Oxyma: An Efficient Additive for Peptide Synthesis to Replace the Benzotriazole-Based HOBt and HOAt with a Lower Risk of Explosion[1]

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

Abstract: Oxyma [ethyl 2-cyano-2-(hydroxyimino)acetate] has been tested as an additive for use in the carbodiimide approach for formation of peptide bonds. Its performance in relation to those of HOBt and HOAt, which have recently been reported to exhibit explosive properties, is reported. Oxyma displayed a remarkable capacity to inhibit racemization, together with impressive coupling efficiency in both automated and manual synthesis, superior to those of HOBt and at least comparable to those of HOAt, and surpassing the latter coupling agent in the more demanding peptide models. Stability assays showed that there was no risk of capping the resin under standard coupling conditions. Finally, calorimetry assays (DSC and ARC) showed decomposition profiles for benzotriazole-based additives that were consistent with their reported explosivities and suggested a lower risk of explosion in the case of Oxyma.

Keywords: additives · calorimetry · oximes · peptide synthesis · solid-phase synthesis

Introduction

The use of additives to support various coupling methodologies is common practice in research laboratories devoted to peptide chemistry. Of these compounds, the most extensively used displayed a benzotriazole core: 1-hydroxybenzotriazole (HOBt), probably the most common reagent found in a peptide synthesis laboratory, was the first to be unveiled in the early 1970s,[2] whereas later on the use of the more powerful 1-hydroxy-7-azabenzotriazole (HOAt)[3] and, more recently, 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) for the same purpose was reported.[4] The presence of any of these compounds in the coupling medium induces the formation of an active ester, which then undergoes aminolysis to yield the desired peptide bond. The acidities of the additives (pKₐ for HOBt: 4.60, pKₐ for HOAt: 3.28, and pKₐ for 6-Cl-HOBt: 3.35)[5,6] are key factors with regard to the stabilities and reactivities of their related active esters, because they are connected with their behavior as leaving groups. In addition, the nitrogen at the 7-position in HOAt provides a classic neighboring group effect that can both increase the reactivity and reduce racemization.[3]

The carbodiimide approach to formation of peptide bonds has long taken advantage of the properties of benzotriazole-based additives, because the active esters formed are less reactive but more stable than the O-acylisoureas. These intermediate species lead to lower levels of racemization and the suppression of other undesired side reactions such as the formation of inactive N-acylisoureas.[7] Other coupling strategies, such as the combination of base and stand-alone coupling reagents, such as immonium (HATU, HBTU/TBTU, and HCTU/TCTU) or phosphonium salts (PyAOP, PyBOP,
Results and Discussion

In our search for a class of safe and efficient additives, we came across a family of strongly acidic oximes that showed properties of interest, reported by Itoh in the early 1970s (Figure 1, from now on referred to as Oxyma) displayed an appropriate balance of availability and ease of handling. Nonetheless, since the publication of those papers, which were not conclusive about its performance relative to those of benzotriazoles, Oxyma has not played an active role in day-to-day peptide chemistry nor has it been used for general amide bond formation.

Deeper investigation into racemization and the compound’s effectiveness for coupling yields were therefore carried out. For study of racemization, peptide models (Z-Phg-Pro-NH2 and Z-Phe-Val-Pro-NH2 in solution and H-Gly-Cys-Phe-NH2 on solid-phase) more reliable than those chosen in the previous papers were used for comparison of HOAt, HOBt, N-hydroxy-2-pyridinone (HOPO), and Oxyma.[17]

With regard to stepwise coupling (Z-Phg-OH onto H-Pro-NH2), the non-benzotriazole-based additive HOPO (selected due to its association with low levels of racemization in two-phase systems)[18] although giving a higher yield of target dipeptide than HOAt and HOBt (Table 1, entry 5 vs entries 1 and 2), gave poorer retention of configuration (Table 1), especially when a 2 min preactivation time was used (Table 1, entry 6). In contrast, the performance of Oxyma (Table 1, entry 3) exceeded not only that of HOPO, but also those of HOBt and even HOAt (1.0%). Nevertheless, in the experiment conducted without preactivation, some of the starting acid remained unreacted. This inconvenience was overcome by use of a 2 min preactivation period (Table 1, entry 4). Moreover, the outstanding lack of racemization was maintained (1.1%).

In the case of the more racemization-prone [2+1] segment coupling (Z-Phe-Val-OH onto H-Pro-NH2), a similar trend was observed (Table 2). HOPO was the poorest racemization-suppressing additive out of the compounds tested, with a percentage of dL epimer of nearly 50% (Table 2, entry 4). In view of the results with Oxyma in the stepwise model, only the 2 min preactivation experiment was performed. In the segment model, the degree of racemization was clearly lower than that seen with HOBt and comparable to that achieved with HOAt (Table 2, entry 3 vs entries 1 and 2). Moreover, the yield obtained with Oxyma was higher (almost 90%).

