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

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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 (pK_a for HOBt: 4.60, pK_a for HOAt: 3.28, and pK_a 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,

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and PyClock) have also been enhanced by the use of these additives.[8–10]

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_a 4.60).[15] 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.

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 and by Izdebsky a few years later.[15,16] Out of all the oximes tested in those studies, ethyl 2-cyano-2-(hydroxyimino)acetate (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.

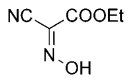


Figure 1. Structure of ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma).

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-NH_2 and Z-Phe-Val-Pro-NH_2 in solution and H-Gly-Cys-Phe-NH_2 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-NH_2), 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%).

Table 1. Yields and racemization during the formation of Z-Phg-Pro-NH_2 in DMF (stepwise solution-phase synthesis).[a]

Entry	Coupling reagent	Yield [%]	DL [%]
1	HOAt/DIC	81.4	3.3
2	HOBt/DIC	81.9	9.3
3	Oxyma/DIC[b]	89.9	1.0
4	Oxyma/DIC[c]	88.2	1.1
5	HOPO/DIC	88.2[d]	17.4
6	HOPO/DIC[c]	83.4	26.1

[a] Couplings were conducted without preactivation, except for entries 4 and 6. LL and DL epimers of the test dipeptide have been described elsewhere.[17] The t_R 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%).

In the case of the more racemization-prone [2+1] segment coupling (Z-Phe-Val-OH onto H-Pro-NH_2), 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%).

Table 2. Yields and racemization during the formation of Z-Phe-Val-Pro-NH_2 in DMF (segment solution-phase synthesis).[a]

Entry	Coupling reagent	Yield [%]	DL [%]
1	HOAt/DIC	86.1	2.1
2	HOBt/DIC	78.8	8.9
3	Oxyma/DIC[b]	89.8	3.8
4	HOPO/DIC	88.5	45.1

[a] Couplings were conducted without preactivation, except in the case of entry 3. LLL and LDL forms have been described elsewhere[17] and were coinjected with authentic and pure samples. [b] A 2 min preactivation time was used.

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

that purpose, the model tripeptide H-Gly-Cys-Phe-NH_2 was assembled in stepwise fashion on a Fmoc-RinkAmide-MBHA-PS resin (0.45 mmol g^{-1}), with use of a 5 min preactivation time and Cys(Trt) as protecting group.[19,20] Levels of racemization were generally much lower than in the Z-Phg-Pro-NH_2 or Z-Phe-Val-Pro-NH_2 models, with $<1\%$ of DL epimer being obtained (Table 3). Comparison between HOAt, HOBt, and Oxyma showed the same trend as observed 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 tripeptide in a higher yield than the other additives (Table 3, entries 1 and 2 vs entry 3).

Table 3. Yields and racemization during the elongation of H-Gly-Cys-Phe-NH_2 in DMF (stepwise solid-phase synthesis).[a,b]

Entry	Coupling reagent	Yield [%]	DL [%]
1	HOAt/DIC	88.4	0.1
2	HOBt/DIC	84.1	0.2
3	Oxyma/DIC	90.8	0.1

[a] A 5 min preactivation time at room temperature was used in all experiments. The t_R values for LL and DL forms were identified by coinjection with authentic and pure samples. [b] An extra peak at 3.5 min, corresponding to the disulfide-bonded dimer (~1%), was found in all experiments.

The effectiveness of Oxyma in terms of yields in the solid-phase approach was tested by the assembly of the ACP (65–74) decapeptide (H-Val-Gln-Ala-Ala-Ile^{69}-Asp-Tyr-Ile^{72}-Asn-Gly-NH_2) on a Fmoc-RinkAmide-Aminomethyl-PS resin (0.63 mmol g^{-1}).[9c] For this purpose, an ABI 433 A peptide synthesizer and standard Fmoc/*t*Bu protocols were used. The relative effectivenesses of Oxyma, HOAt, and HOBt were tested in terms of percentage of target decapeptide and deletion peptides obtained. Before comparison of the additives, it was necessary to find suitable conditions 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 potencies were more emphasized. No problems were encountered 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 challenging. Those originating from misincorporation of a single amino acid (i.e., des-Ile^{72}, des-Ile^{69}, des-Val, des-Ala, des-Gly, or des-Asn) were the most common, but misincorporations 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 percentages of some deletion peptides were lower with HOBt, the decapeptide 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).

