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Article *in* Biopolymers · January 2012

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Use of Oxyma as pH Modulatory Agent to be Used in the Prevention of Base-Driven Side Reactions and Its Effect on 2-Chlorotrityl Chloride Resin

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Received 1 May 2011; revised 5 June 2011; accepted 6 July 2011
Published online 31 August 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bip.21713

ABSTRACT:

The presence of low pKa N*-hydroxylamines is beneficial in peptide chemistry as they reduce some base-mediated side reactions. Here we evaluated the applicability and buffering capacity of Ethyl 2-cyano-2-(hydroxyimino) acetate (Oxyma) in the prevention of aspartimide/ piperidide formation and Pro-based overcoupling and compared it with the performance of HOBt and HOAt. In addition, the compatibility of these additives with the highly acid-labile 2-chlorotrityl chloride resin is examined. © 2011 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 98: 89–97, 2012.*

Keywords: aspartimide; side-reactions; peptide; solid-phase; coupling reagent

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

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Contract grant sponsor: Centro de Investigación Científica y Tecnológica (CICYT)
Contract grant number: CTQ2009-07758
Contract grant sponsor: Generalitat de Catalunya
Contract grant number: 2009SGR 1024
Contract grant sponsor: Luxembourg Bio Technologies, Ltd. (Rehovot)
Contract grant sponsors: Institute for Research in Biomedicine and the Barcelona Science Park, Ministerio de Educación y Ciencia

INTRODUCTION

N-hydroxylamines are among the most potent additives to carbodiimides in synthetic peptide chemistry.[1] Compounds containing such a moiety show unusually high acidity in the context of organic chemistry (pKa = 3–10) and, therefore, are excellent leaving groups in acylation reactions, such as amide bond formation.[1] The inclusion of *N*-hydroxylamines in stand-alone coupling reagents, like onium salts, results in an even more powerful strategy.[2] Among the various templates proposed with this highly acidic group (such as benzotriazines, succinimides or triazoles), benzotriazoles have undoubtedly been the most extensively studied and cited.[3] 1-hydroxybenzotriazole (HOBt, **1**) and 7-aza-1-hydroxybenzotriazole (HOAt, **2**), with a pKa of 4.60 and 3.28, respectively, have found general acceptance and are routinely used additives (Figure 1 left).[3a,4] Nevertheless, safety issues associated with these heterocyclic compounds arose several years ago, thus prompting the search for additives based on alternative cores.[5] It has recently been demonstrated that stable and acidic oximes bearing electron-withdrawing substituents, like ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma, **3**), developed in our group, are a safe and efficient approach for peptide bond formation (Figure 1 right).[6]

However, the application of *N*-hydroxylamine-based compounds in peptide chemistry is not limited merely to assisting the assembly of amino acids and peptide fragments, but might also be beneficial in other steps of peptide/protein elongation, especially when using Fmoc/tBu protection. Although the Fmoc/tBu strategy is now the predominant protection approach due to its fully orthogonal character and milder α-amino deprotection and peptide-resin cleavage

X= CH, HOBt (**1**) Oxyma (**3**)

X= N, HOAt (**2**)

FIGURE 1 Structure of *N*-hydroxylamine-type additives.

conditions, superseding the classical Boc/Bzl method, it is not exempt of detrimental side reactions.[7,8] Most of these occur as a result of the basic conditions required to remove the Fmoc temporary protecting group, such as dehydration of Cys to dehydroalanine or unwanted DKP formation at the dipeptide step.[9] Due to their unique acidic nature, *N*-hydroxylamines reduce the impact of some of these base-driven unwanted reactions, and thus act as pH regulators.[4a,10]

Since Oxyma (**3**) and derivatives induce highly reactive ester formation, we sought to extend the scope of application of this outstanding additive to other methodological aspects of peptide chemistry, such as the prevention of some of the base-mediated undesired reactions in Fmoc/tBu chemistry. Thus, we focused our efforts on the minimization of one of the most critical and frequently encountered side reactions, namely aspartimide formation, and also on the rarely documented but troublesome Pro-based overcoupling.[11] Using the peptide models shown in Figure 2, we evaluated the impact of the acidity of Oxyma (**3**) and compared it with the performance of classical benzotriazoles HOBt (**1**) and HOAt (**2**), not only in the reduction of base-driven impurities but also in the premature release of peptides bound to Barlos (2-chlorotrityl chloride) resin.[12]

METHODS

Aspartimide/Piperidide Formation

The target Fmoc-hexapeptide **4** was manually elongated on 3 g scale of Fmoc-protected Rinkamide-MBHA-PS-resin ($\delta = 0.45$ mmol/g), using Orn(Boc), Asp(OtBu), and Tyr(OtBu) as side chain protected amino acids. 30 min-couplings were conducted by using 0.4*M* solutions of Fmoc-amino acids (3 eq. excess) and Oxyma (3 eq.) in DMF, with 3-min preactivation with DIC (3 eq.), prior to addition to the resin. Fmoc removal cycles were performed by treatment of the peptide-resin with 20% piperidine in DMF for 1+5+5 min. Final

H-Ala-Orn-Asp-Gly-Tyr-Ile-NH_2 (**4**)

H-Tyr-Pro-Pro-Phe-Leu-NH_2 (**5**)

H-Gly-Phe-Leu-OH (**6**)

FIGURE 2 Model peptides used to perform comparative studies between the additives.

