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Tetrahedron

Tetrahedron 61 (2005) 10827-10852

Tetrahedron report number 740

Amide bond formation and peptide coupling

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Received 2 August 2005

Available online 19 September 2005

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Keywords: Amide; Carboxamide; Peptide; Coupling; Condensation; Ligation; Amidation; Aminolysis; Acyl halide; Acyl chloride; Acyl azide; CDl; Acylimidazole; Anhydride; Mixed anhydride; Ester; Activated ester; Activated acid; Phosphonium salt; Uranium salt; Ammonium salt; Protease; Amidase; Lipase; Enzyme; *N*-Carboxyanhydride; Acylboron; Coupling reagent; Polymer-supported; Solid-phase.

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1. Introduction

The amide functionality is a common feature in small or complex synthetic or natural molecules. For example, it is ubiquitous in life, as proteins play a crucial role in virtually all biological processes such as enzymatic catalysis (nearly all known enzymes are proteins), transport/storage (haemo-globin), immune protection (antibodies) and mechanical support (collagen). Amides also play a key role for medicinal chemists. An in-depth analysis of the Comprehensive Medicinal Chemistry database revealed that the carboxamide group appears in more than 25% of known drugs.¹ This can be expected, since carboxamides are neutral, are stable and have both hydrogen-bond accepting and donating properties.

In nature, protein synthesis involving a sequence of peptide coupling reactions (amide bond formation between two α -amino acids or peptides) is very complex, probably to safeguard the unique and precisely defined amino acid sequence of every protein. This barrier is overcome in vivo by a selective activation process catalysed by enzymes, where the required amino acid is transformed into an intermediate amino ester. This intermediate is then involved in a process mediated by the coordinated interplay of more than a hundred macromolecules, including mRNAs, tRNAs, activating enzymes and protein factors, in addition to ribosomes.²

Amide or ester bond formation between an acid and, respectively, an amine or an alcohol are formally condensations. The usual esterifications are an equilibrium reaction, whereas, on mixing an amine with a carboxylic acid, an acid-base reaction occurs first to form a stable salt. In other words, the amide bond formation has to fight against adverse thermodynamics as the equilibrium shown in Scheme 1 and lies on the side of hydrolysis rather than synthesis.³

The direct condensation of the salt can be achieved at high temperature (160-180 °C),⁴ which is usually quite incompatible with the presence of other functionalities (see also Section 2.6.3). Therefore, activation of the acid, attachment

of a leaving group to the acyl carbon of the acid, to allow attack by the amino group is necessary (Scheme 2).



Scheme 2. Acid activation and aminolysis steps.

Hence, a plethora of methods and strategies have been developed and these are now available for the synthetic, medicinal or combinatorial chemist. Relevant examples of these methods are indicated in this report. The chemist might have to screen a variety of such conditions to find the method best adapted to his situation. For example, due to poor reactivity or steric constraints in some extreme cases, the challenge will be to get the amide formed at all. In other situations, the chemist will require the reaction to avoid racemisation. In general, the aim could also be to optimise the yield, to reduce the amount of by-products, to improve selectivity, to facilitate the final purification, to define a scalable process or to exploit more economical reagents. In the last two decades, the combined rapid development of solid-phase technologies and coupling methods has enabled parallel synthesis to become a tool of choice to produce vast amounts of diverse compounds for early discovery in the pharmaceutical industry.

2. Amide bond formation: methods and strategies

Carboxy components can be activated as acyl halides, acyl azides, acylimidazoles, anhydrides, esters etc. There are different ways of coupling reactive carboxy derivatives with an amine:

• an intermediate acylating agent is formed and isolated then subjected to aminolysis

RCOOH + R'OH \longrightarrow RCOOR' + H₂O pKa~4-5 pKa~-2 RCOOH + R'NH₂ \longrightarrow R'NH₃+ + RCOO- $\xrightarrow{}$ RCONHR' + H₂O pKa~4-5 pKa~10-11

Scheme 1. Ester bond versus amide bond formation.

- a reactive acylating agent is formed from the acid in a separate step(s), followed by immediate treatment with the amine
- the acylating agent is generated in situ from the acid in the presence of the amine, by the addition of an activating or coupling agent.

