COMU: A Safer and More Effective Replacement for Benzotriazole-Based Uronium Coupling Reagents**

Ayman El-Faham,*[a, b, c] Ramon Subirós Funosas,[a, d] Rafel Prohens,[e] and Fernando Albericio*[a, d, f]

Abstract: We describe a new family of uronium-type coupling reagents that differ in their iminium moieties and leaving groups. The presence of the morpholino group in conjunction with an oxime derivative—especially ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma)—had a marked influence on the solubilities, stabilities, and reactivities of the reagents. Finally, the new uronium salt derived from Oxyma (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.

Keywords: coupling reagents · Oxyma · peptides · solid-phase synthesis · uronium salts

Introduction

Peptide synthesis is based on an appropriate combination of protecting groups and a suitable choice of coupling method. Nowadays, almost all peptide bonds are formed in the presence of 1-hydroxybenzotriazole (HOBt, 1, Figure 1, left)[2] or its derivatives (HOAt, 2; 6-Cl-HOBt, 3). HOBt derivatives are therefore either used in combination with a carbodiimide or another coupling agent or are built into a stand-alone reagent such as an immonium/HATU (4), HBTU (5), HCTU, (6), Figure 1, right) or phosphonium (PyAOP, PyBOF, PyClock) salt.[3, 4] An oxime salt consists of two parts: a leaving group (YL) and the iminium moiety (Figure 1, center).

Recently we showed that the incorporation of a hydrogen bond acceptor in the iminium part resulted in performances superior to those described previously.[5] As reported in our previous work, the presence of an oxygen in the iminium...
moiety confers more solubility on the reagent, enhances coupling yields, and decreases racemization, thereby allowing the use of just 1 equiv of base. HDMA (7), HDMB (8), and 6-HDMCB (9) are thus more efficient in terms of coupling efficiency and reduction of racemization than their counterparts HATU (4), HBTU (5), and HCTU (6). Importantly, 6-HDMCB (9), which is consistently superior to HDMB (8), often performs in a similar manner to HATU (4), which is one of the most powerful commercially available immonium coupling reagents known to date.[7] It is important to note that all these reagents exist in their N-forms,[8] which are less reactive than the O-forms (Figure 2).[9]

Recent reports have confirmed the explosive properties of HOBt derivatives.[10] In our preceding paper,[11] we showed that Oxyma (10, Figure 3) is an excellent replacement for HOBt and its analogues. Here we report a new uranium salt, COMU (11, Figure 3), which represents the combination of a morpholium-based immonium moiety, introduced in our previous work, and Oxyma (10) as leaving group, as a superior and safe coupling reagent for amide formation.

**Results and Discussion**

Scheme 1 shows the uronium salts prepared for the first screening. We tested four distinct oximes (17a–d) and several iminium moieties, including dimethyl-morpholino, pyrrolidino-morpholino, and dimethylpyrrolidino systems, the last of these as a reference for the role of the pyrrolidino moiety. The corresponding unsymmetrical uronium salts were prepared by treating N,N-dialkyl carbamoyl chlorides 12a or 12b with morpholine (13a) or pyrrolidine (13b) to give the urea derivatives 14a–c (Scheme 1). Urea derivatives (14a–c) were treated with phosgene or oxalyl chloride to yield the corresponding chloro salts, which were stabilized by the formation of hexafluorophosphate salts (15a–c). Subsequent treatment with oxime derivatives (17a–d), obtained by nitrosation from the active methylene compounds 16a–d, in the form of their potassium salts or in the presence of Et,N provided the target compounds (18a–l, Scheme 1).

Interestingly, the 13C NMR spectra of these compounds indicated displacements of the carbocationic carbon of 156.11 ppm for HOTU (18a, the hexafluorophosphate counterpart of TOTU, already described in the literature)[12] and 156.14 ppm for COMU (18c). These displacements are consistent with those reported for this kind of compound in the O-form.[13,9] X-ray crystallography confirmed this hypothesis (Figure 4).

![Figure 2. General structures of the dimethyl-morpholino immonium salts, which are superior to their tetramethyl counterparts.](image-url)

![Figure 3. Structures of Oxyma and the new uronium salt.](image-url)

Scheme 1. Procedure followed for the preparation of the oxime-based uronium-type coupling reagents.
To determine the compatibilities of the new coupling reagents with peptide synthesis in both manual and automatic modes, their solubilities and stabilities in solution and in the solid state were examined by $^1$H NMR analysis. The presence of the oxygen in the iminium structure increased the stabilities of the coupling reagent relative to their tetramethyl derivatives (entries 3 vs. 4, Table 1). Furthermore, Oxyma derivatives have greater stabilities than the benzotriazole derivatives HATU (4) and HBTU (5) (Figure 1). All these reagents showed stabilities greater than 95% in a closed vial. These observations are of practical relevance for both solid-phase and solution strategies: when the activation of a carboxylic acid is slow and the coupling reagent is not stable, it is degraded and no longer able to activate the carboxylic function. This feature is crucial in cyclization steps or for segment coupling steps in convergent strategies, in which the excess of the carboxylic function is either absent (cyclization) or low (segment coupling) and couplings are therefore very slow.

Table 2 indicates that the presence of the oxygen atom in the carbon skeleton and the leaving group are of marked relevance for the solubilities of the compounds. Thus, all dimethyl-morpholino derivatives (18c, 18g, and 18l) were more soluble than their tetramethyl counterparts (18a, 18e, and 18k) (Table 2, entries 3 vs. 4, 5 vs. 6, and 7 vs. 8). Furthermore, ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma, 10) derivatives were more soluble than dicyano and cyano-pyridinyl ones (Table 2, entries 3, 4 vs. 5, 6 and 7, 8). Thus, COMU (18c) and HDMODC (18g) were the most soluble. They were used to prepare solutions of up to 1.5 M and showed clearly higher solubilities than the benzotriazole derivatives 4 and 5 (Table 2, entries 4, 6 vs. 1, 2). This increased solubility can be used to prepare more concentrated solutions in order to enhance coupling yields and to facilitate the removal of the excess of coupling reagent and the urea side-products during the workup in a solution-mode approach.

