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The role of HOBt in coupling reactions

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]. Last year HOBt monohydrate, the standard form of this reagent, was reclassified by the United Nations as a desensitized explosive (UN3380). 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. Consequently, we have had no choice but to withdraw this product. In this innovation we examine the implications of this decision on customers using uronium or phosphonium reagents like HBTU or PyBOP®, where it is current practice to include HOBt in the coupling reaction. We find that omitting HOBt from such reactions has no observable impact on coupling efficiency or on the chiral integrity of the enantiomerization-prone residue cysteine.

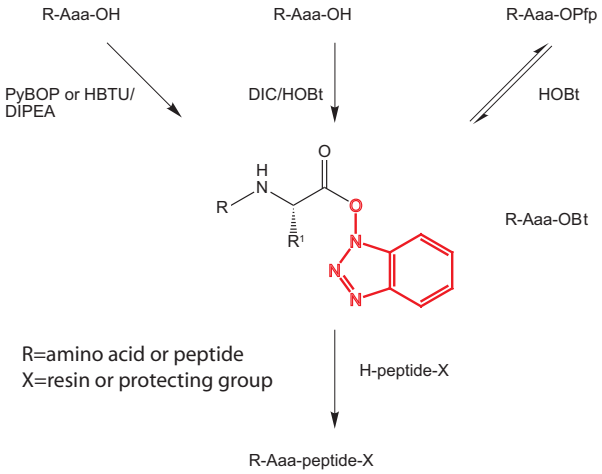

Fig. 1: Generation and use of Bt esters in peptide synthesis.

HOBt in phosphonium and uronium-mediated coupling reactions

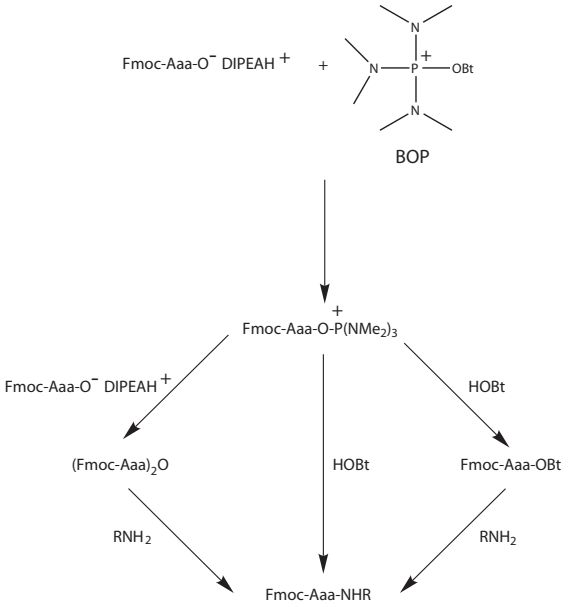

Fig. 2: Proposed mechanism for benzotriazolyl ester formation with BOP.

In 1988, Hudson published a seminal paper which described a competition method that allows the efficiency of different coupling methods to be easily compared [2]. One of the conclusions of this paper was that the initial product of the reaction of a protected amino acid with BOP is the symmetrical anhydride, which then reacts in a second step with the liberated HOBt to form the more reactive Bt ester (Figure 2). He argued that including HOBt into the reaction mixture from the outset would promote the formation of the Bt ester and thereby lead to more efficient coupling. This approach was quickly adopted by others and became standard practice for manual and automated synthesis using both phosphonium- and uronium-based coupling reagents.

However, whether it is really necessary to use HOBt in conjunction with HOBt-based uronium or phosphonium coupling reagents is debatable. The hypothesis that the initial product of the reaction is the symmetrical anhydride is contradicted by ^{13}C NMR data which indicate that activation with BOP is complete within 2 minutes and that the sole product of the reaction is the Bt ester [3]. In the original publications of Castro describing the use of BOP [4] and PyBOP® [5], coupling was conducted highly effectively without any additional HOBt, using only protected amino acid, phosphonium reagent, and base. Indeed, Carpino and Albericio [6] found that, with the possible exception of Asn(Trt), the addition of the HOXt to HXTU-mediated couplings (X=A, B) did not significantly improve yields (Table 1). Furthermore, for some fragment condensation reactions, the inclusion of HOXt appeared to enhance epimerization [7]. In addition, HOAt is rarely added to HATU-mediated couplings as due to its explosive nature, it is not readily commercially available. This result is a confusing situation where HOXt is generally included in HBTU and PyBOP® coupling reactions but not in those using HATU.

Table 1: Percentage of deletion peptides observed in the synthesis of ACP(65-74) (Figure 3) using 1.5 min coupling time and 1.5-fold excess of Fmoc-amino acids activated with either HXTU or HXTU/HOXt (X=A, B) [6].

coupling method	-2Ile	-Ile^{72}	-Ile^{69}	-Val	-Asn
HATU	3	6	12	3	16
HATU/ HOAt	5	9	12	2	8
HBTU	16	11	19	2	7
HBTU/ HOBt	18	12	18	3	3

Evaluation of the effects of added HOBt on PyBOP®-mediated couplings

The coupling of Fmoc-Val-OH to H-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-Rink amide resin (ACP 66-74) was used as a model system to determine the influence of added HOBt on the efficiency of PyBOP®-mediated amide-bond formation. This sequence was selected as it was the same as used by Albericio and Carpino (Figure 3) and the difficulty of the Val coupling is well documented [8]. In order to magnify any differences between the reaction conditions, only 1.5-fold excesses of Fmoc-Val-OH and PyBOP® were used (Figure 4). Reactions were carried out with *in situ* activation or a 5

Table 2: The influence of added HOBt on the coupling of Fmoc-Val to ACP(66-74). Preactivation time in minutes is given in brackets.