As illustrated in the Section 1, amide bond formation can often present difficulties such as low yields, racemisation, degradation, difficult purification etc. To face these challenges, numerous mild coupling reagents and methods have been developed that not only are high yielding, but that potentially help to prevent racemisation of neighbouring chiral centres. A classical example of racemisation is encountered in peptide synthesis when the terminal acid peptide is activated, leading to the formation of the corresponding oxazolone 1a. Under mild basic conditions, the oxazolone undergoes racemisation via the formation of conjugated anionic intermediate 2. The resulting oxazolone 1a, 1b mixture reacts then with a nucleophile, explaining the loss of chiral integrity of the coupled material 3a, 3b (Scheme 3). Therefore, peptides are usually grown at the N-terminus and mild activation conditions are needed. In this latter approach, the activation is advantageously performed on an N-protected α -amino acid, thus avoiding the oxazolone formation.

2.1. Acyl halides

2.1.1. Acyl chlorides. Acyl chlorides (also called acid

chlorides) are one of the easiest methods to activate an acid and numerous acyl chlorides are commercially available. This is usually a two-step process, involving first the conversion of the acid into the acyl halide followed by the coupling itself.

2.1.1.1. Acyl chloride formation. Thionyl chloride SOCl₂ **4**,⁵ oxalyl chloride (COCl)₂ **5**,^{6.7} phosphorus trichloride PCl₃,⁸ phosphorus oxychloride POCl₃⁹ and phosphorus pentachloride PCl₅¹⁰ are commonly used to generate acyl chlorides from their corresponding acids. Phoshonium pentachloride is generally used for aromatic acids, which contains electron-withdrawing substituents and which do not react readily with thionyl chloride **4**.¹¹ The mechanism of acid chloride formation using thionyl chloride **4** or oxalyl chloride **5** is illustrated in Scheme 4. **Caution**: it is important to note that the use of oxalyl chloride **5** is accompanied by the stoichiometric production of two molecules of gas, one of which is carbon monoxide.¹² The generated volume of gas and resulting chemical or safety hazards should always be taken into consideration before setting up these reactions.¹³

These reactions are often promoted by the addition of a drop of dimethylformamide (DMF).¹⁴ The catalytic role of DMF is described in Scheme 5.

One of the major disadvantages of the previously cited chlorinating agents is the production of HCl. Some substrates (e.g., those containing Boc-protected amines)







Scheme 4. Mechanism for acyl chloride formation using oxalyl chloride 5 or thionyl chloride 4.



Scheme 5. Activation with DMF: catalytic cycle.

are acid sensitive and require non-acidic conditions. For example, cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) **6** is used to carry out acyl chloride formation in the presence of triethylamine.¹⁵ The presence of this organic base maintains the basic pH conditions throughout the reaction. The proposed mechanism involves an initial aromatic nucleophilic substitution that generates the corresponding activated aromatic ester **7** and the chlorine anion. The following step is the nucleophilic attack of the chlorine anion on the activated ester to generate the desired acyl chloride (Scheme 6).

Cyanuric chloride **6** is a suitable activating agent for the large-scale manufacture of amides.¹⁶ The process presents many advantages. It involves only 0.33 equiv of triazine promoter, which minimises reagent utilisation and by-product generation. Inexpensive inorganic bases may be used instead of amine bases and the reaction tolerates water. The resulting cyanuric acid by-product can be easily removed by filtration and with a basic wash.

Neutral conditions have also been developed and provide mild conversion of carboxylic acid into acyl chloride. For example, triphenylphosphine (TPP) and a source of chloride have been studied. Carboxylic acids are converted by TPP and carbon tetrachloride into the corresponding acyl chloride,¹⁷ analogous to the conversion of alkyl alcohols into alkyl chlorides.¹⁸ It is suggested that initial formation of triphenyltrichloromethylphosphonium chloride **8** occurs with further reaction yielding chloroform and triphenyl-acyloxyphosphonium chloride (Scheme 7).