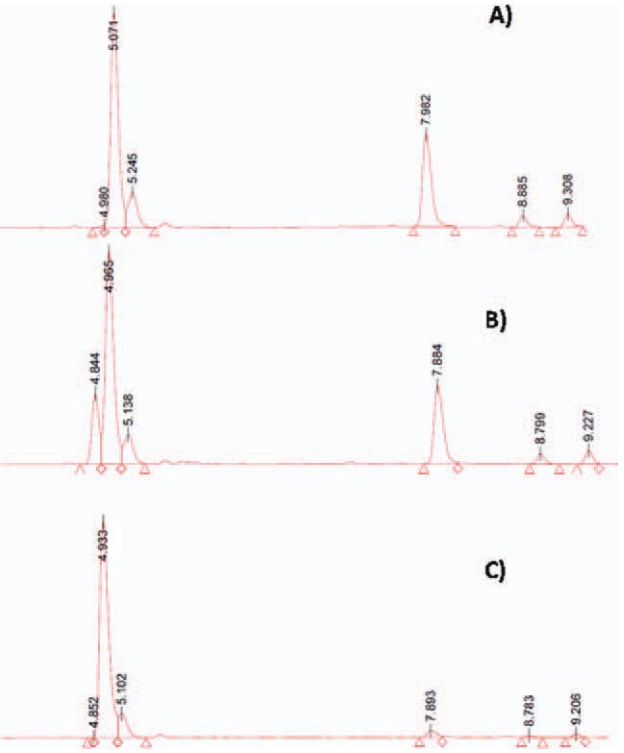


FIGURE 3 Reverse HPLC analysis on mixtures obtained after treatment of peptide model **4** with 20% piperidine in DMF for 6 + 6 h at rt, without additive (A) or containing 1*M* Oxyma (C). A coinjection with the pure synthesized β-peptide was performed to indentify this byproduct (B). The desired hexapeptide is observed at 5.0 min (see Materials and Methods for more details).

test deprotections were conducted with 30 mg of Fmoc-hexapeptide **4**, with a double 6 + 6 h treatment with the corresponding piperidine-additive mixture in DMF (1 ml each). In most experiments a white solid appeared after prolonged exposure with the basic cocktail. After DMF and DCM washings, the peptide chain was cleaved from the resin after addition of a TFA/H_2O (19:1) cocktail for 2 h at room temperature. The solution was then filtered and the resin was washed with DCM (1 ml × 2), which was removed along with TFA under nitrogen flow. The crude peptide was precipitated with cold diethyl ether (2 ml × 3) and after lyophilization, purity of target peptide and percentage of aspartimide and derived byproducts was checked on reverse phase HPLC (see Figure 3 and Table I), with the following conditions: Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm), linear gradient 7 to 20% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm; t_R (β-peptide) = 4.8 min, m/z = $[M+H]^+$ = 651.4; t_R (α-peptide) = 4.9 min, m/z = $[M+H]^+$=651.4; t_R (aspartimide) = 5.1 min, m/z = $[M+H]^+$ = 633.3; t_R (piperidide)= 7.9, 8.8, and 9.2 min, m/z = $[M+H]^+$ = 718.4. Byproducts were identified by coinjection with pure samples.

Pro-Mediated Overcoupling

Tripeptide H-Pro-Phe-Leu-resin was assembled on Fmoc-Rinkamide-AM-PS resin (δ = 0.59 mmol/g), using Tyr(OtBu) as side chain protected amino acid. Thirty minute couplings were

Table I Reduction of Aspartimide and Derived Byproducts During Basic Treatment of Fmoc-Ala-Orn-Asp-Gly-Tyr-Ile-Rinkamide-PS Resin in the Presence of Various Additives[a]

Entry	Concentration of Additive in 20% Piperidine/DMF	Additive	Target Peptide and Aspartimide-Derived Byproducts (%)			
			α-Peptide	β-Peptide[b]	Aspartimide	Piperidides[c]
1	—	—	55.62	0.23	11.06	33.09
2	0.1*M*	HOBt (**1**)	59.72	0.38	9.88	30.02
3		HOAt (**2**)	60.59	0.22	10.43	28.76
4		Oxyma (**3**)	62.75	0.26	9.36	27.62
5	0.5*M*	HOBt (**1**)	65.87	0.23	12.75	21.16
6		HOAt (**2**)	67.11	0.26	13.54	19.08
7		Oxyma (**3**)	74.74	0.51	8.08	16.67
8	1*M*	HOBt (**1**)	79.00	0.25	14.03	6.72
9		HOAt (**2**)	79.68	0.13	14.36	5.82
10		Oxyma (**3**)	85.59	0.10	9.60	4.67

