutions as an explanation for these inconsistencies. We observed that the hydrolytic stability of COMU in DMF was approximately 5 h in an open vial, which makes this coupling reagent unsuitable for automated SPPS with open vials. HBTU and HATU showed a significantly higher hydrolytic stability than COMU, but the coupling efficiency of HBTU was lower. The hydrolytic stability of COMU was increased by using anhydrous DMF, albeit it was still completely hydrolyzed within 24 h in an open vial. The stability of COMU was increased in closed vials, which makes it better suited as a coupling reagent in closed-vial synthesizers or synthesizers with inert-gas options. Thus, COMU can still be one of the most efficient in situ coupling reagents when used under conditions to prevent its hydrolysis.

Furthermore, we investigated the stability of the activated esters that were formed from HBTU, HATU, and COMU and discovered that the activated Oxyma ester that was derived from COMU had a half-life below 4 min at 75 °C. As an alternative, we tested DIC-Oxyma and showed that this coupling reagent provided a high coupling efficiency, relatively low cost (compared to HATU), and high hydrolytic stability in DMF, which makes this a good choice for automated SPPS.

We believe that these findings will help optimize the chemistry for the assembly of difficult peptide sequences, as well as for small proteins.

Experimental Section

General: Analytical HPLC was performed on a Dionex Ultimate 3000 with Chromeleon 6.80SP3 software. Peptides were analyzed on a Phenomenex Jupiter 300 Å C4 column (5 μm, 4.6×150 mm) or a Phenomenex Gemini 110 Å C18 column (3 μm, 4.6×50 mm) at a flow of 1 mL min^{-1} with a linear gradient with increasing amount of buffer for 10–20 min. Buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in CH_3CN. MS (ESI) was performed on an MSQ Plus Mass Spectrometer, Thermo. NMR spectra were recorded on a Bruker Avance spectrometer (^{1}H frequency 300.13 MHz, ^{13}C frequency 75.47 MHz) that was equipped

with a 5 mm BBO probe; the spectra were processed with MestReNova version 6. Chemicals were purchased from Sigma–Aldrich, Fluka, Novabiochem, Iris Biotech GmbH, or Cambridge Isotope Laboratories. N^{α}-Fmoc amino acids contained the following side-chain protecting groups, unless otherwise stated: Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu), Fmoc-Glu(O*t*Bu), Fmoc-Gln(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*Bu), Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp-(Boc)-OH, and Fmoc-Tyr(*t*Bu)-OH. A standard Fmoc-RAM-TG resin (Rapp Polymer GmbH; loading: 0.23–0.24 mmol g^{-1}) was used as the preferred resin. Peptide synthesis was performed on a fully automated peptide synthesizer from Biotage AB (Syro *Wave*™).

Solution stability studies

NMR spectroscopy: The stability of HBTU, HATU, and COMU were measured as 0.13 M solutions in [D_7]DMF by ^{1}H NMR spectroscopy. The open-vial solutions were stored in 4 mL HPLC vials (no stirring) between measurements and the closed-vial solutions were stored in the NMR tube between measurements, both at RT.

General procedure for the synthesis of H-WFTTLISTIM-NH$_2$ (JR sequence, Table 2): In all cases, the peptide syntheses were performed on Fmoc-RAM-TG resin on a 0.05 mmol scale with different coupling reagents, as shown in Table 2. N^{α}-Fmoc deprotection was performed in two stages: 1) piperidine in DMF (2:3) for 2 min at 60 °C and 2) piperidine in DMF (1:4) for 2 min at 60 °C. This deprotection was followed by washing the resin with NMP (4×45 s). The coupling reactions were performed by using N$^{\alpha}$-Fmoc amino acids (5.2 equiv, 0.5 M), coupling reagents HBTU, HATU, COMU, or DIC (5 equiv, 0.44 M) in DMF, and DIEA (10.4 equiv, 2 M) in NMP. Additives, such as HOBt, HOAt, and Oxyma, were added to the amino acids stock solution (5.2 equiv, 0.5 M) when required. In all cases, the coupling reactions were performed for 5 min at 75 °C, followed by washing the resin with NMP (3×45 s). The overall synthesis time was 4 h and 33 min for each peptide. The peptide was released from the resin by using TFA/TES/H_2O (95:2.5:2.5) for 2 h at RT. The crude peptide was precipitate with Et_2O (when possible) and analyzed by LCMS with a C18 column. MS (ESI): *m*/*z* calcd for $C_{58}H_{90}N_{12}O_{14}S$ (average): 1211.5 Da [*M*+H]$^+$; found: 1211.6.