Difficulties to separate the product from the phosphoruscontaining by products can be avoided by the use of polymer-supported phosphine–carbon tetrachloride reagent. **Caution**: the toxicity and environmental risks¹⁹ associated with carbon tetrachloride render this procedure less attractive. Carbon tetrachloride can be substituted by hexachloroacetone.²⁰ Villeneuve has demonstrated that carboxylic acids could be converted by hexachloroacetone and TPP at low temperature into the corresponding acyl chloride. This method was also applied to generate highly reactive formyl chloride. Alternatively, trichloroacetonitrile and TPP also provide mild and efficient conditions.²¹

Other neutral conditions are described by Ghosez et al. using tetramethyl- α -chloroenamine 9.²² During this process, the formation of hydrogen halides is avoided. Thus,



Scheme 6. Acyl chloride formation using cyanuric chloride 6.



Scheme 7. Acyl chloride formation using TPP and carbon tetrachloride.



Scheme 8. Use of Ghosez chlorination agent 9.

this method is extremely useful when acid-labile protecting groups are present (Scheme 8).

2.1.1.2. Coupling reactions with acyl chlorides. The amide bond is formed by reacting the acyl chloride with the desired amine (Scheme 9). An additional base is usually required to trap the formed HCl and to avoid the conversion of the amine into its unreactive HCl salt. Couplings are usually performed in inert dry solvents, in the presence of a non-nucleophilic tertiary amine (NEt₃,²³ *i*Pr₂NEt [also called Hünig's base], or *N*-methylmorpholine). Having said that, acyl chlorides are often robust enough to be coupled to amines under aqueous conditions, for example, in the presence of NaOH²⁴ (Schotten–Baumann conditions).



Scheme 9. Aminolysis.

These reactions can be accelerated with a catalytic amount of pyridine or N,N-dimethylaminopyridine (DMAP).²⁵ In some cases, pyridine is used as the solvent. The formation of an intermediate acylpyridinum salt **10** is stipulated (Scheme 10).

The use of metallic zinc can also accelerate the coupling at room temperature. The method is applicable to alkyl, aryl, heterocycles, carbohydrates and amino acids and leads to high yields.²⁶

2.1.1.3. Limitations of acyl chlorides. Nevertheless, acyl chlorides have limited value in peptide coupling because of the danger of hydrolysis, racemisation, cleavage of protecting groups and other side reactions (e.g., *N*-carboxy anhydride formation, see Section 2.4.2.3). The tendency of acyl chlorides to racemise under basic conditions can be illustrated by the standard synthesis of ketenes.²⁷ Ketenes **11** are formed by reacting an acyl chloride containing an α proton with NEt₃. The ketene **11** can further react with a nucleophile such as an amine to yield the corresponding addition product with an obvious loss of chiral integrity (Scheme 11).

2.1.2. Acyl fluorides. Racemisation and side reaction problems can sometimes be avoided by using acyl fluorides as active intermediates.²⁸ Acyl fluorides are, indeed, less moisture sensitive than acyl chlorides and more reactive towards amines. Another advantage is that they are compatible with Fmoc or Cbz *N*-protections and even with *t*Bu esters or other acid-labile ester groups, and thus they are useful in peptide chemistry.²⁹ They react in the same way as acyl chlorides.

$$R \xrightarrow{O}_{Cl} + (N) \xrightarrow{N}_{N} + RNH_{2} \longrightarrow \begin{bmatrix} O \\ R \xrightarrow{O}_{H} \\ O \\ Cl \end{bmatrix} + RNH_{2} \longrightarrow \begin{bmatrix} O \\ R \xrightarrow{O}_{H} \\ NHR \end{bmatrix} + (N)$$

Scheme 10. Catalytic role of pyridine.



Scheme 11. Potential racemisation via ketene formation.



Scheme 12. Acyl fluoride formation using cyanuric fluoride 12.



Scheme 13. Acyl fluoride formation using TFFH 13.



Scheme 14. Acyl bromide formation using TPP and NBS 14.

Acyl fluorides are commonly formed using cyanuric fluoride 12^{30} in the presence of pyridine and react in a similar way to cyanuric chloride 6 (Scheme 12).