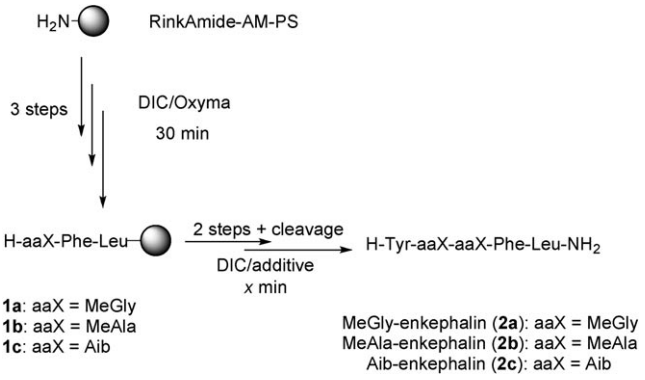
The performances of HOAt, HOBt, and Oxyma were further tested in the manual synthesis of Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-NH_2) analogues.[10] The modifications included the replacement of consecutive Gly amino acids by MeGly, MeAla, or Aib residues. These *N*-methylated or α,α-disubstituted residues were selected because they show steric hindrance suitable for emphasizing differences in reactivity between the active esters. The strategy followed in setting up the experiment is illustrated in Scheme 1.

Resin-bound tripeptides **1a**, **1b**, and **1c** of general structure 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 incorporation 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

Table 4. Percentages of ACP (65–74) decapeptide (H-Val-Gln-Ala-Ala-Ile^{69}-Asp-Tyr-Ile^{72}-Asn-Gly-NH_2) and various deletion peptides obtained during automated synthesis using different additives.[a]

Entry	Coupling reagent	des-2Ile [%]	des-Ile-Ala [%]	des-Ile^{72} [%]	des-Ile-Gly [%]	des-Ile^{69} [%]	des-Val [%]	des-2Ala [%]	des-Ala [%]	des-Gly [%]	ACP [%]	des-Asn [%]
1	HOAt/DIC	1.7	3.9	5.9	–[b]	5.8	1.8	2.9	4.2	0.5	71.8	1.5
2	HOBt/DIC	0.5	2.5	12.6	7.8[c]	4.9	3.2	–[b]	6.0[d]	0.2	62.3	–[b]
3	Oxyma/DIC	0.6	2.6	9.9	–[b]	7.9	2.1	2.7	5.1	0.1	68.7	0.3

[a] HPLC-MS showed the right mass for the ACP decapeptide at 1062.7. [b] No misincorporation was detected. [c] The observed mass could also correspond to that of the des-Ala-Val product. [d] Along with the mass of des-Ala, that of des-Gln was also detected.



Scheme 1. Strategy followed in the manual synthesis of Leu-enkephalin analogues to test the different additives.

times ranged from 5 min to 1 h depending on the Leu-enkephalin analogue, under conditions that afforded the best scenarios for comparing the additives. Pentapeptides **2a**, **2b**, and **2c** of general sequence H-Tyr-aaX-aaX-Phe-Leu-NH_2 were obtained after cleavage from the resin with concentrated TFA. The efficiency of each additive was measured in terms of percentages of pentapeptide and deletion tetrapeptides (and occasionally also starting tripeptide) obtained.

The pentapeptide model **2a** (H-Tyr-MeGly-MeGly-Phe-Leu-NH_2) was, compared to **2b** and **2c**, the least difficult to assemble. Therefore, it is not surprising that a 1 h couplings for the incorporation of both MeGly and Tyr were not the most suitable conditions to highlight the relative efficiencies of the different additives, because quantitative yields of **2a** were obtained. Coupling times were thus shortened to 5 min for this purpose, although even then high and similar percentages were detected with all three additives (see Table 5). Nevertheless, the deletion tetrapeptides des-MeGly 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.