A further characteristic of the Oxyma derivative 18c is that the course of reaction can be followed due to a change in color, which depends on the type of base used. Thus, 2 min after the addition of the coupling reagent, a solution of 18c has turned orange-red when DIEA is used as a base, and pink in the case of TMP. Once the reaction is complete, the solutions become colorless and yellow, respectively (Figure 5).

A preliminary screening on the efficiency of Oxyma-based coupling reagents 18a and 18c, in the coupling of hindered amino acids, was examined with two model systems (Fmoc-Val-OH + H-Val-NH$_2$ and Z-Aib-OH + H-Val-OMe) in solution. The reaction mixtures were followed by HPLC; the $t_{R}$ values for the starting Fmoc-Val-OH and Z-Aib-OH were 20.89 min and 18.25 min, respectively, and those for the products (Fmoc-Val-Val-NH$_2$ and Z-Aib-Val...
OMe) were 19.88 min and 20.90 min, respectively. The Oxyma-based coupling reagents were more reactive than the benzotriazole derivatives (Tables 3 and 4, 18c, 18a vs. 7).

Table 3. Levels of coupling of Fmoc-Val-Val-NH2 with use of different coupling reagents and a range of equivalents of DIEA in DMF as a solvent.[a]

<table>
<thead>
<tr>
<th>time [min]</th>
<th>HATU (4) yield [%]</th>
<th>HDMA (7) yield [%]</th>
<th>COMU (18c) yield [%]</th>
<th>HOTU (18a) yield [%]</th>
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<td>2 equiv 1 equiv</td>
<td>2 equiv 1 equiv</td>
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<tr>
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</table>

[a] HPLC conditions: linear gradient of 10 to 90% CH3CN/0.1% TFA in H2O/0.1% TFA over 30 min, detection at 200 nm. Flow rate = 1 mL min⁻¹.

Column: Waters C18 5 μm, 4.6×150 mm (Waters Dual Wavelength Detector and Waters 717 Plus auto sampler).

Again, the morpholine derivatives were superior to their tetramethyl counterparts (Tables 3 and 4, 18c, 7 vs. 18a, 4). In both cases, COMU (18c) was superior to HATU (4), the most potent of the currently commercially available coupling reagents. This superiority was more remarkable when only 1 equiv of base was used (Table 3 and Table 4), thereby reaffirming the hydrogen bond acceptor role of the oxygen in the morpholine moiety.

Once these encouraging results with the Oxyma-based COMU (18c) had been obtained, a deeper study using several oxime derivatives were carried out. Two model peptides, Z-Phg-Pro-NH2 and Z-Phe-Val-Pro-NH2, were used to study the retention of configuration achieved with the new coupling reagents.[3]

The novel uronium coupling reagents were tested and compared with classical immonium salts (including the benzotriazole derivatives 19, 20, and 21, containing pyrrolidino-morpholino systems, Figure 6) with the aid of these models, which involve stepwise and also [2+1] segment coupling (Tables 5 and 6). For the stepwise coupling of Z-Phg-OH to H-Pro-NH2 to produce Z-Phg-Pro-NH2, the oxime-based COMU (18c), HOTU (18a), HTODC (18e), and HDMODC (18g) gave better conservation of chirality than the benzotriazole-based HATU (4), HBTU (5), HDMA (7), HDMB (8), and 6-HDMCB (9) (Table 5).[7] The dimethyl-morpholino derivative COMU (18c) induced less racemization than other Oxyma derivatives containing different iminium moieties (18a, 18b, 18d) and than other uronium salts containing different oxime substituents (18g, 18l). In the oxime series, the worst results were obtained

Table 4. Levels of coupling of Z-Aib-Val-OMe with use of different coupling reagents and a range of equivalents of DIEA in DMF as a solvent.[3]

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<tr>
<th>time [min]</th>
<th>HATU (4) yield [%]</th>
<th>HBTU (5) yield [%]</th>
<th>COMU (18c) yield [%]</th>
<th>HOTU (18a) yield [%]</th>
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<td>2 equiv 1 equiv</td>
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[a] HPLC conditions: linear gradient of 10 to 90% CH3CN/0.1% TFA in H2O/0.1% TFA over 30 min, detection at 200 nm. Flow rate = 1 mL min⁻¹.

Column: Waters C18 5 μm, 4.6×150 mm (Waters Dual Wavelength Detector and Waters 717 Plus auto sampler).

Table 5. Yields and racemization for the formation of Z-Phg-Pro-NH2 in DMF (solution-phase synthesis).[5]

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<th>Coupling reagent</th>
<th>Base (equiv)</th>
<th>Yield [%]</th>
<th>d,l [%]</th>
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[a] tL and tD forms of the test dipeptide are described elsewhere.[7] The tL values for tL and tD were identified by co-injection with authentic and pure samples of tL HPLC system: linear gradient of 20 to 50% CH3CN/0.1% TFA in H2O/0.1% TFA over 30 min, detection at 200 nm Water Symmetry C18 5 μm 4.6×150 mm, tL = 26.01 min., tD = 27.40 min.
Table 6. Yields and racemization for the formation of Z-Phe-Val-Pro-NH₂ (2+1) in DMF (solution-phase synthesis).[41]

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<th>Coupling reagent</th>
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<th>Yield [%]</th>
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[³] The coupling was performed with Fmoc-Pro-Rink amide-PS-resin and 3 equiv of Z-Phe-Val-OH. 3 equiv of coupling reagent, 6 equiv of base (TMP), and preactivation for 10–30 s in DMF at RT. The peptide was recovered after deblocking with water in TFA (10%) for 1 h at RT. The solvent was removed under vacuum and then washed with hexane. The crude peptide was injected into the HPLC system by using a previously reported method.[39] Extra peaks related to the starting material (Z-Phe-Val-OH) were observed in 3–5% percentages. LLL and LDL forms of the test tripeptide are described elsewhere.[7] Samples were co-injected with authentic and pure samples of LLL.

