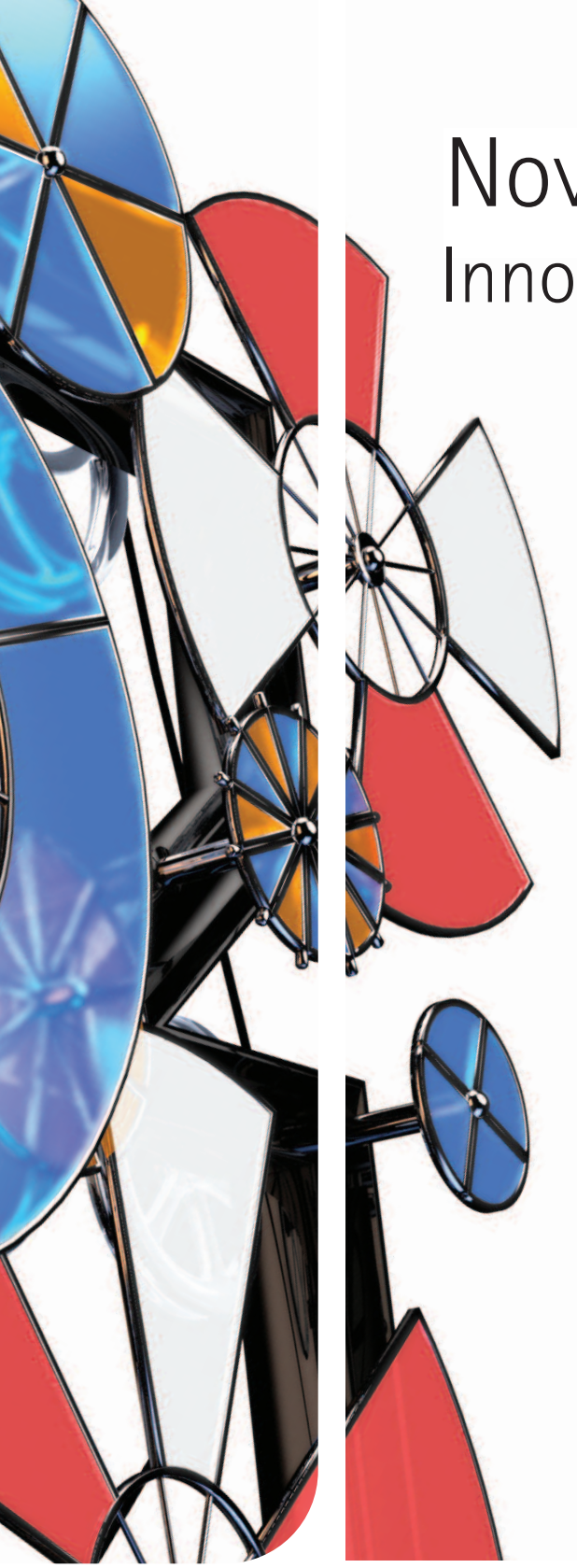

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Innovations: 2/09

Non-explosive replacement for HOBt

1-Hydroxybenzotriazole (HOBt) is one of the most widely used reagents in peptide synthesis, owing to the excellent reactivity and chiral stability of benzotriazolyl (Bt) esters of amino acids and peptides (Figure 1) [1]. Recently, HOBt monohydrate, the standard form of this reagent, was reclassified by the UN as a class 1c explosive [2]. This measure, unfortunately, means that we can no longer ship the product by air or sea and has the effect of making land shipment prohibitively expensive.

The lack of availability of HOBt has caused enormous difficulties to peptide chemists and has led to a search for a non-explosive alternative to HOBt. One candidate, ethyl 2-cyano-2-(hydroxyimino)acetate, also known as Oxyma Pure, shows particular promise. Subiros-Funosas, *et al.* [3] have shown this reagent to be more effective and give lower racemization than HOBt in carbodiimide-mediated coupling reactions. In this Innovation, we present their results supplemented with work from our laboratories demonstrating the efficacy of Oxyma Pure.