[a] Fmoc-hexapeptide **4** was treated with 20% piperidine in DMF in the absence or presence of 0.1–1.0*M* additive for 6 + 6 h at rt.
[b] The mass of the β-peptide would match that of the epimer of the α-peptide. However, the identity of the β-peptide is confirmed by coinjection with a pure sample, manually synthesized using Fmoc-Asp-OtBu.
[c] Up to 3 peaks containing the mass of the corresponding piperidide were observed in a ratio 10:1:1.5 (t_R = 7.9, 8.8, and 9.2 min, see Figure 3).

performed by preactivating 0.4*M* solutions of Fmoc-amino acids (3 eq. excess) and Oxyma (3 eq.) in DMF, with DIC (3 eq.) for 5 min, prior to addition to the resin. Fmoc removal steps were carried out by treatment of the peptide-resin with 20% piperidine in DMF for 1 + 5 + 5 min. 100 mg of the tripeptide were swelled in DCM for 20 min, conditioned in DMF and treated with 0.1*M* solutions of additives (1 ml × 2) for 1 min. Resins turned bright yellow with HOAt and bright yellow–orange with Oxyma. After DMF washings to eliminate the excess of additive solution (1 ml × 5; 30 sec.) a 0.5*M* solution of Fmoc-Pro-OH or Fmoc-Tyr-OH was added and stirred for 2 h to promote early Fmoc removal. Then, DIC (3 eq.) and additive (3 eq.) in DMF were added to the mixture for a final concentration of reagents of 0.3*M*. After a 3-h coupling, resin was washed with DMF and DCM and the peptide chain was cleaved from the resin after addition of a TFA/H_2O (9:1) cocktail for 2 h at room temperature. The solution was then filtered and the resin was washed with DCM (1 ml × 2), which was removed along with TFA under nitrogen flow. The crude peptide was precipitated with cold diethyl ether (3 ml × 3) and lyophilized in H_2O, prior to HPLC analysis (see Table II), using a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm), linear gradient 15 to 21% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 30 min at 40°C (to suppress the presence of different conformations), with detection at 220 nm; t_R (des-Pro) = 13.6 min, m/z = $[M+H]^+$ = 538.3; t_R (pentapeptide)= 17.6 min, m/z = $[M+H]^+$ = 635.3; t_R (+Pro) = 18.9 min, m/z = $[M+H]^+$ = 732.4; t_R (piperidide) = 27.6 min, m/z = $[M+H]^+$ = 798.4. Byproducts were identified by coinjection with pure samples.

Compatibility with 2-Chlorotrityl-Chloride Resin

The target tripeptide H-Gly-Phe-Leu-OH (**6**) was manually assembled on a 2 g scale on 2-chlorotritylchloride-PS-resin (δ = 1.55 mmol/g). First residue was introduced with standard protocols with this resin in DCM, adding a large excess of DIEA with respect to Fmoc-Leu-OH (10 eq.) and let it mix for 55 min (3 eq. of the base were added simultaneously with the amino acid, and extra 7 eq. 10 min later). Then MeOH was added to effect capping of the resin. Loading was recalculated (0–48 mmol/g) after Fmoc removal with 20% piperidine in DMF (1 + 5 + 5 min treatment). Fmoc-Phe-OH and Fmoc-Gly-OH were introduced by means of 30 min-couplings, using 0.4*M* solutions of Fmoc-amino acids (3 eq. excess) and Oxyma (3 eq.) in DMF, with 3-min preactivation with DIC (3 eq.), prior to addition to the resin. Sample and reference experiments of compatibility of resin **15** with *N*-hydroxylamines were conducted with 25 mg of tripeptide-resin. A full cleavage reference was performed by treating the resin with 5% TFA in DCM (1 ml × 5; 5 min), followed by DCM washings (1 ml × 2) and removal of solvent under nitrogen flow. To evaluate premature cleavage with various *N*-hydroxylamines, 0.1*M* solutions in DMF (1 ml) were mixed with resin **15**, previously swelled in DCM (20 min.) and conditioned in DMF (1 ml × 5). After treatment with the resin, filtrates were collected and resin was washed with DMF and DCM. Solvents

Table II Minimization of Pro-Driven Overcoupling of Activated Residues by Previous Treatment of Pro-Peptide-Resin with Various Additives[a]

Entry	Additive	Pentapeptide (%)	+Pro (%)[b]	+Tyr (%)[b]
1	—	96.86	0.56	2.58
2[c]	HOBt (**1**)	98.37	0.45	1.18
3[c]	HOAt (**2**)	98.69	0.44	0.87
4[c]	Oxyma (**3**)	98.44	0.39	1.17

[a] Misincorporation of Pro was observed in all experiments: 1.9% (no additive), 2.7% (HOBt), 1.2% (HOAt) and 0.9% (Oxyma).
[b] To assure complete incorporation of any accidentally unprotected residues in solution into the peptide chain, 3-h couplings were performed and completion was monitored by chloroanyl visual tests.
[c] Initial 1-min (x2) washings with a 0.1*M* solution of the corresponding additive in DMF were performed, carrying out the coupling in presence of the same additive.