General procedure for the synthesis of H-YGGFL-NH$_2$: The peptide synthesis was performed on Fmoc-RAM-TG resin on a 0.05 mmol scale on a Biotage Syro*Wave*™. The first N^{α}-Fmoc deprotection was performed manually with piperidine/DMF (1:4) for 2+10 min followed by washing with NMP. The resin was then placed inside the microwave cavity of the Syro *Wave*™ and the automated peptide synthesis was started with a coupling time of 3 min and N^{α}-Fmoc deprotection for 2+2 min at 60 °C with piperidine/DMF (1:4). The coupling reactions were performed by using N^{α}-Fmoc amino acids (5.2 equiv, 0.5 M), COMU (5 equiv, 0.44 M) in DMF, and DIEA (10.4 equiv, 2 M) in NMP. All of the coupling reactions were performed by using a stock solution of COMU, which was dissolved at the start of the first synthesis and placed in an open vial. The resin was replaced before the start of each synthesis: t=0, 2, 4, 6, and 24 h after COMU was dissolved in DMF. Each peptide synthesis took 1 h and 24 min to complete. A similar experiment was performed in which the stock solution of COMU was placed in a closed vial. The experiment was then started at t=0, 2, 4, 6, 24, 48 h, and 6 days after COMU was dissolved in DMF. The peptides were released from the resins by using TFA/TES/H_2O (95:2.5:2.5) for 2 h at RT. The crude peptides were precipitate with Et_2O and analyzed by LCMS with a C18 column. MS (ESI): *m*/*z* calcd for $C_{28}H_{38}N_6O_6$ (average): 554.6 Da [*M*+H]$^+$; found: 555.5.

Aib coupling reactions: The relative conversion of Aib onto resin-bound H-Aib-Ile-Asp(O*t*Bu)-Tyr(O*t*Bu)-Ile-Asn(Trt)-Gly was performed in a Biotage Initiator at 75 °C for 20 min. The resin-bound H-Aib-Ile-Asp-(O*t*Bu)-Tyr(O*t*Bu)-Ile-Asn(Trt)-Gly peptide was synthesized by using conventional SPPS. Fmoc-Aib-OH was coupled by using either HATU, COMU, or DIC-Oxyma (1.1 or 4 equiv) on a 0.025 mmol scale. During the HATU- and COMU-activated coupling reactions, DIEA (4.4 or 8 equiv) was used. The DIC-Oxyma coupling reactions were performed without the use of base. Subsequently, the resin was washed (3×NMP, 3×CH_2Cl_2, and 3×NMP), the Fmoc group was removed by using 20% piperidine in DMF (2×5 min), and finally another wash cycle was performed as above. The peptides were released from the resin by using TFA/TES/H_2O (95:2.5:2.5) for 2 h at RT. The crude peptide was precipitate with Et_2O and analyzed by LCMS with a C18 column. MS (ESI): *m*/*z* calcd for $C_{39}H_{62}N_{10}O_{12}$ (average): 863.0 Da [*M*+H]$^+$; found: 863.0.

Variable-temperature NMR experiments

Sample preparation: ^{13}C-labeled Boc-Ala-OH (5 mg, 0.026 mmol) and the coupling reagent (0.029 mmol) were dissolved in [D_7]DMF (1 mL) and transferred into a 5 mm NMR tube. The samples were placed in the NMR spectrometer and heated to the appropriate reaction temperature. Each sample was locked, tuned, matched, and shimmed manually before acquiring a ^{13}C NMR spectrum that represented time point t, while these solution were stable. The sample was subsequently ejected from the NMR instrument and DIEA (8.9 μL, 0.052 mmol) was added. The time was started upon the addition of DIEA and the sample was re-inserted into the magnet and allowed a short time for temperature equilibration and shimming. The total time for ejection of the sample, addition of DIEA, and re-insertion was about 30–45 s; thus, the reaction temperature was considered to be relatively stable during this short interval. ^{13}C-spectra were acquired with 64 transients and fixed receiver gain for all samples, by using a ^{13}C experiment with power-gated decoupling, 30° flip angle, 64k spectral width, and 3 s repetition time to allow for full relaxation. Experiments were repeated with intervals of 3, 5, 30, 60, or 120 min (starting with shortest values at the beginning of the reaction) to allow for the acquisition of sufficient data points to characterize the reaction.

Acknowledgements

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[1] Abbreviations for the coupling reagents: *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU), 1-hydroxybenzotriazole (HOBt), 1-hydroxy-7-azabenzotriazole (HOAt), diisopropylcarbodiimide (DIC), 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate (COMU), 1-[(dimethylamino)-(morpholino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (HDBA), 1-[(dimethylamino)(morpholino)-methylene]-1*H*-[1,2,3]triazolio-[4,5-b]pyridinium hexafluorophosphate 3-oxide (HDMA), *O*-[cyano(ethoxy-carbonyl)-methylidene]amino-1,1,3,3-tetramethyluronium hexafluorophosphate (HOTU).
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[21] Estimated prices of the coupling reagents that are needed for one coupling of 0.1 mmol peptide with 5 equiv coupling reagent: HBTU 0.015 €, HBTU/HOBt 0.02 €, HATU 0.76 €, HATU/HOAt 0.80 €, COMU 0.34 €, COMU/Oxyma 0.37 €, DIC/Oxyma 0.046 €.

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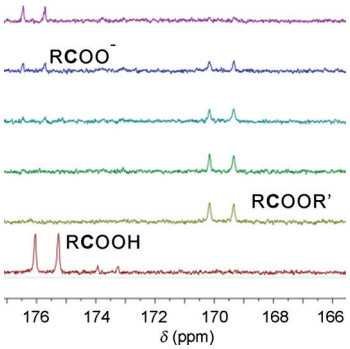
Peptide couplings: Systematic studies of the rate of formation of activated esters by NMR spectroscopy (see figure) and their N-acylation during peptide synthesis were performed by using ^{13}C-labeled Boc-Ala-OH. Study of the amount of residual water in the solvents revealed a significant effect on the electrophilic reagents and intermediates.



Peptides