Further racemization experiments were carried out with regard to loss of configuration during elongation of Cys-containing peptides in the solid-phase approach (Table 3). For

Table 1. Yields and racemization during the formation of Z-Phg-Pro-NH2 in DMF (stepwise solution-phase synthesis).[a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling reagent</th>
<th>Yield [%]</th>
<th>dl [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HOAt/DIC</td>
<td>81.4</td>
<td>3.3</td>
</tr>
<tr>
<td>2</td>
<td>HOBt/DIC</td>
<td>81.9</td>
<td>9.3</td>
</tr>
<tr>
<td>3</td>
<td>Oxyma/DIC[^b]</td>
<td>89.9</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>Oxyma/DIC[^c]</td>
<td>88.2</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>HOPO/DIC</td>
<td>88.2[^d]</td>
<td>17.4</td>
</tr>
<tr>
<td>6</td>
<td>HOPO/DIC[^e]</td>
<td>83.4</td>
<td>26.1</td>
</tr>
</tbody>
</table>

[a] Couplings were conducted without preactivation, except for entries 4 and 6. lL and dl epimers of the test dipeptide have been described elsewhere[^f]. The t1/2 values of lL and dl were identified by coinjection with pure samples of lL. [b] Unreacted Z-Phg-OH was detected. [c] A 2 min preactivation time was used. [d] Extra peak was found at 22.4 min (6.0%).

Table 2. Yields and racemization during the formation of Z-Phe-Val-Pro-NH2 in DMF (segment solution-phase synthesis).[a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling reagent</th>
<th>Yield [%]</th>
<th>dl [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HOAt/DIC</td>
<td>86.1</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>HOBt/DIC</td>
<td>78.8</td>
<td>8.9</td>
</tr>
<tr>
<td>3</td>
<td>Oxyma/DIC[^b]</td>
<td>89.8</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>HOPO/DIC</td>
<td>88.5</td>
<td>45.1</td>
</tr>
</tbody>
</table>

[a] Couplings were conducted without preactivation, except in the case of entry 3. lL,L and lD,L forms have been described elsewhere[^f] and were coinjected with authentic and pure samples. [b] A 2 min preactivation time was used.

and PyClock) have also been enhanced by the use of these additives.[6–10]

However, a potentially explosive character of HOBt and its related additives has recently been reported.[11] This observation has led to their reclassification under a Class 1 explosive category and has consequently increased transportation difficulties. In view of the relevance of these compounds in day-to-day peptide chemistry, it became evident that there was a need for another family of safe and efficient additives, based on a different template. This poses a difficult challenge if it is borne in mind that the performances of additives based on other compounds, such as N-hydroxysuccinimide (HOSu) or pentafluorophenol (HOPfp), are not comparable to those of HOBt and HOAt, because active esters of OSu or OPfp esters are less reactive than benzotriazole esters.[12–14]

Here we report an exhaustive study of ethyl 2-cyano-2-(hydroxyimino)acetate, an oxime first described in the 1970s with an acidity similar to those of the above additives (pK\text{a} 4.60).[15,16] The suitability of this compound as a substitute for benzotriazole-based additives is discussed in terms of its capacity to control racemization, its effectiveness in difficult couplings either in manual or automated synthesis, and its stability in the presence of growing peptide chains. We have also evaluated the safety profile of this additive by calorimetric techniques.

![Figure 1. Structure of ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma).](Image)

that purpose, the model tripeptide H-Gly-Cys-Phe-NH₂ was assembled in stepwise fashion on a Fmoc-RinkAmide-
MBHA-PS resin (0.45 mmol g⁻¹), with use of a 5 min preac-
tivation time and Cys(Trt) as protecting group.[19,20] Levels
of racemization were generally much lower than in the Z-
Phg-Pro-NH₂ or Z-Phe-Val-Pro-NH₂ models, with <1% of
DL epimer being obtained (Table 3). Comparison between
HOAt, HOBt, and Oxyma showed the same trend as ob-
served in solution phase: Oxyma performed with a level of
inhibition of racemization similar to that of HOAt (Table 3,
entry 1 vs entry 3) and superior to that of HOBt (Table 3,
entry 2 vs 3 entry). Moreover, Oxyma afforded the tripep-
tide in a higher yield than the other additives (Table 3, en-
tries 1 and 2 vs entry 3).

The effectiveness of Oxyma in terms of yields in the solid-
phase approach was tested by the assembly of the ACP (65–
74) decapptide (H-Val-Gln-Ala-Ala-Ile⁶⁹-Asp-Tyr-Ile⁷²-Asn-
Gly-NH₂) on a Fmoc-RinkAmide-Aminomethyl-PS resin
(0.63 mmol g⁻¹).[21] For this purpose, an ABI 433A peptide syn-
thetizer and standard Fmoc/iBu protocols were used. The rela-
tive effectiveness of oxyma, HOAt, and HOBt were tested in
terms of percentage of target decapptide and deletion peptides obtained. Before compar-
ison of the additives, it was necessary to find suitable condi-
tions under which differences would be clearly detectable.
Five- or 10-fold excesses of reagents (Fmoc-amino acids,
DIC, and additive) were not satisfactory for achieving this
objective because nearly quantitative yields of ACP were
obtained with HOBt. Use of twofold excesses, however,
yielded mixtures of various deletion peptides, so relative po-
tencies were more emphasized. No problems were en-
countered in the preparation of a solution of Oxyma in DMF.