Table 7. Yields and racemization for the formation of Z-Phe-Val-Pro-NH₂ (2+1) in DMF (solid-phase synthesis).[46]

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</tbody>
</table>

[4] The coupling was performed with Fmoc-Pro-Rink amide-PS-resin and 3 equiv of Z-Phe-Val-OH. 3 equiv of coupling reagent, 6 equiv of base (TMP), and preactivation for 10–30 s in DMF at RT. The peptide was recovered after deblocking with water in TFA (10%) for 1 h at RT. The solvent was removed under vacuum and then washed with hexane. The crude peptide was injected into the HPLC system by using a previously reported method.[39] Extra peaks related to the starting material (Z-Phe-Val-OH) were observed in 3–5% percentages. LLL and LDL forms of the test tripeptide are described elsewhere.[7] Samples were co-injected with authentic and pure samples of LLL.

with the cyano-pyridinyl system (18k, 18l) because of the higher acidity of the corresponding oxime.

For the same model but with the reaction carried out on solid-phase, COMU (18c) gave the best coupling yield, together with levels of racemization similar to those seen with the HOBt derivatives (Table 7).

To check the effectiveness of the new reagents, the demanding Leu-enkephalin derivative H-Tyr-Aib-Aib-Phe-Leu-NH₂[46] was manually assembled on Fmoc-RinkAmide-AM-resin with the use of amino acid/activator (3 equiv), DIEA (6 or 3 equiv) and 30 min coupling times, except in the case of Aib-Aib, for which 1 h double coupling was used. Percentages of incorporation for the coupling of Fmoc-Aib-OH onto the Aib-containing resin were determined by reversed-phase HPLC analysis, after cleavage of the peptide from the resin (Table 8). The best results were obtained with the OxaM-based COMU (18c) and HOTU (18a), with higher percentages of target pentapeptide being obtained during solid-phase assembly of the pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH₂).[38]
obtained than with HDMA (7) or HATU (4). Synthesis with COMU (18c) led to only a 0.26% yield of des-Aib when 2 equiv of DIEA were used, whereas its tetramethyl derivative 18a gave a 1% yield under the same conditions. For a 30 min coupling, 18c gave a 0.14% yield of des-Aib whereas 18a gave a 12.5% yield. This observation indicates that the morpholino moiety increases the reactivities of uronium salts relative to tetramethyl derivatives. These results are consistent with what is discussed above.

In view of the superiority showed by the morpholino-containing uronium salts over their tetramethyl counterparts, the effect of the leaving group was further tested by comparing the benzotriazole-based HDMA (7) and HDMB (8) and the Oxyma-based COMU (18c) in the manual solid-phase assembly of H-Tyr-MeLeu-MeLeu-Phe-Leu-NH$_2$ on Fmoc-RinkAmide-AM-PS-resin. The strategy followed for the assay began with the elongation of resin-bound tripeptide H-MeLeu-Phe-Leu-resin by use of DIC/Oxyma (10) in 30 min couplings. Quantitative yields were verified by means of the Kaiser test for primary amines. After this preliminary step, comparisons were made for the stepwise incorporation of the two last residues, with use of the corresponding immonium/uronium salt and Fmoc-amino acid. Samples were preactivated for 20–30 s with DIEA (2 or 1 equiv relative to uronium salt/Fmoc-amino acid) in order to avoid guanidylation of the growing peptide chain. The coupling times were shortened to 5 min, so that significant differences in the reactivities of the coupling reagents could arise. After cleavage from the resin with 90% TFA/10% H$_2$O and lyophilization, relative performances were checked in terms of percentages of pentapeptide and deletion peptides, as determined by reversed-phase HPLC analysis (Table 9).

The experiments were carried out either in standard (99.8% purity, as determined by GC) or in treated (anhydrous, dried over molecular sieves and bubbled with N$_2$ to remove Et$_3$NH) DMF, as in the rest of experiments, in order to examine the effect of the solvent’s purity (entries 1–6 vs. 7–12). The assay with standard DMF reflected the huge difference in reactivity between HOAt- and HOBt-derived uronium salts, with an impressive—for such demanding conditions—91% yield of pentapeptide being obtained with HDMA (7), whereas HDMB (8) only afforded a poor 7% yield (entries 1, 2). The Oxyma-based COMU (18c) gave a much higher purity than HDMB but, unlike in the previous synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH$_2$, it was far from that afforded by HDMA (43%, entry 3). The experiment was repeated, but with preactivation with only 1 equiv of base, a second equiv being added once the coupling mixture had been added onto the resin, in order to examine whether the base had any effect on the stability of the active ester during preactivation. The results showed no significant variation, except in the case of COMU (18c), for which the yield rose from 43% to 55% (due to the higher rate of coupling for the MeLeu residue), suggesting a high reactivity of the Oxyma-derived active ester (Table 9, entries 4, 5, and 6). The percentage of pentapeptide also increased to the same extent with COMU (18c) when the experiment with 2 equiv DIEA was carried out with treated, instead of standard, DMF, confirming the positive effect of the higher purity of the solvent, although this was not noticeable in the experiments with HDMA and HDMB (Table 9, entries 7, 8, and 9). Finally, an experiment was conducted with only 1 equiv DIEA, displaying the same tendency as observed previously: the performance of COMU (18c) was superior to that of the HOBt derivative but not as potent as that of the HOAt one (Table 9, entries 10, 11, and 12).

