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Coupling reagents

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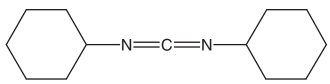
1. Coupling methods

Efficient and unambiguous peptide-bond formation requires chemical activation of the carboxyl component of the N-α-protected amino acid. The activating group or reaction must be chosen carefully to achieve a very high coupling efficiency and at the same time avoid potential side reactions.

There are basically four major types of coupling techniques currently employed for step-wise introduction of N-α-protected amino acids in solid phase synthesis. The most frequently used coupling reagents are shown in Table 1. For a recent review on coupling methods, see [1].

1.1 Carbodiimides



Carbodiimides have been some of the most popular *in situ* activating reagents in peptide synthesis. Dicyclohexylcarbodiimide (DCC), first described in the 1950s, remains the most popular and is a particularly appropriate choice for the apolar environment of polystyrene resins [2]. Reactions are carried out at room temperature in DCM for 12 hours. Besides being somewhat apolar, DCM is also an excellent swelling agent for polystyrene supports. DMF can be added to increase the solubility of reactive species. It can, however, slow down the rate of activation so minimal quantities should be used.

The principal limitation in using carbodiimides is the dehydration of Asn and Gln residues. The addition of HOBt to the reaction mixture will prevent dehydration and has the added benefit of acting as a catalyst [3, 4, 5]. The principal drawback to the use of DCC is the formation of the DCM-insoluble dicyclohexylurea (DCU) during activation and acylation. Other carbodiimides used in SPPS such as diisopropylcarbodiimide (DIPCDI) [6, 7], *t*-butyl-methylcarbodiimide [8, 9] and *t*-butylethylcarbodiimide [8] form ureas which are more soluble in DCM than DCU.

1.2 Preformed symmetrical anhydrides

Preformed symmetrical anhydrides (PSA) [10, 11] have been used by many research groups, mainly in Boc chemistry, because of their high reactivity. They are generated *in situ* using two equivalents of protected amino acid and one equivalent of DCC in DCM. The urea formed is removed by filtration and the couplings then proceed in DMF.

The symmetrical anhydride of Boc-Arg(Tos)-OH should not be used for coupling because it has been found to cause an undesirable insertion reaction. Fmoc-amino acid PSAs have been well studied [12]. Some Fmoc amino acids, such as Gly, Ala, Nle, Cys(Acm), Gln(Mbh), Asn(Mbh) among others, are not readily soluble in DCM [13] and require significant amounts of DMF for solubilization, but DMF slows down the rate of activation.

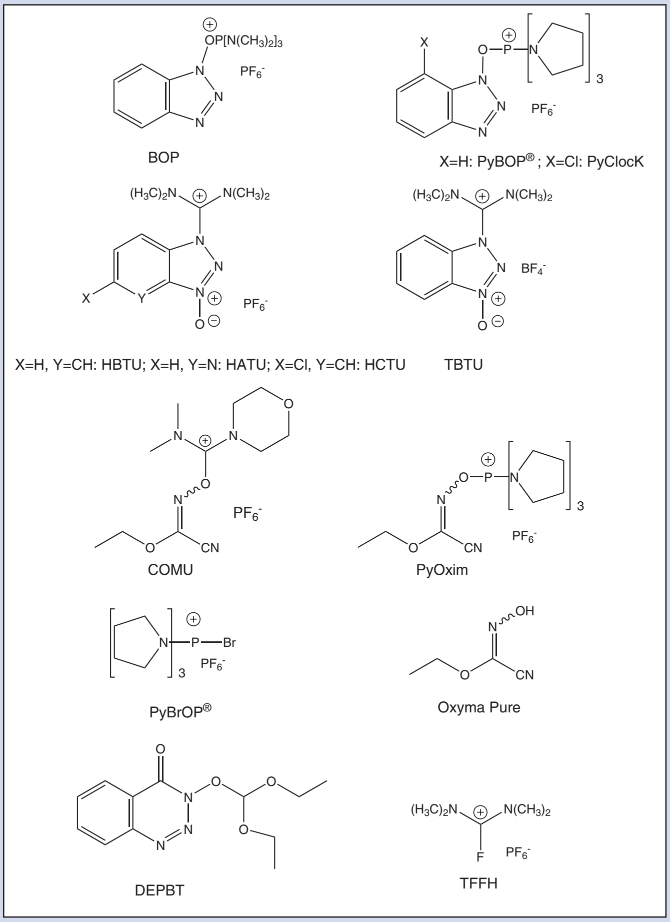


Table 1: Coupling reagents.

Another drawback is that the preparation and use of PSA is quite wasteful, since two equivalents of protected amino acids are required to form one equivalent of activated species and the latter has to be used in excess. It is also recommended that preformed symmetrical anhydrides should be made fresh prior to use.