Several deletion peptides were detected with use of the
different additives, which made separation highly challeng-
ing. Those originating from misincorporation of a single
amino acid (i.e., des-Ile⁶⁹, des-Ile⁷², des-Val, des-Ala, des-
Gly, or des-Asn) were the most common, but misincorpora-
tions of two amino acids also occurred (see Table 4). HOAt
was the most efficient additive, with more than a 70% yield
of ACP being obtained (Table 4, entry 1). Although the per-
centages of some deletion peptides were lower with HOBt,
the decapptide was produced only in about 60% yield
(Table 4, entry 2). In contrast, the performance of Oxyma
was clearly closer to that of HOAt than to that of HOBt
(69%, entry 3, Table 4).

The performances of HOAt, HOBt, and Oxyma were fur-
ther tested in the manual synthesis of Leu-enkephalin (H-
Tyr-Gly-Phe-Leu-NH₂) analogues.[10] The modifications
included the replacement of consecutive Gly amino acids by
MeGly, MeAla, or Aib residues. These N-methylated or
α,α-disubstituted residues were selected because they show
stERIC hinderance suitable for emphasizing differences in reac-
tivity between the active esters. The strategy followed in set-
up the experiment is illustrated in Scheme 1.

Resin-bound tripeptides 1a, 1b, and 1c of general struc-
ture H-aaX-Phe-Leu-resin were manually assembled on a
Fmoc-RinkAmide-AM-PS resin (0.63 mmol g⁻¹) by means of
30 min couplings with use of DIC/Oxyma. A quantitative
yield was verified by use of the Kaiser test for primary
amines. There was no need for recoupling. Stepwise incorpo-
ration of the last two residues was then studied with each
additive. Amino acids were preactivated for 3 min to ensure
full formation of the active esters being compared. Coupling

![Scheme 1. Strategy followed in the manual synthesis of Leu-enkephalin analogues to test the different additives.](image)

![Image](image)
Peptide Synthesis Additives

Table 5. Percentages of H-Tyr-MeGly-MeGly-Phe-Leu-NH₂ (2a) and related deletion peptides obtained after solid-phase assembly with 5 min coupling times with the different additives.[4]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling reagent</th>
<th>Pentapeptide [%]</th>
<th>des-MeGly [%]</th>
<th>des-Tyr [%]</th>
<th>Tripeptide [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HOAt/DIC</td>
<td>94.9</td>
<td>3.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HOBt/DIC</td>
<td>84.8</td>
<td>7.5</td>
<td>6.6</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>Oxyma/DIC</td>
<td>91.4</td>
<td>3.8</td>
<td>4.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 6. Percentages of H-Tyr-MeAla-MeAla-Phe-Leu-NH₂ (2b) and related deletion peptides obtained in solid-phase assembly with 30 min coupling times with the different additives.[5]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling reagent</th>
<th>Pentapeptide [%]</th>
<th>des-MeAla [%]</th>
<th>des-Tyr [%]</th>
<th>Tripeptide [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HOAt/DIC</td>
<td>73.6</td>
<td>23.2</td>
<td>3.1</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>HOBt/DIC</td>
<td>46.1</td>
<td>38.1</td>
<td>15.2</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>Oxyma/DIC</td>
<td>79.0</td>
<td>16.9</td>
<td>4.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

[a] HPLC-MS showed the right mass for the pentapeptide at 583.8.

McGly and des-Tyr (and even starting tripeptide 1a) were found in the crude products. The highest percentage of pentapeptide was obtained in the experiment with HOAt (94.9%, entry 1, Table 5), an excellent purity for such extreme conditions. HOBt was not as effective as its aza counterpart, but still provided the pentapeptide 2a in an 84.8% yield (Table 5, entry 2). Under these conditions, the performance of Oxyma was impressive, surpassing that of HOBt (Table 5, entry 3), rising above 90% in percentage of pentapeptide, and showing a similar potency to HOAt. There was almost no starting tripeptide in the crude mixture (0.6%). In some cases, the percentage of des-Tyr was higher than that of des-MeGly, thereby confirming that this model pentapeptide was the least demanding.

In view of our observations of the structural similarities between pentapeptide 2b (H-Tyr-MeAla-MeAla-Phe-Leu-NH₂) and 2a, we applied the same coupling times. In this system, however, very little pentapeptide was formed, but surprisingly Oxyma was almost ten times more effective than HOAt (0.6% for HOAt, 0.2% for HOBt and 5.3% for Oxyma). When 30 min couplings were used, considerable percentages of pentapeptide were observed and, unlike in the previous model, more significant differences arose (see Table 6). Synthesis with HOBt resulted in the least pure crude mixture (below 50%, entry 2, Table 6), whereas the other two additives afforded the pentapeptide 2b in a much more efficient manner. Again, the performance of Oxyma was slightly better than that of HOAt (Table 6, entry 3 vs entry 1), with the purity being increased up to almost 80%. This superiority is attributed to the greater effectiveness of the incorporation of the MeAla residue, whereas the incorporation of Tyr was more even. The high percentage of the des-MeAla product relative to the des-Tyr product confirms the higher complexity of the sequence in relation to that of 2a. The presence of a certain pentapeptide lacking the N-methyl group at the MeAla residues (about 1%), regardless of the additive used, should be mentioned.