The effectiveness of COMU (18c) was compared with that of HOTU (18a) in the synthesis of the common decapeptide model ACP (65–74) on solid-phase (Table 10).[a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling conditions</th>
<th>Coup. reagent</th>
<th>Penta [%]</th>
<th>des-MeLeu [%]</th>
<th>des-Tyr [%]</th>
<th>Trip [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 equiv HDMA</td>
<td>91.4</td>
<td>4.5</td>
<td>3.8</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2 equiv DIEA</td>
<td>7.0</td>
<td>42.2</td>
<td>9.7</td>
<td>41.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>standard COMU</td>
<td>42.9</td>
<td>48.5</td>
<td>4.4</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2 equiv DMF</td>
<td>91.5</td>
<td>5.0</td>
<td>3.1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2 equiv DIEA</td>
<td>6.9</td>
<td>42.4</td>
<td>9.4</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>standard COMU</td>
<td>55.5</td>
<td>39.5</td>
<td>3.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2 equiv HDMA</td>
<td>89.4</td>
<td>6.7</td>
<td>3.6</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2 equiv DIEA</td>
<td>6.8</td>
<td>43.0</td>
<td>9.2</td>
<td>41.0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>treated COMU</td>
<td>56.0</td>
<td>38.5</td>
<td>3.3</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2 equiv DMF</td>
<td>73.7</td>
<td>18.5</td>
<td>7.4</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2 equiv DIEA</td>
<td>5.7</td>
<td>37.8</td>
<td>8.9</td>
<td>47.6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>standard COMU</td>
<td>33.6</td>
<td>51.9</td>
<td>6.3</td>
<td>8.2</td>
<td></td>
</tr>
</tbody>
</table>

[a] HPLC-MS showed the correct mass for the pentapeptide at 695.5. Fmoc-amino acids were preactivated for 20–30 s. [b] Fmoc-amino acids were preactivated with only 1 equiv DIEA, with addition of another 1 equiv onto the resin after the first addition.

The peptide was manually elongated on a Fmoc-Rink Amide-AM-resin (0.7 mmol g$^{-1}$). Coupling times of 2 min were used and excesses of reagents were 2 equiv for coupling reagents and amino acids and 4 equiv for DIEA. Incorporation was detected for Ile$^{72}$ onto Asn and for Ile$^{69}$ onto Asp. Peptide purity was determined by reversed-phase HPLC analysis, after cleavage of the peptide from the resin by treatment with TFA/H$_2$O (9:1) for 2 h at RT. HPLC analysis again showed a better performance of the morpholino-containing derivative than for the tetramethyl analogue: some deletion peptides, such as des-Val, were not observed with COMU (18c) and the percentage of pentapeptide ob-
tained was higher than that obtained with HOTU (18a) (Table 10, entry 1 vs 2).

The safety profile of COMU (18c), the most efficient of the uronium salts tested, was also considered and compared to those of HDMA (7) and HDMB (8). This issue was highly relevant in view of the explosive properties of benzotriazole-based additives and derived stand-alone coupling reagents, which limit their transportation. Although explosivity has never been reported for HDMA (7) or HDMB (8), the fact that they contain HOBt/HOAt and that other immonium salts, such as TBTU, have also shown explosive properties,[15] means that a certain safety risk is assumed. Our main interest was focused on checking whether the novel coupling reagent COMU (18c) would display the common pattern observed in explosive compounds: high release of pressure associated with rapid decomposition.[14]

The thermal risk was assessed through a combination of two calorimetry assays: Differential Scanning Calorimetry (DSC) and Accelerating Rate Calorimetry (ARC). A preliminary assessment of the risk associated with a given decomposition can be determined by means of a dynamic DSC experiment. The heat released during this assay is measured by comparing it with a reference that has undergone the same thermal process. In addition, the relative decomposition kinetics (which are linked to explosivity) are highlighted[16] in this assay, samples are heated in a closed crucible under a flow of N₂ from 30 to 300 °C at a constant heating rate of 10°C min⁻¹. Diagrams displaying the heat flow as a function of time and temperature showed a distinct difference in decomposition behavior between benzotriazole-based HDMA (7) and HDMB (8) and the novel COMU (Figure 7). During the decomposition of HDMA (7) and HDMB (8) the release of heat was slow at the beginning and increased very sharply, reaching a maximum and finally decreasing. This decomposition profile as observed for HDMA (7) and HDMB (8) resembles that of an autocatalytic reaction, in which the product also acts as a catalyst, the rate of the reaction increasing at the same time as the conversion.[17] These self-accelerating reactions warrant special attention because of their great unpredictability, resulting from the starting induction period with no thermal signal and their unexpected steep initiation. Therefore, a temperature alarm in an industrial process is not effective with compounds that show this kinetic behavior. Nevertheless, a dynamic DSC experiment can provide only indications of the autocatalytic nature of decomposition.

In contrast, COMU decomposed in a more constant manner that did not resemble this autocatalytic pattern. The normalized exothermic ΔH values should also be noted: 209 kJ mol⁻¹ for HDMB, 245 kJ mol⁻¹ for HDMA, and 183 kJ mol⁻¹ for COMU. These observations indicate that in the event of an explosion, COMU would have less thermal severity. The ΔTad value (adiabatic temperature rise), calculated from experimentally ascertained exothermic ΔH values, also shows this relative severity: 248°C for HDMB (8), 290°C for HDMA (7), and 214°C for COMU (18c). To conclude with the information that can be extracted from the DSC assay, the onset temperature at which decomposition began was lower in the case of the experiment with COMU (18c) (160°C) than with HDMA (7) or HDMB (8) (177°C and 180°C, respectively).

A further evaluation of the risks associated with these compounds was carried out under adiabatic conditions by the ARC technique.[17] By this approach, the pressure released and the ΔTad can be directly determined. The assay begins with the application of the “heat-wait-seek” method, and when self-heating of the sample at a rate higher than 0.02°C min⁻¹ is detected, the experiment is changed to adiabatic mode. When decomposition occurs, the temperature and the pressure rise, and once the temperature reaches values above 300°C the assay is stopped manually. In all cases the pressure rises detected were relatively low, in comparison with those of related additives.[18] Particularly in the released pressure measured in the COMU (18c) experiment was similar to that seen with HDMA (7) (53 vs. 55 bar) and slightly higher than that seen with HDMB (8) (24 bar) (Figure 8).