Table 5. Percentages of H-Tyr-MeGly-MeGly-Phe-Leu-NH_2 (**2a**) and related deletion peptides obtained after solid-phase assembly with 5 min coupling times with the different additives.[a]

Entry	Coupling reagent	Pentapeptide [%]	des-MeGly [%]	des-Tyr [%]	Tripeptide [%]
1	HOAt/DIC	94.9	1.4	3.2	0.5
2	HOBt/DIC	84.8	7.5	6.6	1.1
3	Oxyma/DIC	91.4	3.8	4.2	0.6

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

In view of our observations of the structural similarities between pentapeptide **2b** (H-Tyr-MeAla-MeAla-Phe-Leu-NH_2) 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.

Table 6. Percentages of H-Tyr-MeAla-MeAla-Phe-Leu-NH_2 (**2b**) and related deletion peptides obtained in solid-phase assembly with 30 min coupling times with the different additives.[a]

Entry	Coupling reagent	Pentapeptide [%]	des-MeAla [%]	des-Tyr [%]	Tripeptide [%]
1	HOAt/DIC	73.6	23.2	3.1	0.1
2	HOBt/DIC	46.1	38.1	15.2	0.6
3	Oxyma/DIC	79.0	16.9	4.0	0.1

[a] HPLC-MS showed the right mass for the pentapeptide at 611.5.

The coupling conditions that had afforded the pentapeptide **2b** in moderate yields were not valid for the **2c** analogue (H-Tyr-Aib-Aib-Phe-Leu-NH_2),[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-

Table 7. Percentages of H-Tyr-Aib-Aib-Phe-Leu-NH_2 (**2c**) and related deletion peptides obtained in solid-phase assembly under various sets of coupling conditions with the different additives.[a]

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

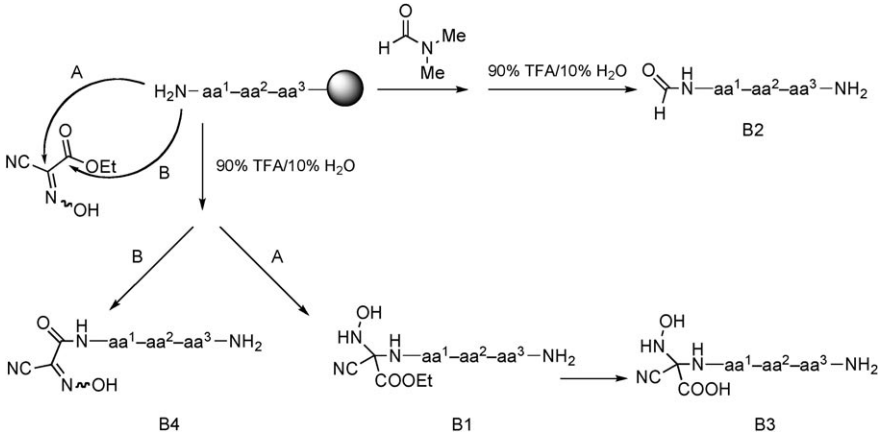
[a] HPLC-MS showed the right mass for the pentapeptide at 611.4. [b] A double coupling was performed.

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% H_2O, 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 formylated 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 byproducts each show a characteristic maximum of absorbance at 235–240 nm, like Oxyma. An unknown impurity with the same retention time as **B1** but with a +43 mu 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-NH_2 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.