The coupling conditions that had afforded the pentapeptide 2b in moderate yields were not valid for the 2a analogue (H-Tyr-MeGly-MeGly-Phe-Leu-NH₂),[9c] because the purities were much lower (see Table 7). The enormous percentages of the des-Aib product present in the crude mixtures confirm that the synthesis of 2c is the most demanding of those of the three analogues. Consistently with the trend observed in the previous tests, it was observed that HOBt was the least efficient additive, capable of affording the pentapeptide only in extremely low purity (3%, Table 7, entry 2). Use of its sister additive HOAt did not greatly increase the level of purity (11%, entry 1, Table 7). The superiority of Oxyma in this model was evident, its difference from HOAt in terms of reactivity being greater than that seen in the synthesis of the 2b analogue (28%, entry 3, Table 7). In view of this general low purity profile, 1 h couplings were also carried out. The same trend in relative effectiveness was observed, although this time the percentages of pentapeptide were enhanced. Not only did HOBt still afford the lowest purity (9.8%, entry 5, Table 7), but it was the only reagent that still gave some of the des-Tyr product and the starting tripeptide under the new conditions. The relative performances of Oxyma and HOAt were maintained (Table 7, entry 6 vs entry 4).

In order to improve the level of coupling of the Aib residue further, double couplings of 30 min and of 1 h were performed. Although use of the former set of conditions did not result in any significant changes in the degrees of purity obtained, use of the latter resulted in enhanced percentages of 2c, especially in the synthesis with Oxyma (69%, entry 12, Table 7). It is noteworthy that des-Tyr was still observed even in the longest coupling when HOBt was used (entry 11, Table 7).

Because Oxyma was clearly the additive that displayed the highest potency in the most difficult sequence, but the coupling to afford 2c could still not be driven to completion under the conditions tested, a 4 h double coupling for the in-
corporation of Aib and a standard 1 h coupling for introducing Tyr was conducted with this additive. A near-quantitative yield (92%) was observed. It is worth noting that the coupling effectiveness of Oxyma in these pentapeptides can be considered similar to that of HOAt, one of the most efficient additives and classed among the most powerful coupling reagents.

The stability of Oxyma in relation to the N terminus of a resin-anchored peptide was studied. Our concern mainly related to the side-reaction involving nucleophilic attack of the N terminus amino group on the ethyl ester of Oxyma, which might generate a resin-bound byproduct. This unwanted reaction would lead to the termination of the peptide chain, one of the most undesirable events in solid-phase peptide synthesis. To check the viability of this side reaction, experiments involving two tripeptides (H-Gly-Phe-Val-resin and the less nucleophilic H-MeGly-Phe-Leu-resin) under various strong reaction conditions were carried out. In all experiments 10 equiv of a solution of Oxyma in DMF or NMP was mixed with the resin, without the rest of the reagents that would be added in a standard coupling, to provoke the appearance of side-reactions. Apart from the expected byproduct (named B4), others were also detected (see Scheme 2).

Experiments 1–3 were carried out with the H-Gly-Phe-Val-resin model tripeptide. Experiment 1 was assisted with microwaves for 10 min at 80°C, the resin being mixed with a solution of Oxyma in DMF. After cleavage from the resin with 90% TFA/10% H2O, samples were analyzed by HPLC-PDA, revealing 43.2% of the unmodified peptide (Figure 2). The major byproduct found was B2 (47.9%), a peptide formulated at the N terminus, which presumably involves the solvent. Byproducts B1 and B3 (2.5 and 2.4%, respectively, Path A, Scheme 2), and the predicted B4 (4.0%, Path B) were also detected. The byproduct B1 was the product of attack at the electrophilic carbon of the oxime group and B3 appears to be its hydrolyzed derivative at the ethyl ester (either during microwave irradiation or in the cleavage step), although it should appear at a lower retention time than B1 because of its higher polarity. The mass of B4 was detected as two close peaks, suggesting that two isomers were present. The UV profiles of these additive-based by-products each show a characteristic maximum of absorbance at 235–240 nm, like Oxyma. An unknown impurity with the +43 mass difference ([M+H]+ = 364.3) with respect to the unmodified tripeptide ([M+H]+ = 321.2) was present in the crude mixture, as detected by electrospray HPLC-MS.

In experiment 2, the same solution of the additive in DMF as used in experiment 1 was mixed with the resin at room temperature and left overnight. These considerably milder conditions afforded H-Gly-Phe-Val-NH2 in 92.3% purity (Figure 2). Unlike in experiment 1, only the byproduct B2 (2.1%), which is not additive-based, was observed, along with a compound (5.4%) showing an unusual UV profile that would suggest that it is not peptide-based. Coinjection of the products from experiment 1 and experiment 2 confirmed that this compound was not present in the products of experiment 1. The unknown compound with the +43

![Scheme 2. Main byproducts found after cleavage of tripeptides used in Oxyma stability experiments from the resin.](image)