	%Val incorporation			
coupling time (min)	PyBOP/DIPEA 1.5:3 (0)	PyBOP/DIPEA /HOBt 1.5:3:1.5 (0)	PyBOP/DIPEA 1.5:3 (5)	PyBOP/DIPEA /HOBt 1.5:3:1.5 (5)
2	28	24	24	28
10	31	31	41	44
60	70	72	70	72

Fig. 3: Sequence of ACP(65-74). Difficult couplings as noted by Albericio and Carpino are indicated by red arrows. The percentage of deletion peptides at these difficult couplings is shown in Table 1.

minute preactivation time to model the coupling protocols used on Symphony and ABi peptide synthesizers, respectively. The extent of coupling was monitored by the quantitative Fmoc release assay or by HPLC analysis of a sample of cleaved product. The results shown in Table 2 indicate that within experimental error added HOBt has little or no influence on the outcome of this PyBOP® coupling reaction, confirming the findings of Albericio and Carpino.

The coupling of Fmoc-Asn(Trt)-OH to H-Gly-Wang resin was also evaluated since Albericio and Carpino [6] found in their study that the yield of this reaction was improved by the addition of HOBt (Table 3). The coupling was carried out using 1 eq. of Fmoc-Asn(Trt)-OH activated with PyBOP® with and without added HOBt (Figure 4) . The results shown in Table 3 indicate that, like the coupling of Val, added HOBt has no effect on coupling efficiency of Asn(Trt).

A protocol for coupling without the use of added HOBt is given in Method 1.

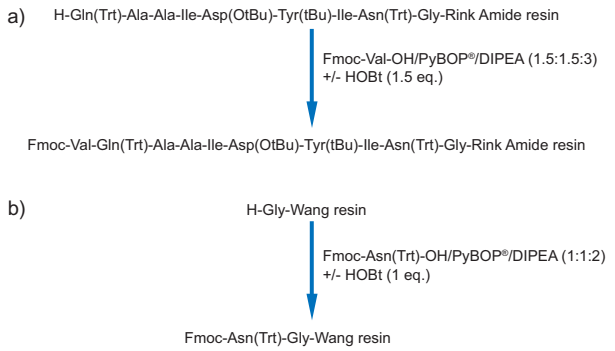

Fig. 4: Models used to evaluate effect of added HOBt on coupling efficiency. a) ACP(65-74); b) Fmoc-Asn(Trt)-Gly-Wang resin.

Table 3: The influence of added HOBt on the coupling of Fmoc-Asn(Trt) to H-Gly-Wang resin.

	%Asn(Trt) incorporation	
coupling time (min)	PyBOP/DIPEA 1:2	PyBOP/DIPEA/HOBt 1:2:1
2	75	72
10	95	93
30	96	95
60	100	100

Influence of added HOBt on the enantiomerization of cysteine

Urethane-based protecting groups offer excellent protection to α-amino acids against oxazolone-mediated enantiomerization during amide-bond formation, so the loss of chiral integrity is generally not an issue in step-wise synthesis. The exception is cysteine which can also enantiomerize via an elimination-addition mechanism. Fmoc-protected cysteine derivatives, in particular, can undergo extensive enantiomerization in the basic milieu of uronium and phosphonium-mediated coupling reactions [8]. Therefore, since omitting HOBt from phosphonium or uronium-mediated couplings will increase the basicity of the reaction medium, it was felt prudent to check the effect of this measure on the levels enantiomerization of this sensitive residue. This was done by preparing the model tripeptide, H-Val-Cys-Phe-OH, with and without the use of added HOBt during the coupling of Fmoc-Cys(Trt)-OH and assessing extent of epimerization by HPLC analysis of the cleaved products (Figure 5). Using this method, the D-Cys content of the peptides prepared with and without added HOBt was found to be 10% and 7%, respectively, indicating added HOBt has little or no influence on the enantiomerization of cysteine.

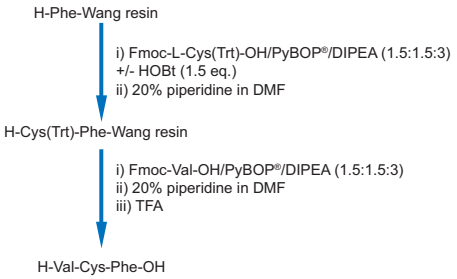

Fig. 5: Synthesis of H-Val-Cys-Phe-OH.

Method 1: PyBOP®/HXTU activation (X=A, B, C)

1. Dissolve the Fmoc-amino acid (3 eq. relative to resin loading) and PyBOP® or HXTU (3 eq.) in the minimum volume of DMF.
2. Add DIPEA (6 eq.) to the amino-acid solution.
3. Stir the mixture and add immediately to resin.

Ordering information

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
01-62-0016	PyBOP®	5 g 25 g 100 g
01-62-0015	TBTU	5 g 25 g 100 g

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