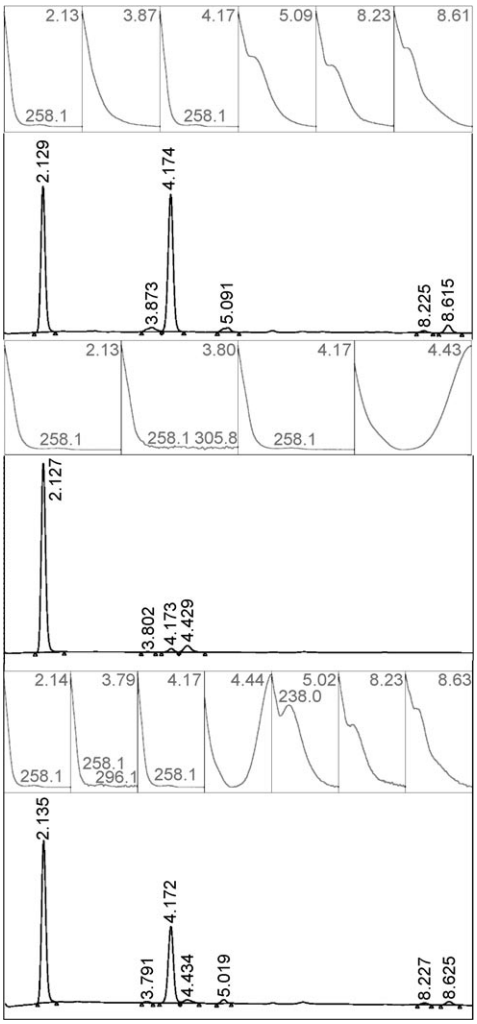


Figure 2. HPLC-PDA and UV profiles of crude mixtures from stability experiments: experiment 1 (above), experiment 2 (center), and coinjection of samples from experiment 1 and experiment 2 (below), showing the tripeptide H-Gly-Phe-Val-NH_2 (2.1 min, $[M+H]^+ = 321.2$) and byproducts **B1** (3.9 min, $[M+H]^+ = 463.3$, experiment 1 and coinjection), **B2** (4.2 min, $[M+H]^+ = 349.2$), **B3** (5.1 min, $[M+H]^+ = 435.3$), and **B4** (8.2 and 8.8 min, $[M+H]^+ = 417.2$). Unknown impurities are found at 3.9 min ($[M+H]^+ = 364.3$, experiments 1, 2, and coinjection) and at 4.4 min ($[M+H]^+ = 620.4$, experiment 2 and coinjection).

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 byproducts 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 mass difference with respect to the unmodified tripeptide. 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-resin 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 N_2 flow, from 30 to 300 °C at a constant heating rate of 10 °C min^{-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 kJ mol^{-1} for HOBt hydrate and 226 kJ mol^{-1} for HOAt, vs 125 kJ mol^{-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

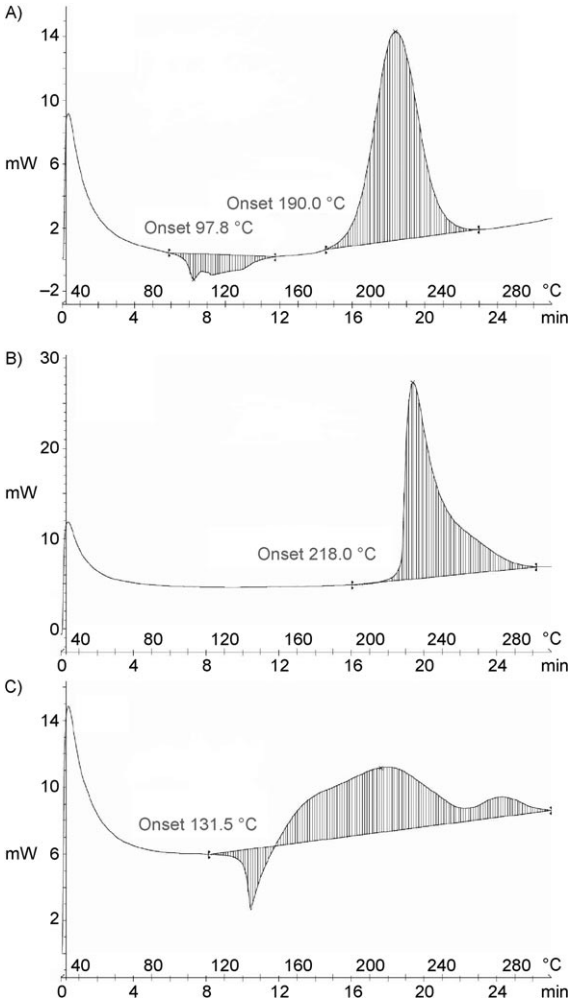


Figure 3. Thermograms showing heat flow versus temperature and time for DSC experiments with A) HOBt hydrate, B) HOAt, and C) Oxyma.