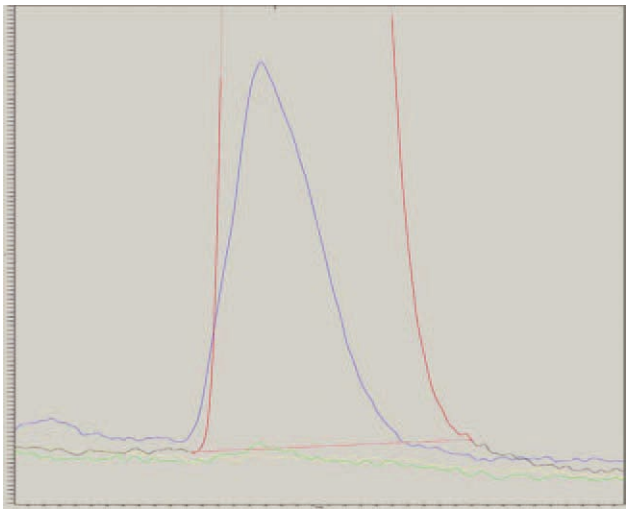


FIGURE 4 Reverse HPLC analysis on samples showing premature cleavage of tripeptide **6** from 2-chlorotrityl chloride resin (**15**), after 5 min (yellow), 1 h (green), and 24 h (blue) treatment with Oxyma (**3**), relative to 5% TFA cleavage (red) as reference.

were evaporated under reduced pressure (DMF coevaporated with toluene). The corresponding white solids (except the runs with Oxyma, in which a yellow oil was obtained) were disolved in H_2O/CH_3CN 1:1 (8 ml) and 23 μl were injected onto reverse-phase HPLC (see Figure 4 and Table III). A Waters SunFire C18 Column (3.5 μm, 4.6 $\times$ 100 mm) was used, using a 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; t_R (H-Gly-Phe-Leu-OH, **6**) = 4.0 min, m/z = $[M+H]^+$ = 336.3. Presence or absence of tripeptide **6** was confirmed after coinjection with a pure sample and by HPLC-MS analysis. The corresponding additives were also observed in the mixture.

Table III Percentage of Premature Cleavage of Peptide H-Gly-Phe-Leu-OH (6) From the Highly Acid Labile 2-Chlorotrityl Chloride Resin (15) Induced by Various Acidic Additives[a,b]

Entry	Additive	Treatment Time	Peptide **6** Released (%)
1	HOBt (**1**)	5 min	0
2		1 h	0.29
3		24 h	5.64
4	HOAt (**2**)	5 min	0.20
5		1 h	0.33
6		24 h	6.30
7	Oxyma (**3**)	5 min	0
8		1 h	0.29
9		24 h	5.51

[a] Percentage of cleavage is shown as relative absorbance, in reference to a sample obtained by treating resin **15** with 5% TFA/DCM. A blank of the treatment was also performed.

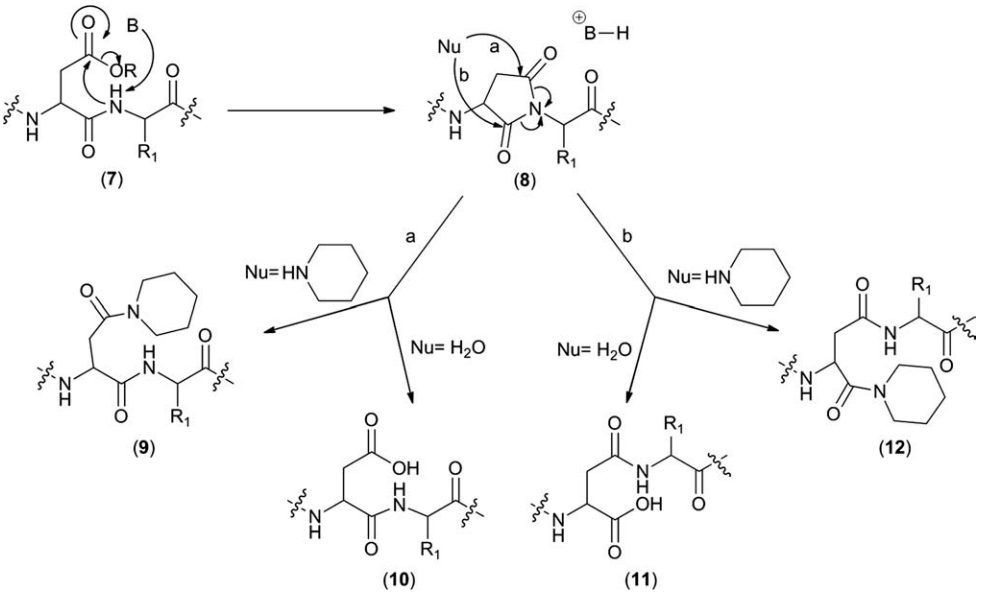
[b] To minimize interference of HCl traces dissolved in DCM, solvent was purified under basic alumina (Al_2O_3).