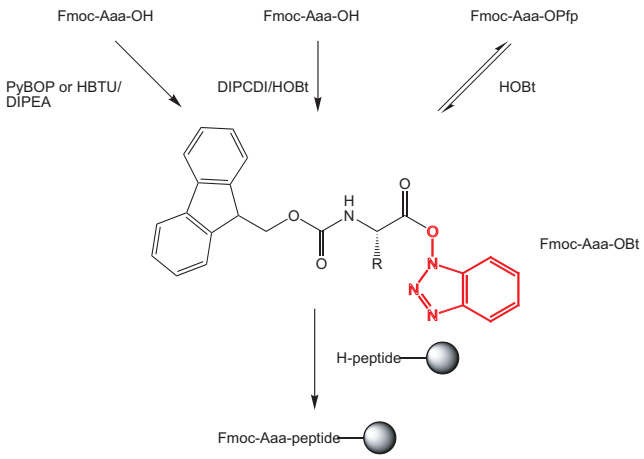
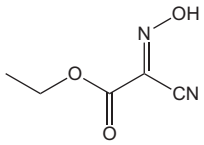


Fig. 1: Common routes to Bt ester formation.

Oxyma Pure



Oxyma Pure was first identified as a potential coupling additive in the 1970s [4, 5]. It has a pK_a of 4.60, the same as HOBt, but lacks the potentially explosive triazole structure of HOBt and analogous compounds such as HOAt [6] and Cl-HOBt [7] (Table 1).

Table 1: pK_a of Oxyma Pure and benzotriazole-based coupling reagents.

Compound	pKa
HOAt	3.28
HOBt	4.60
6-Cl-HOBt	3.35
Oxyma	4.60

Like HOBt, Oxyma Pure reacts with protected amino acids or peptides in the presence of carbodiimides to form active esters capable of acylating amines (Figure 2). The reaction initially generates a highly reactive *O*-isoacylurea. This intermediate can rearrange to form an unreactive *N*-acylurea, undergo racemization *via* oxazolone formation or enolization, or form a symmetrical anhydride. The function of HOBt in such reactions is to convert the *O*-isoacylurea to the less reactive and more chirally stable Bt ester. Therefore, to evaluate Oxyma Pure in the role of HOBt, it is necessary to evaluate its performance both in suppressing racemization and enhancing coupling efficiency.

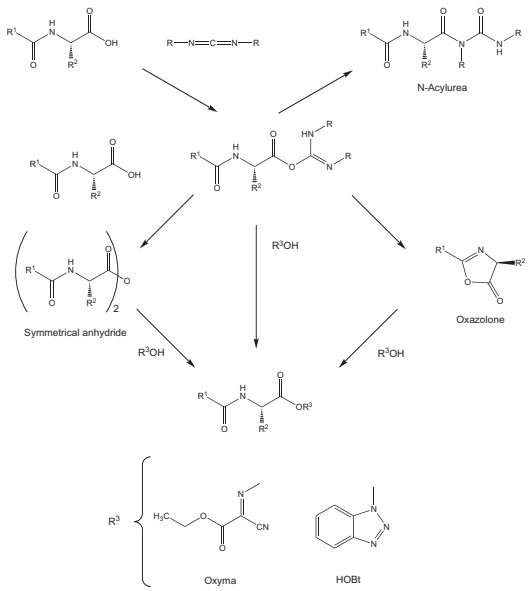


Fig. 2: Possible reaction pathways for carbodiimide activation in presence of Oxyma Pure or HOBt.

Racemization

For racemization studies, Subiros-Funosas, *et al.* [3] selected the coupling of the notoriously racemization prone Z-Phg-OH to H-Pro-NH_2, the fragment condensation of Z-Phe-Val-OH to H-Pro-NH_2 and the solid phase synthesis of H-Gly-Cys-Phe-NH_2 as model systems. In these tests (Tables 2 - 4), Oxyma Pure performed extremely effectively giving levels of racemization comparable to HOAt and better than HOBt. Moreover, the yields of products were higher for Oxyma Pure than both HOBt and HOAt.

Table 2: Product composition obtained from the coupling of Z-Phg-OH to H-Pro-NH_2.