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

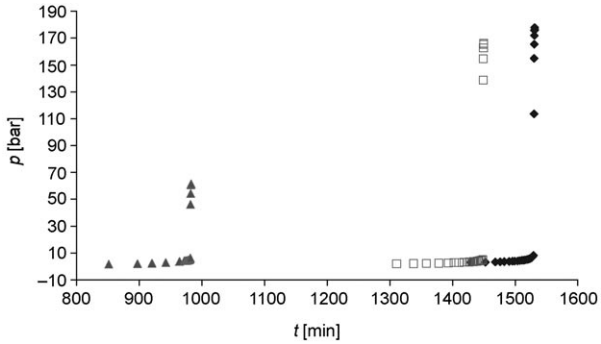


Figure 4. ARC experiments. Decomposition profiles of HOBt hydrate (◆), HOAt (□), and Oxyma (▲) showing released pressure (bar) as a function of time (min).

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

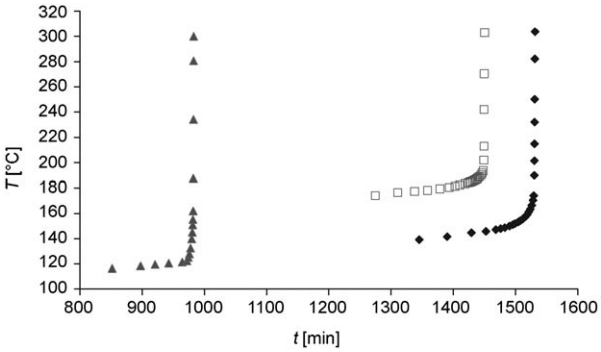


Figure 5. ARC experiments. Decomposition profiles of HOBt hydrate (◆), HOAt (□), and Oxyma (▲) showing temperature (°C) as a function of time (min).

when using Oxyma than in the cases of HOBt hydrate or HOAt.

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 Aib analogues of Leu-enkephalin, its performance was even superior to that of HOAt. The elongation of the ACP decapeptide in an ABI 433 A 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 a 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 HOBt 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 purity. All peptides, des-amino acids, and byproducts were identified by HPLC-MS electrospray mass spectroscopy. The synthesis of ACP was carried out in an Applied Biosystems ABI 433 A automated peptide synthesizer by the Fmoc/*t*Bu protection strategy. Experiments involving side reactions related to Oxyma were conducted in a CEM Discover Microwave. Differential scanning calorimetry assays were performed in a Mettler–Toledo DSC-30 differential scanning calorimeter, with high-pressure crucibles of a capacity of 30 μL. In the adiabatic calorimetry experiment, an accelerating rate calorimeter from Thermal Hazard Technology was used, along with ARCTC-HC-MCQ (Hastelloy) test cells. The sensitivity threshold was set at $0.02\,^{\circ}C\,min^{-1}$.

Racemization tests with model peptides in solution phase: Test couplings were carried out as previously described for Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2.[17] Crude products were analyzed by reverse-phase HPLC (with a Waters Symmetry C18, 5 μm, 4.6×150 mm column), linear gradient over 30 min of 20 to 50% (Z-Phg-Pro-NH_2) or 20 to 80% (Z-Phe-Val-Pro-NH_2) CH_3CN/0.036% TFA in H_2O/0.045% TFA, detection at 220 nm. In the Z-Phg-Pro-NH_2 model, the t_R values of the LL and DL epimers were 26.01 and 27.40 min, respectively, whereas in the Z-Phe-Val-Pro-NH_2 case, the t_R values of the LLL and LDL epimers were 19.98 and 21.05 min, respectively.