Table 7. Percentages of H-Tyr-Aib-Aib-Phe-Leu-NH2 (2c) and related deletion peptides obtained in solid-phase assembly under various sets of coupling conditions with the different additives.[a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling conditions</th>
<th>Coupling reagent</th>
<th>Pentapeptide [%]</th>
<th>des-Aib [%]</th>
<th>des-Tyr [%]</th>
<th>Tripeptide [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30 min</td>
<td>HOAt/ DIC</td>
<td>11.3</td>
<td>86.1</td>
<td>1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>1 h</td>
<td>HOBt/ DIC</td>
<td>3.0</td>
<td>91.0</td>
<td>0.9</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>1 h</td>
<td>Oxyma/ DIC</td>
<td>28.0</td>
<td>70.5</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>1 h</td>
<td>HOAt/ DIC</td>
<td>28.7</td>
<td>71.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>1 h</td>
<td>HOAt/ DIC</td>
<td>9.8</td>
<td>86.9</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>1 h</td>
<td>Oxyma/ DIC</td>
<td>55.7</td>
<td>44.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>1 h</td>
<td>HOAt/ DIC</td>
<td>31.2</td>
<td>68.4</td>
<td>0.4</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>1 h[b]</td>
<td>Oxyma/ DIC</td>
<td>8.0</td>
<td>90.6</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>9</td>
<td>1 h[b]</td>
<td>Oxyma/ DIC</td>
<td>46.5</td>
<td>53.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>1 h[b]</td>
<td>HOAt/ DIC</td>
<td>55.0</td>
<td>45.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>1 h[b]</td>
<td>HOAt/ DIC</td>
<td>18.9</td>
<td>80.6</td>
<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>1 h[b]</td>
<td>Oxyma/ DIC</td>
<td>69.0</td>
<td>31.0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

[a] HPLC-MS showed the right mass for the pentapeptide at 611.4. [b] A double coupling was performed.
mass difference seen in experiment 1 was also detected (<0.2%) in experiment 2. The low mass increase and the different UV profile in relation to the additive-based by-products suggests that this impurity was not generated by Oxyma.

In view of the effect of DMF in the stability experiments, experiment 3 was carried out in the microwave as in experiment 1 but this time with a solution of Oxyma in NMP. As expected, B2 almost disappeared completely (3.6%), with the unmodified tripeptide being obtained in 64.0% purity. Comparison with experiment 1, which afforded only a 43.2% yield of tripeptide, illustrates the impact of the solvent on the purity obtained, although more byproducts were detected, including B3, B4, and the impurity with the +43

As in experiment 2, no B1 was detected. In all these experiments, Oxyma-based byproducts were detected only in those carried out under the more extreme conditions (Exps. 1 and 3).

Unlike Exps. 1–3, which were designed as the worst scenarios possible, including the presence of the less hindered (and therefore more prone to react) amino acid Gly at the N terminus, experiment 4 was conducted with the less nucleophilic H-MeGly-Phe-Leu-resin (which was also used in the coupling efficiency assays). The experiment was carried out in DMF, the regular solvent for SPPS, although the above results had been poorer than those obtained in NMP, and with 10 min irradiation in the microwave. The unmodified tripeptide was obtained in 94.5% purity, much higher than the 43.2% achieved with the H-Gly-Phe-Val-tripeptide. Compound B1 was the only additive-based byproduct detected (1.5%), together with B2 (4.0%).

Having addressed the relative potencies of HOBt, HOAt, and Oxyma and the stabilities of the proposed additives, we next focused on the safety profiles of the additives. In view of the recent reports relating to the potentially explosive properties of benzotriazole-based additives, which restrict their transportation and commercial availability, it was crucial to ensure that Oxyma did not follow the classical pattern observed in explosive substances: fast decomposition with simultaneous large increase in pressure.[21]

To explore the thermal safety of the additives, dynamic Differential Scanning Calorimetry (DSC) and Accelerating Rate Calorimetry (ARC) assays were carried out. DSC allows the heat released by a certain compound to be measured, by comparing it to a reference when both are exposed to the same thermal treatment.[22] This experiment also provides valuable information about relative decomposition kinetics (which are connected with the explosive character of a given compound), when heated in a closed crucible under N2 flow, from 30 to 300°C at a constant heating rate of 10°Cmin⁻¹. As a result, diagrams displaying the heat flow as a function of time/temperature are obtained. In addition to displaying higher normalized exothermic ΔH values (234 kJmol⁻¹ for HOBt hydrate and 226 kJmol⁻¹ for HOAt, vs 125 kJmol⁻¹ for Oxyma), the kinetic profiles of the benzotriazole-based derivatives differed significantly from that of Oxyma. Whereas the former compounds decomposed quickly, the latter decomposed in a much slower and constant manner (Figure 3). This decomposition profile cannot be labeled as non-explosive per se, because the study was not an explosivity test. However, it clearly does not coincide with the standard thermal behavior of an explosive substance, such as those observed for HOBt hydrate and HOAt. Interestingly, at 115°C, Oxyma, unlike HOAt and HOBt hydrate, melted before decomposing, and this endothermic phenomenon can be considered a possible safety barrier. Although this barrier is not very precise, because melting and decomposition processes occur almost simultaneously, it might moderate the exothermic released heat. In the experiment with HOBt hydrate, another endothermic
process, corresponding to the desolvation heat, can be observed. The approximate onset temperature at which decomposition began was 131°C, considerably lower than in the cases of HOBt hydrate (190°C) or HOAt (218°C).