Coupling method	Yield	DL (%)
HOAt/DIPCDI	81	3.3
HOBt/DIPCDI	82	9.3
Oxyma/DIPCDI	90	1.0
Oxyma/DIPCDI	88	1.1

Table 3: Product composition obtained from the coupling of Z-Phe-Val-OH to H-Pro-NH_2.

Coupling method	Yield	DL (%)
HOAt/DIPCDI	86	2.1
HOBt/DIPCDI	79	8.9
Oxyma/DIPCDI	90	3.8

Table 4: Product composition obtained from the solid phase synthesis of H-Gly-Cys-Phe-NH_2.

Coupling method	Yield	DL (%)
HOAt/DIPCDI	88	0.1
HOBt/DIPCDI	84	0.2
Oxyma/DIPCDI	91	0.1

As a further test, step-wise solid phase syntheses of the ABRF test peptide was undertaken on a PTI Symphony using HOBt/DIPCDI and Oxyma Pure/DIPCDI activation. The resulting peptidyl resins were subjected to total acid hydrolysis in deuterium chloride, and the extent of racemization of certain amino acids was determined by chiral-GC/MS. In this case, the results given by HOBt and Oxyma Pure were comparable, with the exception of that for histidine. This result may be a reflection of the slower formation of the Oxyma Pure active ester noted by Subiros-Funosas, *et al* [3].

Table 5: Racemization of key residues during SPPS of ABRF peptide using DIPCDI/HOBt and DIPCDI/Oxyma coupling.

Amino acid	HOBt (%D)	Oxyma (%D)
Arg	0.1	0.1
Asx	0.1	0.1
Cys	0.3	0.3
His	0.4	0.8

Coupling efficiency

Subiros-Funosas, *et al.* [3] compared the coupling efficiencies obtained with Oxyma Pure, HOBt and HOAt in solid phase synthesis of enkephalin analogs containing MeGly, MeAla and Aib residues in place of Gly residues. In all cases, Oxyma Pure/DIPCDI activation gave consistently better results than HOBt/DIPCDI and in some cases as good or if not better results than HOAt/DIPCDI.

Table 6: Product composition obtained from synthesis of H-Tyr-MeGly-MeGly-Phe-Leu-NH_2 with 5 min coupling times.

Coupling	Yield	des-MeGly (%)	des-Tyr (%)
HOAt/DIPCDI	95	1.4	3.2
HOBt/DIPCDI	85	7.5	6.6
Oxyma/DIPCDI	91	3.8	4.2

Table 7: Product composition obtained from synthesis of H-Tyr-MeAla-MeAla-Phe-Leu-NH_2 with 30 min coupling times.

Coupling	Yield	des-MeAla (%)	des-Tyr(%)
HOAt/DIPCDI	74	23.2	3.1
HOBt/DIPCDI	46	38.1	15.2
Oxyma/DIPCDI	79	16.2	4.0

Table 8: Product composition obtained from synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2 with 30 min coupling times.

Coupling	Yield	des-Aib (%)	des-Tyr(%)
HOAt/DIPCDI	11.3	86.3	1.8
HOBt/DIPCDI	3.0	91.0	5.1
Oxyma/DIPCDI	28.0	70.5	0.4

SPPS using Oxyma Pure

In practice, Oxyma Pure can be used in an identical manner to HOBt in carbodiimide mediated couplings. Oxyma Pure can be dissolved in DMF and used as a solution on automated synthesizers in place of the standard HOBt/DMF solution. The instrument can be programmed to deliver Oxyma Pure/DMF and DIPCDI in either DMF or DCM to the amino acid derivative, and mixture allowed to preactivate for 2 - 10 minutes before the activated amino acid solution is transferred to the reaction vessel containing the resin.

Table 9: Sequences prepared by automated synthesis using Oxyma Pure/DIPCDI and HOBt/DIPCDI.