Study of cysteine racemization during assembly of H-Gly-Cys-Phe-NH_2 on solid phase:[19,20] Experiments consisted of the study of the stepwise coupling of Cys and Gly residues onto previously formed H-Phe-RinkAmide-MBHA-PS-resin (0.45 mmol g^{-1}, 50 mg), with use of the Fmoc/*t*Bu and Cys(Trt) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than in the case of des-Gly dipeptides. Coupling times of 1 h were used after 5 min preactivation 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/ethane-1,2-dithiol/H_2O/TIS (94:2.5:2.5:1) for 1 h at room temperature. The colorless solution was filtered and the resin was washed with CH_2Cl_2 (0.5 mL×3). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude pentapeptide was precipitated with cold Et_2O (3 mL×4) and, after being lyophilized, was analyzed by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6×100 mm), linear gradient 5 to 100% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. The t_R values of the LL and DL epimers were 3.37 and 3.60 min, respectively.

Solid-phase automated synthesis of ACP (65–74) (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2):[14] The ACP (65–74) decapeptide model was assembled on Fmoc-RinkAmide-Aminomethyl-PS-resin (0.63 mmol g^{-1}, 0.1 mmol) with use of an automated ABI 433 A 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/H_2O (9:1) treatment over 2 h at room temperature. After filtration of the solution containing the peptidic material, the resin was washed with CH_2Cl_2 (1 mL×2), which was removed under nitrogen along with TFA. The resulting crude peptide was purified with cold Et_2O (2 mL×3) 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 CH_3CN/0.045% TFA in H_2O over 30 min, with detection at 220 nm. Retention times were: t_R decapeptide = 26.88 min, t_R des-2 Ala = 24.48 min, t_R des-Ala = 24.50 min, t_R des-Asn = 32.01 min, t_R des-Gly = 26.33 min, t_R des-Ile^{69}, Ile ^{72} = 2.97 min, t_R des-Ile, Ala = 4.20 min, t_R des-Ile^{72} = 7.17 min, t_R des-Ile^{69} = 9.38 min, t_R 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 preactivation (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/H_2O (19:1) at room temperature for 1 h. The solution was filtered and the resin was washed with CH_2Cl_2 (0.5 mL×2). The solvent and TFA were removed under nitrogen flow. In all cases, reversed-phase HPLC analysis showed purity above 99%.

Solid-phase synthesis of H-Tyr-MeGly-MeGly-Phe-Leu-NH_2 (2a) with use of the different additives: The pentapeptide was manually elongated on a pure previously assembled H-MeGly-Phe-Leu-Rinkamide-Aminomethyl-PS-resin (0.63 mmol g^{-1}, 50 mg) with a preactivation 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/H_2O (9:1) for 2 h at room temperature. The solution was then filtered and the resin was washed with CH_2Cl_2 (1 mL×2), which was removed along with TFA under nitrogen. The crude pentapeptide was precipitated with cold Et_2O (2 mL×3) and, after being lyophilized, was analyzed by reversed-phase HPLC, with a Waters Symmetry C18 column (5 μm, 4.6×150 mm), linear gradient 16 to 16.5% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 30 min, with detection at 220 nm. The t_R value of the pentapeptide was 26.31 min, whereas the t_R values of des-MeGly, des-Tyr, and the tripeptide H-MeGly-Phe-Leu-NH_2 were 25.81, 9.62, and 8.58 min, respectively.

Solid-phase synthesis of H-Tyr-MeAla-MeAla-Phe-Leu-NH_2 (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. Preactivation 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/H_2O (9:1) over 2 h at room temperature. The solution was then filtered and the resin was washed with CH_2Cl_2 (1 mL × 2), which was removed along with TFA under nitrogen. The crude pentapeptide was purified with cold Et_2O (2 mL × 3) and lyophilized. Purity was analyzed by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm), linear gradient 20 to 25% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. The t_R value of the pentapeptide was 8.21 min, whereas the t_R values of des-MeAla, des-Tyr, and the tripeptide H-MeAla-Phe-Leu-NH_2 were 8.81, 4.73, and 2.34 min, respectively.