Complementary to the DSC assays is the ARC technique, which enables study of a given decomposition under adiabatic conditions.[23] The associated pressure rise can be measured and the onset temperatures accurately determined, unlike in DSC experiments, which suffer from uncertainty because of the low amount of sample.[21] The “heat-wait-seek” method is applied until self-heating of the sample is detected and then the experiment is changed to adiabatic mode. Once decomposition begins, pressure and temperature increase. The assay is stopped when the temperature rises above 300°C.

Again, the benzotriazole-based additives showed markedly different behavior from that of Oxyma in terms of the pressure measured (Figure 4). In the case of the former compounds, high pressures were observed (178 and 167 bar for HOBt hydrate and HOAt, respectively). These results are not surprising, because benzotriazoles release N_2 when decomposing. In contrast, the associated pressure detected in the assay conducted with Oxyma was considerably lower (61 bar).

With regard to the onset temperatures, in the case of Oxyma decomposition began at 124°C, whereas in the experiments with HOBt hydrate and HOAt it began at 145°C and 178°C, respectively (Figure 5). In order to work under safe conditions, it is recommended that the temperature of a given compound be kept at values at which the time to maximum rate under adiabatic conditions is greater than 24 h.[24] As a rule of thumb, this temperature value can be estimated in many cases by subtracting 50 K from the decomposition onset observed in an ARC experiment.[25] In the case of Oxyma, this safety value is at a lower temperature than is the case for HOBt hydrate and HOAt (74°C vs 128°C and 95°C, respectively). Nonetheless, under standard conditions for peptide synthesis, the working temperature does not usually rise above 25°C. Moreover, when stability assays were conducted with microwave irradiation at 80°C, no incident was reported. Although in terms of thermal risk assessment Oxyma is less thermally stable, showing a lower decomposition onset temperature, the above results (mainly the pressure rise associated with decomposition) suggest that the risk of this process ending in a thermal runaway is less likely.
when using Oxyma than in the cases of HOBt hydrate or HOBt.

Conclusions

To conclude, here we have extensively tested ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) as an additive for peptide synthesis for use in combination with carbodiimides. Oxyma showed clear superiority to HOBT in terms of suppression of racemization and of coupling efficiency in all the experiments conducted. In some cases, such as the racemization assay carried out on the stepwise model or the demanding assembly of MeAla and Abg analogues of Leu- enkephalin, its performance was even superior to that of HOAT. The elongation of the acp decapeptide in an ABI 433A peptide synthesizer also demonstrated the compatibility and coupling efficiency of Oxyma in an automated synthesis. Stability assays of Oxyma with regard to N-terminal amino groups showed no peptide-bound byproducts when extremely strong conditions were avoided, even with use of 10 equiv in the absence of base, carbodiimide, and Fmoc-amino acid. Last but not least, two calorimetry techniques, DSC and ARC, showed decomposition profiles for HOAT and HOBt that resemble that of an explosive substance, because of their fast decomposition and high derived pressure rises. In contrast, Oxyma did not follow this pattern, and decomposed at a slower rate, releasing only one third of the pressure observed in the experiments with HOAT and HOAT. Therefore, Oxyma can be considered a potent replacement for benzotriazole-based additives showing a lower thermal risk.

Experimental Section

General: Oxyma (ethyl cyanoglyoxylate-2-oxime, 97%) was obtained from commercial sources (Aldrich). DMF was used in peptide grade triazoles showing a lower thermal risk. Oxyma can be considered a potent replacement for benzotriazole-based additives showing a lower thermal risk.

Experiments consisted of the study of the stepwise coupling of Cys and Gly residues onto previously formed H-Phe-RinkA- mide-MBHA-PS-resin (0.45 mmol g⁻¹, 50 mg), with use of the Fmoc/Bu and Cys(Trt) protection strategy. Glycine was introduced in order to achieve better separation of t.t. and t.t. isomers than in the case of des-Gly dipeptides. Coupling times of 1 h were used after 5 min precoupling of a solution of Fmoc-amino acid, Oxyma, and Dic (3 equiv excess) in DMF (0.3 m) at room temperature. Fmoc removal was carried out with piperidine/DMF (1.4, 2 × 5 min). The peptide chain was released from the resin by treatment with TFA/ethan-1,2-dithioleOH/TIS (94:2.5:2.5) for 1 h at room temperature. The colorless solution was filtered and the resin was washed with CHCl₃ (0.5 mL × 3). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude tetrapeptide was precipitated with cold Et₂O (3 mL) and, after being lyophilized, was analyzed by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 mm. 4.6 × 100 mm), linear gradient 5 to 100% of 0.036% TFA in CH₃CN:0.045% TFA in H₂O over 8 min, with detection at 220 nm. The tₚ values of the t.t. and t.t. epimers were 3.57 and 3.60 min, respectively.