Additional, the increase in temperature during decomposition (ΔTad) was considerably lower with COMU (18c) (64°C) than with HDMA (7) or HDMB (8) (164°C and 121°C, respectively), thereby confirming the lower thermal
severity of COMU observed in the DSC assay (Figure 9). Also consistent with the previous assay were the distinct kinetic profiles of COMU (18c) and the benzotriazole-based immonium salts. Whereas the temperatures in the HDMA (7) and HDMB (8) experiments increased slowly at the beginning and rose suddenly to their maxima (similarly to the DSC results, suggesting autocatalytic kinetics), in the COMU (18c) experiment the temperature reached approximately one third of the total ΔTad over a period of time longer than had been required for the whole decomposition of HDMA (7) and HDMB (8). These slower kinetics could enable pressure originating from decomposition to be released into the environment.

With regard to the onset temperatures, ARC allows more accurate determination than DSC, which is known to suffer from uncertainty due to the smaller scale. For COMU (18c), decomposition began at a lower temperature than for HDMA (7) and HDMB (8) (91°C vs. 119°C and 122°C). For safe working, it is recommended that the temperature of a given compound be maintained at values at which the time to maximum rate under adiabatic conditions is longer than 24 h.[19] This temperature can commonly be estimated after running an ARC assay, by subtraction of 50 K from the observed onset.[19] This safety value is at a lower temperature with COMU (18c) than with HDMA (7) and HDMB (8) (41°C vs. 69°C and 72°C). Although peptide chemistry is usually performed at room temperature, and therefore below this safety threshold value, these results show that COMU has less thermal stability than benzotriazole-based immonium salts that also contain the morpholino moiety. The experimentally determined and calculated values determined from calorimetric studies and discussed above are shown in Table 11.

To study further the autocatalytic natures of the decompositions of HDMA (7) and HDMB (8), as suggested by the results obtained from the dynamic DSC and ARC experiments, in contrast with the results obtained for COMU (18c), we performed isothermal DSC assays. This technique is the most reliable way to detect whether decompositions follow autocatalytic or non-autocatalytic kinetics. Temperatures were set at 10°C below the onset observed in the corresponding dynamic DSC assay, and remained constant for 480 min. As a result, we obtained thermograms showing heat flow versus time (Figure 10). As would be expected for an autocatalytic reaction, HDMA (7) and HDMB (8) each defined a bell-shaped heat release curve, reported for this type of kinetics, in which the reaction accelerated, passing through a maximum of heat release and then decreasing. In contrast, COMU (18c) displayed a typical non-autocatalytic, nth-order kinetic profile, in which the rate of heat release decreased uniformly with time.[16] The former distinct kinetic profile strongly suggests the risk of a thermal runaway, which may lead to a sudden explosion, and consequently the safety measures that need to be taken.

### Conclusions

In conclusion, we describe a new class of O-form uronium-type coupling reagents that differ in their immonium moieties and also in the leaving groups. 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. As would be expected, HOAt derivatives were in all cases confirmed to be superior to HOBt ones in terms both of coupling yields and of retention

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**Table 11. Experimentally determined and calculated values obtained from dynamic DSC and ARC assays with the different stand-alone coupling reagents.**

<table>
<thead>
<tr>
<th>Coupling reagent</th>
<th>Onset ΔTad [°C]</th>
<th>DSC ΔH [kJ mol⁻¹]</th>
<th>ARC Δp [bar]</th>
<th>Onset ΔTad [°C]</th>
<th>expΔTad [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDMA (7)</td>
<td>177</td>
<td>245</td>
<td>290</td>
<td>119</td>
<td>55</td>
</tr>
<tr>
<td>HDMB (8)</td>
<td>180</td>
<td>209</td>
<td>248</td>
<td>122</td>
<td>24</td>
</tr>
<tr>
<td>COMU (18c)</td>
<td>160</td>
<td>183</td>
<td>214</td>
<td>91</td>
<td>53</td>
</tr>
</tbody>
</table>

[a] Calculated ΔTad (°C) was obtained from the general formula ΔTad (°C) = ΔH (J mol⁻¹)/[MW (g mol⁻¹)c_p (kJ kg⁻¹°C⁻¹)], with estimated c_p = 2 kJ kg⁻¹°C⁻¹.
Benztiazole-Based Uronium Coupling Reagents

General: TLC was performed on silica plates (8 x 4 cm, Albet) in suitable solvent systems with visualization with a Spectroline Model CM-10 UV lamp (254 nm). Melting points were measured in open capillary tubes with a Buchi B-540 melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded on a Thermo Nicolet series Fourier Transformer instrument as KBr pellets. The absorption bands (Δνmax) are given in wavenumbers (cm⁻¹). A Shimadzu UV-250/PC instrument was used as a UV/Vis spectrophotometer. NMR spectra were recorded on a Varian mercury 400 MHz spectrometer at room temperature. Tetramethylsilane (TMS) was used as reference for all NMR spectra, with chemical shifts reported as ppm relative to TMS. HPLC analyses were carried out with a Waters Symmetry Column C18, 5 μm, 4.6 x 150 mm with dual λ absorbance detector. HPLC-MS analyses were carried out with a Waters Symmetry Column C18, 5 μm, 4.6 x 150 mm with dual detector. All solvents used for recrystallization, extraction, column chromatography, and TLC were of commercial grade, distilled before use, and stored under dry conditions.

N-V-Dimethylmorpholine-4-carboxamide (DMU, 14a) The ura derivative was distilled and collected at 127–129°C as a colorless oil in a yield of 92.4% (73 g from 0.5 mol reaction). 1H NMR (CDCl3): δ = 2.84 (s, 6H; 2CH3), 3.22–3.2 (m, 4H; 2CH2), 3.68–3.70 ppm (m, 4H; 2CH2); 13C NMR (CDCl3): δ = 38.62, 47.51, 51.75, 65.97, 164.96 ppm.

N-V-Dimethylpiperidine-1-carboxamide (DMPyU, 14b) The pure ura was obtained as a colorless oil at 98–100°C in a yield of 92.4% (73 g from 0.5 mol reaction). 1H NMR (CDCl3): δ = 2.84 (s, 6H; 2CH3), 3.22–3.2 (m, 4H; 2CH2), 3.68–3.70 ppm (m, 4H; 2CH2); 13C NMR (CDCl3): δ = 38.62, 47.51, 65.97, 164.96 ppm.