Method 1: Symmetrical anhydride formation

1. Dissolve the Fmoc amino acid (10 eq. relative to resin loading) in dry DCM. One or two drops of DMF may be needed to aid complete dissolution.
2. Add a solution of diisopropylcarbodiimide (5 eq. relative to resin loading) in dry DCM to the amino acid solution.
3. Stir the mixture for 20 min at 0°C, keeping the reaction mixture free of moisture with a calcium chloride drying tube.
4. Remove the DCM by evaporation under reduced pressure using a rotary evaporator.
5. Dissolve the residue in the minimum of DMF and add the solution to the resin.

1.3 Active esters

Extensive studies have been carried out on the use of Fmoc amino acid activated esters. Some of them have become extremely useful and are now routinely used in solid phase peptide chemistry [14].

OBt and Oxyma Pure esters

1-Hydroxybenzotrazole (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 [3]. However, recently HOBt monohydrate, the standard form of this reagent, was reclassified by the UN as a class 1c explosive. 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. Subirós-Funosas, *et al.* [15] have shown this reagent to be more effective and give lower racemization than HOBt in carbodiimide-mediated coupling reactions.

Oxyma Pure was first identified as a potential coupling additive in the 1970s [16, 17]. 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 [18] and Cl-HOBt [19] (Table 2).

Table 2: pK_a of Oxyma Pure and benzotriazole-based coupling reagents.

Compound	pKa
HOAt	3.28
HOBt	4.6
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 1) (Method 2).

Method 2: Oxyma ester formation

1. Dissolve the Fmoc amino acid (5 eq. relative to resin loading) and Oxyma (5 eq.) in DCM/DMF (1:1).
2. Add DIPCDI (5 eq. relative to resin loading) to the amino acid solution.
3. Stir the mixture for 10 min at rt, keeping the reaction mixture free of moisture with a calcium chloride drying tube.
4. Add the solution to the resin.

Racemization

To compare racemization when using Oxyma with HOBt, Subirós-Funosas, et al. [15] 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 3 - 5), 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.

Fig. 1: Possible reaction pathways for carbodiimide activation in presence of Oxyma Pure or HOBt.

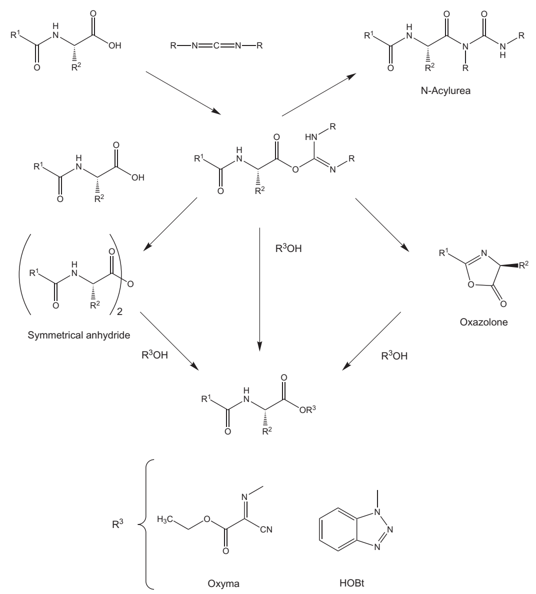


Table 3: Product composition obtained from the coupling of Z-Phg-OH to H-Pro-NH_2.

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 4: 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 5: 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, we undertook the step-wise solid phase syntheses of the ABRF test peptide 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 (Table 6). This result may be a reflection of the slower formation of the Oxyma Pure active ester noted by Subirós-Funosas, *et al* [15].

Table 6: 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

Subirós-Funosas, et al. [15] also 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 (Tables 7 - 9). 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 7: 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 8: 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

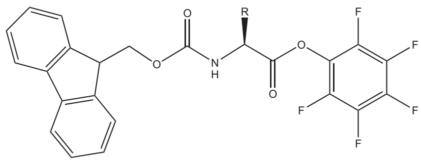
Table 9: Product composition obtained from synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2 with 30 min coupling times.

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.

OPfp esters



Pentafluorophenyl esters [20] are also efficient acylating agents and their chemical structures provide little opportunity for side reactions. They react somewhat slower than symmetrical anhydrides. The addition of HOBt significantly increases the rate of reaction [21, 22], making them very useful activated species for solid phase synthesis. However, not all amino acid OPfps are currently available, for example Fmoc-Ser(tBu)-OPfp and Fmoc-Thr(tBu)-OPfp do not crystallize and are difficult to purify.

Method 3-3: Use of OPfp esters

1. Dissolve the Fmoc amino acid pentafluorophenyl ester (5 eq. relative to resin loading) and HOBt (5 eq.) in DMF.
2. Add the solution to the resin.