Solid-phase automated synthesis of ACP 65–74 (H–Val–Gln–Ala–Ala–Leu–Asp–Tyrl–Asn–Gly–NH₂)³⁰ The decapeptide was assembled on Fmoc-RinkAmide-Aminomethyl-PS-resin (0.63 mmol g⁻¹, 0.1 mmol) with use of an automated ABI 433A peptide synthesizer. Twofold excesses of Fmoc-amino acids and solutions (0.2 m) of the corresponding additive and DIC in DMF were used (1 mL of the solution was added in each coupling step: 0.2 mmol). The peptide chain was cleaved from the resin by TFA/H₂O (9:1) treatment over 2 h at room temperature. After filtration of the solution containing the peptide material, the resin was washed with CHCl₃ (1 mL × 2), which was removed under nitrogen along with TFA. The resulting crude peptide was purified with cold Et₂O (2 mL) and lyophilized. Peptide purity was analyzed by reversed-phase HPLC, with use of a Waters Symmetry C18 column (5 μm. 4.6 × 150 mm), linear gradient 12.5 to 17% of 0.036% TFA in CH₃CN:0.045% TFA in H₂O over 30 min, with detection at 220 nm. Retention times were: tₚ des-Val = 26.68 min, tₚ des-Ala = 24.88 min, tₚ des-Ala = 24.50 min, tₚ des-Asn = 32.01 min, tₚ des-Gly = 26.33 min, tₚ des-Ile = 2.97 min, tₚ des-Val, Ala = 4.20 min, tₚ des-Ile-Gly = 7.17 min, tₚ des-Ile-Gly = 9.38 min, tₚ des-Val = 20.22 min.

Solid-phase general synthesis of H-aaX-Phe-Leu-resin and H-Gly-Phe-Val-resin tripeptides: Peptides were manually assembled on a Fmoc-RinkAmide-Aminomethyl-PS resin (0.63 mmol g⁻¹, 2 g). Coupling times were 30 min, with 3 equiv excess of each Fmoc-amino acid, Oxyma, and DIC. During precoupling (1.5 min), a bright yellow color was observed immediately after addition of DIC. Fmoc removal was carried out with piperidine/DMF (1:4, 2 × 5 min). Tripeptide purity was checked after sample cleavage (10 mg) from the resin with TFA/H₂O (9:1) at room temperature for 1 h. The solution was filtered and the resin was washed with CHCl₃ (0.5 mL × 2). The solvent and TFA were removed under nitrogen flow. In all cases, reversed-phase HPLC analysis showed purity above 95%.

Solid-phase synthesis of H-Tyr–MeGly–MeGly–Phe–Leu–NH₂ (2a) with use of the different additives: The pentapeptide was manually elongated on a pure previously assembled H-MeGly–Phe–Leu–Rinkamide–Amino- methyl-PS-resin (0.63 mmol g⁻¹, 50 mg) with a precoupling time of 3 min and use of Fmoc-amino acids (3 equiv, excess), the corresponding additive (3 equiv), and DIC (3 equiv). The coupling time was 5 min for the introduction of both MeGly and Tyr. The pentapeptide was cleaved from the resin by treatment with TFA/H₂O (9:1) for 2 h at room temperature. The solution was then filtered and the resin was washed with CHCl₃ (1 mL × 2), which was removed along with TFA under nitrogen. The crude pentapeptide was precipitated with cold Et₂O (2 mL) and, after being lyophilized, was analyzed by reversed-phase HPLC, with use of a Waters Symmetry C18 column (5 μm. 4.6 × 150 mm), linear gradient 16 to 16.5% of 0.036% TFA in CH₃CN:0.045% TFA in H₂O over 30 min, with detection at 220 nm. The tₚ value of the pentapeptide was 26.31 min, whereas the tₚ values of des-MeGly, des-Tyr, and the tripeptide H-MeGly–Phe–Leu–NH₂ were 25.81, 9.62, and 8.58 min, respectively.

Solid-phase synthesis of H-Tyr–MeAla–MeAla–Phe–Leu–NH₂ (2b) with use of the different additives: The pentapeptide was manually assembled on a pure previously synthesized H-MeAla–Phe–Leu–Rinkamide–Amino-
methyl-PS-resin (0.63 mmol g⁻¹, 50 mg), with use of Fmoc-amino acids (3 equiv excess), the corresponding additive, and DIC. Precipitation and coupling times for the introduction of both MeAla and Tyr were 3 and 30 min, respectively. The peptide chain was cleaved from the resin by treatment with TFA/H₂O (9:1) for 2 h at room temperature. From the solution, the peptide was purified by reverse-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm), linear gradient 20 to 50% of 0.036% TFA in CH₃CN/0.045% TFA in H₂O over 8 min, with detection at 220 nm. The tₜ value of the peptide was 8.21 min, whereas the tₜ values of des-MeAla, des-Tyr, and the tripeptide H-MeAla-Phe-Leu-NH₂ were 8.81, 4.73, and 2.34 min, respectively.