RESULTS AND DISCUSSION

Aspartimide Formation

In recent years, the advantageous properties of aspartimide-containing compounds have attracted increasing interest because of their application as chemical tools in biotechnology, chemical biology, and material science.[13] However, the undesired formation of such cyclic constructs, originated in Asp residues, is still regarded as one of the major unsolved side reactions during peptide/protein synthesis. This unwanted intramolecular cyclization was originally observed in Boc SPPS, in the strong acidic media required for cleavage of the peptide from the resin (HF or TFMSA) or N^{α}-Boc removal (HBr or TFA) and under basic N^{α}-neutralization and coupling steps with tertiary amines.[4a,14,15] Nevertheless, the extent of this side reaction is dramatic in the Fmoc/tBu approach, since the repetitive piperidine treatments, required for Fmoc removal, increasingly reduce the percentage of unmodified peptide in each cycle after the introduction of Asp in the sequence.[11a,14] Base-mediated formation of aspartimide-peptide structures and derived byproducts in piperidine-containing cocktails is depicted in Scheme 1.

The internal cyclization of the Asp-containing peptide (**7**) is initiated by the nucleophilic attack of the backbone amide group of the preceding residue onto the β–carboxyl moiety of the side chain protecting group of aspartic acid. Basic-catalysis renders this aminosuccinyl-like structure (**8**) faster than in acidic conditions, although during HF cleavage of the peptide, aspartimides are reported to occur even in the unprotected side chain carboxylic acid.[11a,15,16] Therefore, aspartimide units (often referred to as Asi or Asc) result in backbone-modified structures that are at the same time reactive enough to undergo ring opening by nucleophiles.[17] Hydrolysis of Asi residues can take place in both succinimide carboxyl groups, either restoring the original α–peptide (**10**, path A, Scheme 1) or giving rise to the isoaspartyl-β–peptide (**11**, path B, Scheme 1), which permanently modifies the peptide backbone. Given the structural similarity of these two isomers, difficulties arise when attempting to separate them, especially when handling long peptides.[18] In addition, piperidine is nucleophilic enough to remain attached to the Asi cyclic structure, thereby giving rise to the corresponding piperidides of the α– and β-peptide (**9** and **12** respectively, Scheme 1).[10,11a,19] In summary, only 25% of the possible aspartimide-opening byproducts would lead to the unmodified target peptide. The true scenario is even worse, bearing in mind that the percentage of piperidides increases as does the number of Fmoc-removal cycles when Asp is included in the sequence and also that β-peptides (**11** and **12**) are preferentially formed in DMF, the regular solvent used in SPPS



SCHEME 1 Intramolecular base-catalyzed mechanism of aspartimide formation and subsequent breakdown by nucleophiles.

(Solid Phase Peptide Synthesis).[20] Moreover, the aspartimide cyclic ring is more sensitive towards racemization than the starting open sequence and thus even more detrimental impurities are obtained. The presence and amount of aspartimide and its derived opening byproducts are dependent on many factors, including the β–carboxyl group, temperature, solvent, resin, sequence and conformation.[10,11b,17,19b]

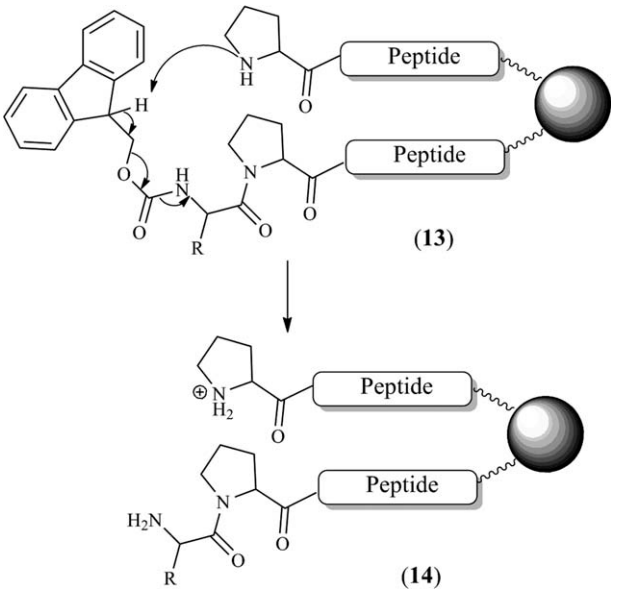
Several strategies have been envisaged to decrease or, at least prevent, the abovementioned cyclization of Asp residues. Some of these consist of enhancing the sterical hindrance of the base or β-carboxy side chain protecting group used, with the idea of impeding the initial attack of the amide backbone moiety.[19b,21] Alternative approaches are based on the pseudo Pro methodology, by the introduction of preformed Asp-X dipeptide building blocks, which avoid the risk of internal cyclization.[17,22] Mild-cleavable orthogonal N^{α}-protecting groups have also been described.[23] However, all these strategies are relatively expensive, time-consuming, or require extra steps, thereby limiting their widespread application. In contrast, the addition of *N*-hydroxylamines in the N^{α}-amino deprotection cocktails is a readily available, efficient, and cost-saving choice to prevent aspartimide formation. The advantages of the presence of HOBt (**1**) or HOSu (*N*-hydroxysuccinimide) in basic-catalyzed Asi formation were initially reported during coupling in the Boc SPSS approach and were later studied in Fmoc-removal cocktails, using Fmoc/tBu protection.[4a] Substantial reduction of aspartimide-derived byproducts can be obtained by using 2% HOBt solutions in 20% piperidine in DMF, resulting in a remarkable increase of the target peptide.[10,19b] Such minimization of Asp cyclization is probably due to competition of the *N*-hydroxylamine with the acidic Asp-X backbone amide group for the base, which is thought to be the crucial step of aspartimide formation.[4a] Markedly acidic non-*N*-hydroxylamine additives, like deactivated phenols, are also known to induce a similar effect, although the mechanism is unclear.[10] In this regard, hypotheses have been proposed about the formation of salt-like adducts when phenols are mixed with tertiary amines, such as Et_3N.[4a]