Morphinolphosynridolin-1-ylmethanone (MPyU, 14c) The ura derivative was distilled and collected at 127–129°C as a colorless oil in a yield of 92.4% (73 g from 0.5 mol reaction). 1H NMR (CDCl3): δ = 2.84 (s, 6H; 2CH3), 3.22–3.2 (m, 4H; 2CH2), 3.68–3.70 ppm (m, 4H; 2CH2); 13C NMR (CDCl3): δ = 38.62, 47.51, 65.97, 164.96 ppm.

General Procedure for the synthesis of chlorouronium salts: Oxalyl chloride (100 mmol) in CH2Cl2 (100 mL) was added dropwise to room temperature over 5 min to a solution of ura derivative (100 mmol) in dry CH2Cl2 (200 mL). 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 x 100 mL) and then bubbled with N2 to remove the excess of the ether. The obtained residue was very hygroscopic, so it was dissolved directly in CH2Cl2 and saturated aqueous potassium hexafluorophosphate (100 mmol in 50 mL water, KPF6) was added at room temperature with vigorous stirring for 10–15 min. The organic layer was collected, washed once with water (100 mL), dried over anhydrous MgSO4, and filtered. The solvent was removed under reduced pressure to give a white solid that was recrystallized from CH2Cl2/ether or acetonitrile/ether to give white crystals.

4-[(Dimethylamino)chloromethylene]morpholin-4-amine hexafluorophosphate (DMCh, 15a) The salt was obtained as white crystals in a yield of 89.6% (28.9 g). M.p. 94–95°C; 1H NMR (CD3COCD3): δ = 3.39 (s, 6H; 2CH3), 3.75 (t, 4H; 2CH2), 3.86 ppm (t, 4H; 2CH2); 13C NMR (CD3COCD3): δ = 44.36, 52.82, 65.99, 162.79 ppm.

N-[Chloro(pyrrolidin-1-yl)methylene]-N-methylmethanaminium hexafluorophosphate (DMPyCh, 15b) The product was obtained as a white solid in a yield of 89.0%. M.p. 93–95°C; 1H NMR (CD3COCD3): δ = 2.00–2.13 (m, 4H; 2CH2), 3.49 (s, 6H; 2CH3), 3.90–4.02 ppm (m, 4H; 2CH2).

1-[Chloro(morpholino)methylene]pyrrolidinium hexafluorophosphate (MPyCh, 15c) The chloro salt was obtained by the method described above. The product was obtained as a white solid in a yield of 69.3%. M.p. 99–100°C; 1H NMR (D2O): δ = 2.10–2.13 (m, 4H; 2CH2), 3.87 (t, 4H; 2CH2), 4.00 (t, 4H; 2CH2), 4.04–4.06 ppm (m, 4H; 2CH2); 13C NMR (CDCl3): δ = 25.80, 51.75, 55.97, 65.97, 154.85 ppm; elemental analysis (% calculated for CH5ClNF3O5: C 31.00, H 32.69, N 8.03; found: C 31.00, H 32.69, N 8.31.

General Procedure for preparation of uranium-type coupling reagents: The chloro (20 mmol) was added at 0°C to a solution of an oxime potassium salt or a benztiazole derivative (20 mmol) in acetonitrile (50 mL). The reaction mixture was stirred at this temperature for 30 min and was then heated to room temperature with stirring over 5 min. The crude product was then filtered and washed with acetonitrile. The solvent was concentrated to a small volume (1/4) under reduced pressure, and dry ether was then added to afford the product as a white solid in a pure state.

Synthesis of the potassium salt of hydroxycarbonimidoyl dicyanide (17a) Sodium nitrite (14.2 g, 206 mmol) was slowly added at 0°C (20–30 min addition) to a solution of malononitrite (9.06 g, 138 mmol) in acetic acid (20 mL) and water (50 mL). This mixture was then stirred at the same temperature for 45 min. After quenching with the reaction with HCl (2N, 100 mL), the compound was extracted three times with ether (3 x 100 mL). The extracts were dried over anhydrous Na2SO4, and the ether was removed under vacuum to give an oily residue. The oily product was added slowly to a cold solution of KOH (8.0 g) in MeOH (100 mL), and then the reaction mixture was stirred for 20 min. Excess ether was then added to afford the potassium salt as yellow crystals in a yield of 14.1 g (77.5%). 13C NMR (DMSO): δ = 107.40, 113.57, 119.78 ppm.

Synthesis of the potassium salt of diethyl 2-hydroxyimino)malonate (17e) A solution of diethyl malonate (16 g, 0.1 mol) in glacial acetic acid (17.5 mL, 0.3 mol) was slowly added at 0°C while a solution of NaNO2 (20.7 g, 0.3 mol) in water (250 mL) was added dropwise over 3–4 h. The ice bath was removed and the mixture was stirred vigorously for a further 20 h. The nitrosation was carried out in three-necked flasks with appropriate fittings and a small vent to allow nitric oxide to escape. The reaction mixture was extracted with CH2Cl2 (400 mL) and then 3 x 100 mL portions. The combined CH2Cl2 extracts were dried over anhydrous Na2SO4. The CH2Cl2 was removed under vacuum and the resulting oily product was dissolved in CH2Cl2 (400 mL) and then stirred with anhydrous K2CO3 (32 g) for 15 min. After filtration, the CH2Cl2 was removed until 200 mL was reached. Ether was added until the solution became cloudy, and the mixture was then kept in the refrigerator overnight to afford off-white crystals in 63.4% yield. M.p. 116–118°C; 1H NMR (CDCl3): δ = 1.24–1.29 (q, 6H; 2CH3), 4.20–4.29 ppm (m, 4H; 2CH2).

Synthesis of 2-pyridyldihydroxyiminoacetanilide (17d) A solution of sodium nitrite (4.5 g, 0.065 mol in 5 mL water) was added slowly, with cooling, to a solution of 2-pyridylacetanilide (2.2 g, 0.019 mol) in glacial acetic acid (4.5 mL). After 12 h standing, the precipitate was filtered off, washed with water, dried, and then recrystallized from ethanol to afford
the product in 65% yield. M.p. 220–222°C (lit. mp. 219–222°C, 68%).