Peptide	Sequence
1	H-Lys-His-Asp-Pro-Cys-Gly-Trp-Asn-Gly-Pro-Arg-Pro-Met-Arg-Gly-NH_2
2	H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2
3	H-Gly-Asp-Phe-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-NH_2

To demonstrate the efficacy in automated solid phase peptide synthesis, Oxyma Pure and HOBt were used in the syntheses of the peptides shown in Table 9. All syntheses were carried out on a PTI Symphony using 4-fold excesses of Fmoc-amino acids coupled for one hour. Activation was carried out by delivering 0.3 M solutions in DMF of Fmoc-amino acids and Oxyma Pure or HOBt to the peptidyl resin, followed by 0.3 M DIPCDI in DMF. Following cleavage and isolation the crude peptides were analyzed by HPLC. The results obtained using HOBt and Oxyma Pure were essentially identical.

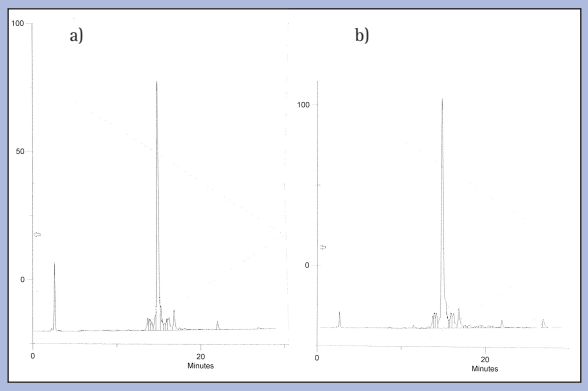


Fig. 3: HPLC profiles of crude peptide 1 prepared with a) HOBt and b) Oxyma.

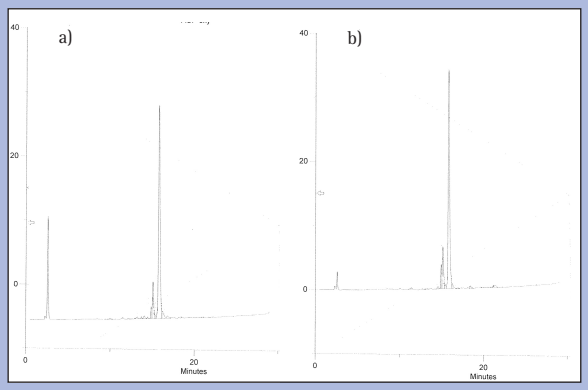


Fig. 4: HPLC profiles of crude peptide 2 prepared with a) HOBt and b) Oxyma.

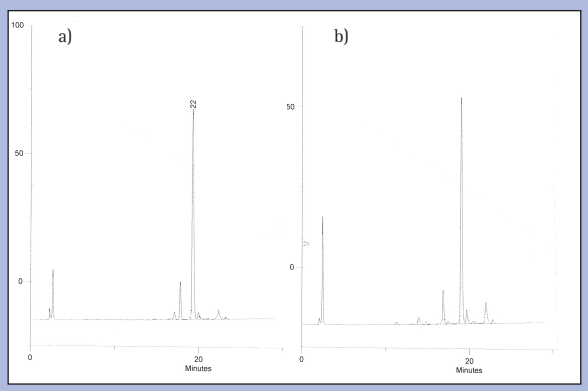


Fig. 5: HPLC profiles of crude peptide 3 prepared with a) HOBt and b) Oxyma.

Ordering information

851086	Oxyma Pure	25 g
NEW		100 g

Oxyma-based coupling reagents

851085	COMU	5 g
NEW		25 g
		100 g
851088	TOTU	5 g
NEW		25 g
		100 g

Other coupling reagents

851087	PyClocK	5 g
NEW		25 g
		100 g
01-62-0001	BOP	5 g
		25 g
		100 g
01-62-0041	HATU	5 g
		25 g
01-62-0010	HBTU	5 g
		25 g
		100 g
01-62-0038	HCTU	5 g
		25 g
		100 g
01-62-0021	MSNT	1 g
		5 g
		25 g
01-62-0016	PyBOP®	5 g
		25 g
		100 g
01-62-0017	PyBrOP®	5 g
		25 g
		100 g
01-62-0015	TBTU	5 g
		25 g
		100 g
01-62-0011	WSC	5 g
		25 g

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