1.4 Coupling reagents

In situ activating reagents are widely accepted because they are easy to use, they give fast reactions, even between sterically hindered amino acids, and their use is generally free of side reactions. Most are based on phosphonium or aminium (formerly known as uronium) salts which, in the presence of a tertiary base, can smoothly convert protected amino acids to a variety of activated species (see this chapter, section 11.1 on page 3.44). The most commonly employed, BOP, PyBOP, HBTU and TBTU, generate OBt esters, and these have found wide application in routine SPPS and solution synthesis for difficult couplings. BOP should be handled with great care as the by-product formed during activation is highly carcinogenic. BOP can be substituted by PyBOP without loss of performance.

Care should be taken when using aminium-based activation reagents not to use an excess relative to the carboxylic acid component as this can lead to capping of the amino terminus through guanidine formation [23]. This side reaction is commonly encountered during on-resin cyclization reactions. Phosphonium-based activating reagents, with the exception of PyBrOP® when used in DMF, do not give rise to such by-products [24].

Couplings reagents are also available which generate esters that are more reactive than OBt. Three such reagents are HATU [25, 26], HCTU [27] and PyClocK, which in the presence of base convert carboxylic acids to the corresponding OAt and 6-ClOBt esters respectively. Such esters are more reactive than their OBt counterparts owing to the lower pKa of HOAt and HOCt compared to HOBt. Furthermore, HOAt has the added benefit of the pyridine nitrogen which provides anchiomeric assistance. to the coupling reaction [25]. HATU is the most efficient coupling reagent of this series. However, owing to its cost, its use is generally recommended for difficult and hindered couplings and the synthesis of long peptides.

Table 10: Product composition obtained from the solid phase synthesis of H-Tyr-MeLeu-MeLeu-Phe-Leu-NH_2 using various coupling reagents. The last tow residues were coupled for only 5 minutes to exaggerate differences in efficiencies between reagents.

% Compostion of products by HPLC				
Coupling reagents	MeLFL	YMeLFL	MeLMeLFL	YMeLMeLFL
PyBOP	60	31	5	4
PyOxim	10	45	5	40
HBTU	57	31	8	4
HCTU	31	35	9	25
HATU	0	16	7	77
TOTU	5	58	2	35
COMU	9	47	4	40
Ezracom	9	50	5	36
HDMC	24	46	7	21

Recently, coupling reagents based on the Oxyma Pure leaving group have been introduced, the most useful of which are COMU [28] and PyOxim [29]. However, oxyma-based coupling reagents generate esters as almost as reactive (Table 10) [30] as HATU but are much less expensive. Furthermore, Oxyma-based coupling reagents are not-based onpotentially explosive triazole reagents. COMU is an excellent reagent for solution phase synthesis as the by-products of using it are water soluble and easily removed. COMU is not suitable for use with synthesizers that require pre-prepared solutions of activator as its solution stabililty is poor (Table 11). PyOxim on the otherhand has solution stability comparable to PyBOP, mediates couplings with high efficiency and gives a 1.5 M solution in DMF, compared to 0.5 M for HATU and HBTU (Table 12). The latter has important practical implications, it enables reactions can be performed at higher concentrations with concomitant improvements in efficiency. Epimerization during coupling of fragments also appears to be less when using COMU or PyOxim than with HBTU or HATU.

Table 11: Closed vialstability in d7-DMF as determined by ^{1}H nmr.

Coupling reagent	2 days	7 days	14 days
HDMC	99	99	98
COMU	67	46	36
PyOxim	90	88	86
TOTU	89	85	74
Ezracom	90	85	79
PyBOP	88	85	81
HATU	99	99	98

COMU and PyOxim are used in exactly the same way as PyBOP®, HBTU or HATU (Method 4), but instead of generating a benzotriazolyl ester it forms the corresponding ester of Oxyma Pure. In situations where racemization is an issue, COMU can be used with as little as one equivalent of base since the morpholino oxygen acts as an integral base.

Table 12: Solubility of couplng reagents in DMF.

Coupling reagent	Molarity
HATU	0.45
HBTU	0.5
HCTU	0.8
HDMC	0.75
COMU	1.5
TOTU	0.8
PyBOP	1.5
PyOxim	1.5
Ezracom	0.4

The coupling of N-methyl amino acids can be a difficult and slow process. PyBrOP [31] allows efficient, fast and enantiomerization-free couplings, providing a significant improvement over existing reagents.

Method 4: COMU/PyBOP/PyOxim/HATU/HBTU/HCTU activation

1. Dissolve the Fmoc amino acid (5 eq. relative to resin loading), COMU, PyBOP, HATU, HCTU or HBTU (4.9 eq.) in DMF.
2. Add DIPEA (10 eq. relative to resin loading) to the amino acid solution.
3. Stir the mixture and add the solution immediately to the resin.

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