To further test the capacity of *N*-hydroxylamines to decrease the internal cyclization of Asp residues in piperidine-based cocktails and the effect of the acidic group-containing template, here we compared the performance of Oxyma (**3**) with that of the benzotriazoles HOBt (**1**) and HOAt (**2**). On the basis of previous articles reporting their sensitivity towards this side reaction, hexapeptide Fmoc-Ala-Orn-Asp-Gly-Tyr-Ile-NH_2 (**4**, Figure 2) was selected as the peptide model to conduct the comparison.[24] Aspartimide formation is severe in Asp-Gly sequences, since the absence of C^{α}-alkyl side chain in the preceding residue to Asp enhances the nucleophilicity of the amide backbone nitrogen.[11] Furthermore, the Asp-Gly-Tyr-Ile domain originally contained within the 1–6 fragment of toxin II of scorpion *Androctonus australis Hector* (H-Val-Lys-Asp-Gly-Tyr-Leu-NH_2) gives extra propensity to this internal cyclization.[4a,14,16] The nature of the β-carboxy-protecting group also affects the extent of the side reaction, as this moiety is the leaving group on the determining step.[14,18a] In the

present synthesis, Asp(OtBu) was used because it is easily available and known to produce a significant percentage of aspartimide-derived impurities, in comparison with the more bulky OAda and OPhiPr or with OMpe and ODie, which are highly flexible and efficient in preventing Asp to Asi conversion.[11b]

Synthesis of target peptide **4** took place by means of standard DIC/Oxyma (**3**), coupling cycles starting from Fmoc-Rinkamide-PS resin. Once the Fmoc-protected hexapeptide was successfully assembled, the extent of intramolecular cyclization of Asp (and consequently, the amount of derived impurities) was maximized in order to achieve clearer comparison of the effects of the additives. Thus a 6 + 6 h double treatment of model peptide **4** with a 20% piperidine in DMF cocktail, mimicking the effect of repetitive Fmoc-removal steps, was found to cause up to 45% of aspartimide-related side products. Since increasing amounts of additive induced greater suppression of this undesired event, experiments conducted in the presence of various *N*-hydroxylamine concentrations, in the range 0.1*M*–1*M*, were performed. Results are summarized in Table I.

Unexpectedly, under the experimental conditions, three piperidides were observed in the ratio 10:1:1.5 (see peaks at 7.9, 8.8, and 9.2 in Figure 3). Taking into account the preferred ring-opening via path B in Scheme 1 when DMF is used as solvent, major piperidide is expected to be based on the isoaspartyl-β-hexapeptide (**11**). Epimerization on the C^{α} could lead to an extra form of piperidides. On the one hand, treatment of the Fmoc-hexapeptide **4** with 0.1*M* solutions of the additives induced a slight increase on the target α–peptide purity (4–7%, entries 2–4). On the other hand, higher concentrations (0.5*M* and especially 1*M*) achieved major improvements on the percentage of desired peptide, by substantially decreasing the amount of aspartimide and piperidides (entries 5–10). Experiments conducted in the presence of Oxyma (**3**) clearly produced a higher content of desired unmodified hexapeptide **4** than analogous tests with HOBt (**1**) and HOAt (**2**), which rendered similar purities. Oxyma (**3**) successfully reduced the severity of aspartimide and piperidide formation under all the concentrations tested (entries 4, 7, and 10). Of note was the minimization of internal Asp cyclization when 1*M* solutions of Oxyma (**3**) in 20% piperidine in DMF were used as Fmoc-removal cocktails (see Figure 3), thus enhancing the α–peptide purity by 30% compared with the test conducted in the absence of additive (entries 1 vs. 10). It is also worth noting that the superiority of Oxyma (**3**) over benzotriazoles cannot be explained by the relative acidities of Oxyma (**3**, pKa = 4.60), HOBt (**1**, pKa = 4.60) and HOAt (**2**, pKa = 3.28), suggesting the influence of additional, undisclosed factors on aspartimide prevention. In this regard, the lower bulkiness of Oxyma than the benzotriazoles could result in stronger competition with the acidic backbone peptide proton for piperidine.