General Procedure for the preparation of urea derivatives:

The N,N-dialkylcarbamoyl chloride (0.6 mol) was added dropwise at 0°C to a stirring mixture of secondary amine (0.5 mol) and triethylamine (TEA, 0.5 mol) in CH2Cl2 (400 mL). When the addition was complete, the mixture was stirred for 3–4 h at room temperature. The reaction mixture was basified with NaOH (10%), and the organic layer was then collected and the aqueous layer was washed with CH2Cl2 (100 mL). The combined CH2Cl2 solution was washed with H2O (2×100 mL) and saturated solution of NaCl (2×100 mL). Finally, the organic solvent was dried over anhydrous MgSO4 and filtered, and the solvent was removed under reduced pressure. The crude residue obtained was purified by vacuum distillation.

O-(Cyano(ethoxy carbonyl)methylidene)amino)-1,1,3,3-tetramethyluronium hexafluorophosphate (HOTU, 18a): The product was obtained as a white solid in a yield of 6.32 g (82.1%). M.p. 135–137°C (dec). The triethylamine/HOAc mixture gave a lower yield (68.7%) than the potassium salt strategy, maybe due to the washing with water. 1H NMR ([D 6]acetone): δ = 1.37 (1 H, CH3), 3.37 (s, 12 H; 4 CH2), 4.82 ppm (q, 2 CH2; CH3); 13C NMR ([D 6]acetone): δ = 13.46, 40.71, 64.56, 106.78, 135.09, 156.11, 161.43 ppm; elemental analysis (%) calcd for C12H24F6N3O6P: 386.25; C = 31.10, H = 4.44, N = 14.51; found: C = 31.34, H = 4.35, N = 14.75.

1-(Cyano(ethoxy carbonyl)methylidene)amino)-2-oxo-2-oxazolidinone (18b): The product was obtained as a white solid, in a yield of 6.8 g (82.7%). M.p. 126–127°C (dec). 1H NMR ([D 6]acetone): δ = 1.37 (1 H, CH3), 2.10, 2.13 (m, 4 H; 2 CH2), 3.36 (s, 6 H; 2 CH2), 3.95–3.99 (m, 4 H; 2 CH2), 4.48 ppm (q, 2 CH2; CH3). 13C NMR ([D 6]acetone): δ = 13.47, 25.09, 40.40, 51.58, 64.52, 106.74, 134.82, 156.12, 165.68 ppm; elemental analysis (%) calculated for C9H14F2N2O3P: (328.27) C = 34.96, H = 4.65, N = 13.59; found: C = 35.07, H = 4.79, N = 13.73.

1-(Cyano(ethoxy carbonyl)methylidene)amino)-2-oxo-2-oxazolidinone (18c): The product was obtained as white crystals in a yield of 7.6 g (88.8%), and decomposes without melting at 159.90°C according to dinitamic (Figure 3). 1H NMR ([D 6]acetone): δ = 1.38 (1 H, 3 CH3), 3.41 (s, 6 H; 2 CH3), 3.82 (t, 4 H; 2 CH2), 3.89 (t, 4 H; 2 CH2), 4.48 ppm (q, 2 CH2; CH3). 13C NMR ([D 6]acetone): δ = 13.48, 40.70, 49.94, 65.49, 66.04, 106.76, 135.03, 156.14, 161.60 ppm; elemental analysis (%) calculated for C9H14F2N2O3P: (328.27) C = 33.65, H = 4.47, N = 13.08; found: C = 33.79, H = 4.59, N = 13.30.

1-(Cyano(ethoxy carbonyl)methylidene)amino)-1,3,3-tetramethyluronium hexafluorophosphate (HMPOy, 18d): The product was obtained as white crystals in a yield of 8.2 g (90.3%). M.p. 171–172°C; 1H NMR ([D 6]acetone): δ = 1.26 (1 H, 3 CH3), 1.98–2.02 (m, 4 H; 2 CH2), 3.65–3.68 (m, 4 H; 2 CH2), 3.74–3.76 (m, 4 H; 2 CH2), 3.84–3.87 (m, 4 H; 2 CH2), 4.37–4.40 ppm (q, 2 CH2; CH3); 13C NMR ([D 6]acetone): δ = 13.49, 25.09, 49.20, 51.57, 55.98, 51.76, 64.54, 106.69, 134.63, 156.11, 157.88 ppm; elemental analysis (%) calculated for C9H14F2N2O3P: (454.31) C = 37.01, H = 4.66, N = 12.33; found: C = 37.25, H = 4.78, N = 12.50.

O-(Cyano(ethoxy carbonyl)methylidene)amino)-1,1,3,3-tetramethyluronium hexafluorophosphate (HOTDO, 18e): The product was obtained as a white solid in a yield of 5.0 g (74.0%). M.p. 180–181°C (dec); 1H NMR ([D 6]acetone): δ = 3.27 (s, 12 H; 4 CH2) ppm; 13C NMR ([D 6]acetone): δ = 40.80, 105.10, 108.21, 119.65, 160.67 ppm; elemental analysis (%) calculated for C9H14F2N2O3P (393.18): C = 38.33, H = 3.57, N = 20.65; found: C = 38.32, H = 3.56, N = 20.88.

1-(Cyano(methylidene)amino)-dimethylpyrolydine-5[4,5]-pyridined 3-oxide hexafluorophosphate (HMPyA, 19): The product was obtained as a white solid in a yield of 7.3 g (81.3%). M.p. 206–208°C (dec); 1H NMR ([D 6]acetone): δ = 2.11–2.15 (m, 2 CH3), 2.18–2.30 (m, 2 CH2); 3.48–3.63 (m, 2 CH2); 3.79–4.16 (m, 10 H; 5 CH2); 8.02 (dd, 1 H), 8.53 (dd, 1 H), 8.58 ppm (dd, 1 H); 13C NMR ([D 6]acetone): δ = 25.09, 41.74, 42.35, 50.52, 51.58, 66.19, 66.42, 123.74, 127.84, 149.65 ppm; elemental analysis (%) calculated for C9H14F2N2O3P (448.30): C = 37.51, H = 4.27, N = 18.75; found: C = 37.75, H = 4.42, N = 19.02.