Solid-phase synthesis of H-Tyr-Alb-Alb-Phe-Leu-NH₂ (2c) with use of the different additives:

The peptide was manually elongated on a previously synthesized H-Aib-Phe-Leu-Rinkamine-Aminoethyl-PS-resin (0.63 mmol g⁻¹, 50 mg), with use of Fmoc-amino acids (3 equiv, excess), the corresponding additive (3 equiv), and DIC (3 equiv). Residues were preactivated for 3 min prior to addition to the resin. Coupling times are displayed in Table 7. The peptide chain was cleaved from the resin by treatment with TFA/H₂O (9:1) for 2 h at room temperature. The solution was then filtered and the resin was washed with CH₂Cl₂ (1 mL/C148). In both types of experiments, the resin-bound compounds were cleaved from the resin by treatment with TFA/H₂O (9:1) for 2 h at room temperature. The solu-
tion was then filtered and the resin was washed with CH₂Cl₂ (1 mL/C148). It was removed along with TFA under nitrogen. The crude peptide was purified with cold Et₂O (2 mL×3) and lyophilization. Purity was checked by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm) and a linear gradient of 20 to 35% of 0.036% TFA in CH₃CN/0.045% TFA in H₂O over 8 min, with detection at 220 nm. The tₜ value for the pentapeptide was 5.75 min, whereas, as the tₜ values for des-Aib, des-Tyr, and the tripeptide H-Aib-Phe-Leu-NH₂, were 6.03, 3.93, and 2.25 min, respectively.

General procedure for stability assays with Oxyma:

Stability experiments 1–4 were conducted on two resin-bound tripeptides: H-MeGly-Phe-Val-resin and H-Gly-Phe-Val-resin. The resin was weighed into a 2 mL solid-phase syringe, swelled in CH₂Cl₂ (C1485), and then conditioned with a solution of Oxyma in DMF (C14810) and CH₂Cl₂ (C14810). In both types of experiments, the resin-bound compounds were cleaved from the resin by treatment with TFA/H₂O (9:1) for 2 h at room temperature. The solu-
tion was then filtered and the resin was washed with CH₂Cl₂ (1 mL/C148). In the microwave-assisted experiment, the resin was first transferred into a 3 mL CEM Discover Microwave. The resin was then transferred back into the syringe and washed with NMP or DMF (0.02 mL/C148). For each experiment, the resin was weighed into a 2 mL solid-phase syringe, swelled in CH₂Cl₂ (C1485), and then conditioned with a solution of Oxyma in DMF (C14810) and CH₂Cl₂ (C14810). In both types of experiments, the peptide chain was cleaved from the resin by treatment with TFA/H₂O (9:1) over 2 h at room temperature. The solu-
tion was then filtered and the resin was washed with CH₂Cl₂ (1 mL×2), which was removed along with TFA under nitrogen. The crude peptide was purified with cold Et₂O (2 mL×3) and, after lyophilization, purity was checked by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm) and a linear gradient of 20 to 35% of 0.036% TFA in CH₃CN/0.045% TFA in H₂O over 8 min, with detection at 220 nm. The tₜ value for the pentapeptide was 5.75 min, whereas, as the tₜ values for des-Aib, des-Tyr, and the tripeptide H-Aib-Phe-Leu-NH₂, were 6.03, 3.93, and 2.25 min, respectively.

General procedure for dynamic differential scanning calorimetry assays:

The thermal behavior of HOAt, HOBt hydrate, and Oxyma was tested.

General Procedure for Accelerating Rate Calorimetry (ARC) from Thermal Hazard Technology, in ARCTIC-HC-MCQ (Hatelloy) test cells. Samples (2.08 g of HOBt hydrate, 1.605 g of HOAt, and 3.451 g of Oxyma) were introduced into the calorimetric test cell at room temperature, without stirring. The cell was heated at the initial temperature (30°C) and the “heat-wait- seek” method was applied; this 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 (default sensitivity threshold). When self-heating was detected, the sample was changed to adiabatic mode. After decomposition, the assay was stopped when the temperature rose above 300°C. The phi factors were 2.6058 (HOBt hydrate), 3.04373 (HOAt), and 1.95575 (Oxyma).

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[1] Abbreviations not defined in text: Aib, a-aminoisobutyric acid; ACP, acyl carrier protein decapetide (65–74); AM-PS, aminomethyl-polyethylene resin; ARC, accelerating rate calorimetry; 6-Ctt, HOAT, 6-chloro-1-hydroxybenzotriazole; DIC, N,N-dimisopropylcarbodiimide; DIAE, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DSC, Differential Scanning Calorimetry; HATU, N-[(dimethylamino)methylene]-N,N,N′-trimethylhexahydrophosphoramide N-oxide; HBTU, N-[(benzotriazol-1-yl)-dimethylaminomethylene]-N-methylhexahydrophosphoramide N-oxide; HCTU, N-[(1H-6-chlorobenzotriazol-1-yl)(dimethylamino)methylene]-N-methylhexahydrophosphoramide N-oxide; HOAT, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; HODbt, 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazin-6-ol; HOPO, 2-hydroxypyridine-N-oxide; HOOP, pentfluorophenol; HOSu, N-hydroxysuccinimide; NMP, N-methyl-2-pyrrolidinone; PyAOP, azabenzotriazol-1-yl-(dimethylamino)methylene hexafluorophosphoramide; TBTU, benzotriazol-1-yl-N-oxysulfonylpyridin-2-ylidene)-methylaminium hexafluorophosphoramide; TCTU, N-[(1H-6-chlorobenzotriazol-1-yl)(dimethylamino)methylene]-N-methylhexahydrophosphoramide N-oxide; TFA, trifluoroacetic acid; Z, benzoyloxycarbonyl. Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature (J. Biol. Chem. 1972, 247, 977).


Peptide Synthesis Additives


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