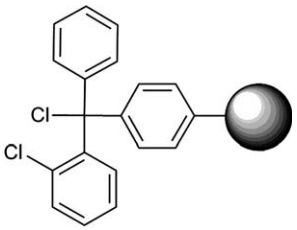
SCHEME 2 Mechanism of accidental Fmoc removal of recently incorporated residues by Proline.

Pro-Based Overcoupling

Fmoc N^{α}-amino protection is generally regarded as compatible with standard basic coupling conditions, generally carried out in the presence of DIEA (pKa = 10.1), which is less basic than the piperidine required for the deprotection step (pKa = 11.1).[25] However, the coupling cocktail may contain other basic amines that are also capable of removing the Fmoc protecting group to some extent. This is the case of proline, whose secondary amine character (pKa = 10.6) confer it the highest α-amino basicity among proteinogenic amino acids. Although scarcely reported to date, accidental Pro-mediated overcoupling of the residue being incorporated may occur as result of the high basicity of this amino acid. This undesired deprotection is expected to be more pronounced when the Fmoc-amino acid is already introduced in the resin-bound peptide, due to the higher effective concentration of peptide and spatial proximity between growing peptide chains (Scheme 2). Once the corresponding activated amino acid has been incorporated into a proline, a vicinal, still unacylated proline at the *N*-terminus of the elongated peptide might act as basic center substracting the 9-fluorenyl proton, thereby initiating the removal of Fmoc (**13**). Consequently, another molecule of activated Fmoc-amino acid can be introduced on the now N^{α}-amino unprotected H-aa-Pro-peptide (**14**), thereby resulting in overcoupling.

Given the role of Pro as basic center in this premature deprotection, and bearing in mind the remarkable basicity/nucleophilicity-masking properties exhibited by *N*-hydroxylamines **1–3** in the prevention of aspartimide formation, their capacity to reduce the impact of Pro-mediated overcoupling was analyzed during elongation of H-Tyr-Pro-Pro-Phe-Leu-NH_2 (**5**, Figure 2). This Leu-enkephaline modified peptide model presents two consecutive prolines in central positions and therefore, two distinct overcoupling-promoting sites. The experimental design was to quantitatively obtain the resin-bound H-Pro-Phe-Leu tripeptide, using Fmoc-Rinkamide-AM-PS and DIC/Oxyma standard couplings, and to study the extent of overcoupling at Pro^3 and Pro^4 after short treatment of the resin with 0.1*M* solutions of various *N*-hydroxylamines. In the absence of previous *N*-hydroxylamine washings, hexapeptides presenting extra Pro and Tyr were observed under regular coupling conditions (0.12% combining both byproducts). Although apparently insignificant, such a low percentage can cause major problems when synthesizing APIs (Active Pharmaceutical Ingredients). However, for comparison purposes, the percentage of +Pro and +Tyr was exaggerated by mixing the *N*-hydroxylamine-treated peptide-resin with a solution of the Fmoc-amino acid in DMF for 2 h, prior to addition of the coupling cocktail (DIC and corresponding *N*-hydroxylamine as additive). Results are shown in Table II. The possible effect of the acidity of Fmoc-Pro/Tyr-OH in the reduction of Pro-overcoupling is included in the experiment without additive (entry 1), therefore, results obtained in the experiments performed after treatment of the peptide-resin with 0.1*M* *N*-hydroxylamine are unequivocally attributted to the presence of these additives (entries 2, 3, 4).

An appreciable increase in the purity of the peptide was achieved with an initial double 1-min treatment of the Pro-peptide with HOBt (**1**), HOAt (**2**) or Oxyma (**3**), compared to a control experiment without any previous washing under DIC couplings (entry 1 vs. 2–4). Overcoupling of Tyr occurred to a higher extent than Pro in all the assays, but no simultaneous +Pro,Tyr heptapeptide was detected. Oxyma (**3**) was the most efficient *N*-hydroxylamine in reducing the presence of +Pro hexapeptide and presented a similar percentage of +Tyr to that detected in the experiment with HOBt (**1**). The 7-aza analogue of HOBt (**2**) afforded the lowest amount of the hexapeptide with an additional Tyr, with an overall percentage of pentapeptide close to 99%. Oxyma (**3**) showed an intermediate overall performance between HOBt (**1**) and HOAt (**2**). Nevertheless, a certain degree of des-Pro deletion peptide was observed in all cases, Oxyma (**3**) being the most remarkable agent in terms of coupling efficiency (see Table II footnote b) and consequently achieving a similar overall purity to that attained by HOAt (**2**), and at a lower cost. However, the exact mechanism of action of *N*-hydroxylamines remains unclear. Resins became brightly colored after initial washings (yellow with HOAt, yellow-orange with Oxyma), thereby suggesting the presence of the anion as result of acid-base exchange with Pro. However, this interaction did not completely suppress the nucleophilicity of Pro, since couplings were quantitative in 3 h or less.



(**15**)

FIGURE 5 Structure of polystyrene-based 2-chlorotrityl chloride resin.