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Benzotriazole-Based Uronium Coupling Reagents

5-Chloro-1-[morpholinopyrrolidinyl-1-ylidene]methyl]-1H-benzo[d]-[1,2,3]triazole 3-oxide hexafluorophosphate (HMPyC, 21): The product was obtained as a white solid in a yield of 7.96 g (82.7%). M.p. 217–218° (dec); 1H NMR (D2DMSO): δ = 2.10–2.15 (m, 2H; CH2), 3.18–3.25 (m, 5H; CH), 3.81–4.09 (m, 10H; CH2), 7.99 (d, 1H), 8.02 (d, 1H), 8.12 ppm (dd, 1H); 13C NMR (D2DMSO): δ = 25.95, 52.89, 53.36, 66.09, 66.42, 115.79, 115.84, 132.57, 132.62, 134.06, 134.12, 147.38 ppm; elemental analysis (% calculated for C15H19ClF6N5O2P (481.76): C 37.40, H 3.98, N 14.54; found: C 37.65, H 3.89, N 14.60.

Synthesis of H-Tyr-MeLeu-MeLeu-Phe-Leu-NH2 on solid phase: Trispeptide H-MeLeu-Phe-Leu-NH2 was manually assembled on Fmoc-RinkAmine-Aminomethyl-PS-resin (0.63 mmol g⁻¹), after Fmoc removal with piperidine in DMF (20%, 2 × 5 min). The resin was washed with DMF (10×), CH2Cl2 (1×), and DMF (1×); after 30 min coupling, with preactivation of Fmoc-amino acids (3 equiv) with Oxyma (3 equiv) and DIC (3 equiv) in DMF for 1.5 min. Quantitative incorporation was checked at each step by use of the Kaiser test for primary amines. Sample cleavage (10 mg) with TFA/H2O (9:1) confirmed the trispeptide in >99.5% purity, as analyzed by reversed-phase HPLC and ESI-MS ([M+H]⁺ = 405.32). The two last residues, Tyr and MeLeu, were introduced by preparation of 0.3 m solutions of coupling reagent (3 equiv) and Fmoc-amino acid (3 equiv) in standard or treated DMF, and preactivation of the mixture with DIEA (3 or 6 equiv) for 20–30 s. The peptide chain was cleaved from the resin with TFA/H2O (9:1) over 2 h at room temperature. The solution was filtered and the resin was washed with CH2Cl2 (1 mL × 2), which was removed together with TFA under nitrogen flow. The crude peptide was purified with cold Et2O (2 mL × 3) and after lyophilization, purity was checked on reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm), with a 20% to 50% linear gradient of 0.036% TFA in CH3CN/0.045% TFA in H2O over 8 min, with detection at 220 nm. The ms of the pentapeptide was 5.0, 5.7, and 3.0 min respectively.

Synthesis of ACP (65–74) (H-Val-Glu-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH2): The pentapeptide was elongated manually on an Fmoc-Rink Amide-AM-resin (0.7 mmol g⁻¹). Coupling times of 2 min were used, and excesses of reagents were 2 equiv for Fmoc-amino acids and coupling reagents and 4 equiv for DIEA. Incorporation was determined for Ile72 onto Asn and for Ile72 onto Asp. Peptide purity was determined by reversed-phase HPLC analysis (Symmetry Waters C18 (4.6 × 150 mm, 4 μm), linear gradient over 30 min of 10 to 90% CH3CN in H2O, 0.1% TFA, flow rate 1 mL min⁻¹, tR decapsidepeptide = 10.43 min, tR Asn = 10.5 min, tR des-Ile72 = 7.5 min, tR des-Ile72−Ile72 = 9.1 min, tR des-Val = 8.43 min), after cleavage of the peptide from the resin by treatment with TFA/H2O (9:1) for 2 h at room temperature.

General Procedure for dynamic differential scanning calorimetry assays: The thermal behavior of HDMA (7b), HDMB (8), and COMU (18c) was tested. Samples (1 mg) were heated from 30°C to 300°C at a rate of 10°C min⁻¹ in a closed high-pressure crucible with N2 flow in a Mettler-Toledo DSC-30 differential scanning calorimeter. Diagrams showing heat flow as a function of temperature and time were obtained.

General Procedure for isothermal differential scanning calorimetry assays: The autocatalytic natures of HDMA (7b), HDMB (8), and COMU (18c) was tested. Samples (1 mg) were heated to 10°C below the onset temperatures detected in the dynamic DSC [HDMA (7): 167°C, HDMB (8): 170°C, and COMU (17c): 150°C] for 480 min in a closed high-pressure crucible with N2 flow in a Mettler-Toledo DSC-30 differential scanning calorimeter. Diagrams showing heat flow as a function of time were obtained.

General Procedure for AR experiments: Assays were carried out on an Accelerating Rate Calorimeter (ARC) from Thermal Hazard Technology, with use of ARCTIC-HC-MCQ (Hastelloy) test cells. Samples [1.837 g of HDMB (8), 1.605 g of HDMA (7), and 2.350 g of COMU (18c)] were introduced into the calorimetric test cell at room temperature, without stirring. The cell was heated at the initial temperature (50°C) and the “heat-wait-seek” method was applied. This procedure consisted of heating the sample by 5°C and, after 15 min of equilibrium, measuring whether self-heating was occurring at a rate higher than 0.02°C min⁻¹ (default sensitivity threshold). When self-heating was detected, the system was changed to adiabatic mode. After decomposition, the assay

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Uronium coupling reagents: A new family of uronium-type coupling reagents differing in their iminium moieties and leaving groups (see Figure) is described. The presence of the morpholino group in combination with an oxime derivative, especially ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma), had a marked influence on the solubilities, stabilities, and reactivities of the reagents. Furthermore, the Oxyma moiety offers a lower explosion risk than benzotriazole derivatives.