Solid-phase synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2 (2c) with use of the different additives:[14] The pentapeptide was manually elongated on a pure previously synthesized H-Aib-Phe-Leu-Rinkamide-Aminomethyl-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/H_2O (9:1) for 2 h at room temperature. The solution was then filtered and the resin was washed with CH_2Cl_2 (1 mL × 2), which was removed along with TFA under nitrogen. The crude peptide was purified with cold Et_2O (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 CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. The t_R value for the pentapeptide was 5.75 min, whereas the t_R values for des-Aib, des-Tyr, and the tripeptide H-Aib-Phe-Leu-NH_2 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-Leu-resin and H-Gly-Phe-Val-resin. The resin was weighed into a 2 mL solid-phase syringe, swelled in CH_2Cl_2 (×5), and then conditioned in the reaction solvent, DMF or NMP (×5). In the overnight experiment, a solution of Oxyma in DMF or NMP (0.02 M, 10 equiv) was directly added to the resin. After 12 h at room temperature, the resin was filtered and washed with DMF or NMP (×10) and CH_2Cl_2 (×10). In contrast, in the microwave-assisted experiment, the resin was first transferred into a suitable microwave-compatible vial. The solution of Oxyma in DMF or NMP (0.02 M, 10 equiv) was then added to the resin and the mixture was irradiated at 80 °C for 10 min in a CEM Discover Microwave. The resin was then transferred back into the syringe and washed with NMP or DMF (×10) and CH_2Cl_2 (×10). In both types of experiments, the resin-bound compounds were cleaved from the resin by treatment with TFA/H_2O (9:1) for 1 h at room temperature. The solution was filtered and the resin was washed with CH_2Cl_2 (0.05 mL × 3), which was removed together with TFA under nitrogen. The crude peptide was purified with cold Et_2O (2 mL × 3) and lyophilized. The byproduct content of the samples was checked by reversed-phase HPLC. Exps. 1–3 (H-Gly-Phe-Val-resin) were analyzed by use of a SunFire C18 Column (3.5 μm, 4.6 × 100 mm), linear gradient 15 to 30% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. Retention times were: t_R unmodified tripeptide = 2.13 min, t_R byproduct **B1** = 3.87 min, t_R byproduct **B2** = 4.17 min, t_R byproduct **B3** = 5.09 min, t_R byproduct **B4** = 8.22 and 8.61 min, t_R impurity with detected mass M+43 = 3.80 min, t_R unknown impurity = 4.43 min. experiment 4 (H-MeGly-Phe-Val-resin) was analyzed with use of a Waters Symmetry C18 column (5 μm, 4.6 × 150 mm), linear gradient 5 to 100% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 15 min, with detection at 220 nm. Retention times were: t_R unmodified tripeptide = 6.25 min, t_R byproduct **B1** = 6.88 min, t_R byproduct **B2** = 7.68 min.

General Procedure for dynamic differential scanning calorimetry assays: The thermal behavior of HOAt, HOBt hydrate, and Oxyma was tested. Samples (1 mg) were heated from 30 °C to 300 °C at a heating rate of 10 °C min^{-1} in a closed high-pressure crucible with N_2 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 ARC experiments: Assays were carried out on an Accelerating Rate Calorimeter (ARC) from Thermal Hazard Technology, in ARCTC-HC-MCQ (Hastelloy) test cells. Samples (2.083 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^{-1} (default sensitivity threshold). When self-heating was detected, the system was changed to adiabatic mode. After decomposition, the assay was stopped when the temperature rose above 300 °C. The phi factors[26] were: 2.6058 (HOBt hydrate), 3.04373 (HOAt), and 1.95557 (Oxyma).

Acknowledgements

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[1] Abbreviations not defined in text: Aib, α-aminoisobutyric acid; ACP, acyl carrier protein decapeptide (65–74); AM-PS, aminomethyl-polystyrene resin; ARC, accelerating rate calorimetry; 6-Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; DIC, *N,N*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DSC, Differential Scanning Calorimetry; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; HODhbt, 3-hydroxy-3,4-dihydro-4-oxo-l,2,3-benzotriazine; HOPO, 2-hydroxypyridine-*N*-oxide; HOPfp, pentafluorophenol; HOSu, N-hydroxysuccinimide; NMP, *N*-methyl-2-pyrrolidinone; PyAOP, azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyClock, 6-chloro-benzotriazol-1-yl-*N*-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]*N*-methylmethanaminium tetrafluoroborate *N*-oxide; TCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl. 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).
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