Compatibility with 2-Chlorotrityl Chloride Resin

The convenience of the acidic nature of *N*-hydroxylamines discussed here might depend on the synthetic strategy chosen. Thus, a highly acid-labile resin or linker is sometimes required to obtain a fully side chain-protected peptide upon cleavage, to be used in the next fragment coupling step or cyclization. For this purpose, 2-chlorotrityl chloride resin (**15**, Figure 5, alternatively referred to as Barlos resin) has become popular as a *C*-terminus peptide acid-generating solid support, subject to cleavage upon diluted (1–5%) TFA treatment.[12] In this context, premature cleavage of the peptide from this resin, caused by the acidic pH induced by *N*-hydroxylamines, such as HOBt (**1**) or HOAt (**2**), has been generally accepted to occur to a low extent, but has never been tested or proved. Therefore, the additional goal of the present study was to study the ratio of undesired peptide release from the mild acid-cleavable resin **15**, when various *N*-hydroxylamines, including Oxyma (**3**), are present in the media

Tripeptide H-Gly-Phe-Leu-OH (**6**, Figure 2), optimally assembled in Barlos resin (**15**) by means of DIC/Oxyma (**3**) couplings, was selected as peptide model to study the drawbacks associated with HOBt (**1**) and HOAt (**2**), and Oxyma (**3**), when such an acid-labile solid support is used. To maximize the potential adverse effects of these acidic additives, 0.1*M* solutions in DMF were mixed with resin **15** in the absence of other reagents commonly found in coupling cocktails such as Fmoc-aa-OH or carbodiimide. With the aim to monitor the progressive release of peptide **6**, various treat-

ment times were examined (5 min, 1 h and 24 h), measuring the absorbance of filtrates at 220 nm, compared to a full cleavage reference. Table III shows the percentages of premature cleavage from 2-chlorotrityl chloride resin (**15**) with the distinct additives.

For each *N*-hydroxylamine used, progressive cleavage was observed with time (see Figure 4). After short 5-min treatments, only a minimum amount of peptide **6** was detected in the experiment conducted in the presence of HOAt (**2**) (entry 4 vs. 1, 7). Higher rates of cleavage were also observed with this additive than with HOBt (**1**) or Oxyma (**3**) after more prolonged exposure to Barlos resin (entries 5, 6 vs. 2, 3, 8, 9). This tendency is in agreement with the expected behavior regarding the relative pKa values, HOAt (**2**) being the most acidic *N*-hydroxylamine (3.28 vs. 4.60) and so the one rendering the highest percentage of released peptide **6**. The similar acidity of HOBt (**1**) and Oxyma (**3**) resulted in almost identical values. However, it must be noted that after 24 h of contact with 2-chlorotrityl chloride resin (**15**), percentages of undesired cleavage of >5% were observed, regardless of the type of additive used.

CONCLUSIONS

N-hydroxylamines with pKa in the range 3–10 have been extensively used as additives to carbodiimides, assisting the formation of the native peptide bond. However, their capacity to reduce the impact of a wide range of base-driven side reactions, also as a result of their unusual high acidity, makes them a much more versatile tool than a simple catalyst in the context of peptide chemistry. Here, we show that addition of *N*-hydroxylamines to a Fmoc-removal cocktail or short treatments, prior to couplings onto Pro-peptide resins, greatly minimizes the presence of undesired byproducts. It has been observed that more concentrated solutions further increase the potential of these additives, as noted in the case of aspartimide prevention, where the percentage of unmodified α–peptide can increase up to 30%. Thus, *N*-hydroxylamines are a cost-saving, easily available alternative to full suppression methods. In addition, we have demonstrated that the low pH induced by these *N*-hydroxylamines causes minimal loss of peptide during syntheses carried out in mild acid-labile resins, such as 2-chlorotrityl chloride. However, caution should be taken during long treatments with this solid support.

Among these *N*-hydroxylamines, the recently rediscovered ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) shows promising results as a suppressing agent of these base-derived side reactions. Furthermore, its low cost and safe decomposition make Oxyma an attractive alternative to classical benzotriazoles. The minimization capacity of piperidides and other aspartimide-related byproducts supersedes that of HOBt and even HOAt, in the proposed hexapeptide model at all the concentrations tested. A substantial increase in the purity was achieved when using 1*M* solutions of Oxyma in 20% piperidine in DMF. With regard to the Pro overcoupling, Oxyma showed a similar performance to HOBt, with a much greater coupling efficiency. Interestingly, although the pKa of Oxyma is the same as that of HOBt (4.60) and higher than that of HOAt (3.28), the performances observed in the reduction of base-driven unwanted reactions did not follow this acidity trend. This observation suggests that other factors participate in the interaction between the *N*-hydroxylamine and the basic center. The sterical hindrance of the benzotriazole ring, in comparison to the much smaller α,α–disubstituted oxime, may be responsible for this behavior. However, in the premature cleavage of peptide from acid-labile Barlos resin, which depends only on the pH generated, the expected tendency between the additives is observed.

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