Alternatively, *N*,*N*-tetramethylfluoroformamidinium hexafluorophosphate (TFFH) **13** can be used in the presence of Hünig's base.³¹ This salt is advantageous in being nonhygroscopic and stable to handling under ordinary conditions. The postulated two-step mechanism is described in Scheme 13. The TFFH **13** activation uses the urea formation as the driving force. Very similar reagents are used as onepot coupling reagents and do not require the isolation of the intermediate acyl chloride.³²

Diethylaminosulphur trifluoride (DAST) $Et_2NSF_3^{33,34}$ and deoxofluor (MeOEt)₂NSF₃³⁵ have been used to convert carboxylic acid or acyl chloride into carbonyl fluoride. These fluorinating agents have the advantage of reacting in the absence of a base. Differential scanning calorimetry (DSC) studies suggest that deoxofluor is safer to use on a large scale than DAST, as its exotherm is gradual over a wider temperature range and easier to control.

2.1.3. Acyl bromides. Acyl bromides are used on some rare occasions to generate amide bonds. α -Bromoacetyl bromide is one of the most common examples. Acid bromides prepared with phosphorus pentabromide usually also undergo α -bromination.³⁶ Other ways to prepare acyl bromides in situ are to use Ph₃P/*N*-bromosuccinimide NBS **14**³⁷ (see Scheme 14 and Section 2.5.2.2.1), PPh₃/Br₂,³⁸ SOBr₂,³⁹ BBr₃/Al₂O₃⁴⁰ or even (BrCO)₂⁴¹ (see Section 2.1.1). More recently, they have also been prepared under mild conditions using 1-bromo-*N*,*N*-2-trimethyl-1-propenylamine.^{42,43}

2.2. Acyl azides

The acyl azide⁴⁴ route is one of the first developed for peptide coupling by Curtius.⁴⁵ Acyl azides can be prepared from the corresponding methyl esters via a two-step synthesis. The methoxy group is displaced with hydrazine to generate the acyl hydrazide **15**, which then undergoes a nitrosation reaction to yield the final acyl azide **16** (Scheme 15).

formation



Scheme 15. Historical mutistep amide synthesis via acyl azide preparation.

This is usually an efficient coupling method with almost no racemisation, but an occasional side reaction is a Curtius rearrangement, leading to the formation of the unwanted corresponding isocyanate (Scheme 16).⁴⁶



Scheme 16. Possible side reaction: Curtius rearrangement.



Scheme 17. One-pot amide preparation using DPPA 17.



Scheme 18. One-pot amide preparation using CDI 18.



Scheme 19. Multistep carbamoylimidazolium salt 20 strategy.

A more convenient one-pot process has been developed using diphenylphosphonic azide (DPPA) 17^{47} (see Section 2.5.2.2.1). If no nucleophile is present, the acyl azide 16 can rearrange to yield the corresponding isocyanate (Scheme 17).

2.3. Acylimidazoles using CDI

Carbonyl diimidazole (CDI) 18^{48} is a useful coupling reagent that allows one-pot amide formation. Acyl carboxy imidazole and imidazole are initially formed but readily react together to yield the activated species as the acylimidazole 19 (Scheme 18). Practically, the acylimidazole is preformed for 1 h and then the amine is added. This reaction, which generates imidazole in situ, does not need an additional base and is even compatible with HCl salts of the amine.^{49,50} This reagent is commonly used on a large scale⁵¹ in peptide chemistry and its use can be extended to the formation of esters and thioesters. reaction of secondary amines with N,N'-carbonyldiimidazole, followed by methylation with methyl iodide, have also been used for the preparation of tertiary amides and proved to be efficient. A proposed mechanism has been described. The carbamoylimidazolium salt serves as both the source of the amine donor and as the activation reagent for the carboxylic acid acceptor (Scheme 19).

With a similar activation step to that in the use of CDI **18**, N,N'-carbonylbis(3-methylimidazolium) triflate (CBMIT) **21** has been described as an efficient aminoacylating reagent for peptide synthesis (Fig. 1).⁵³



Carbamoylimidazolium salts 20^{52} obtained from the

2.4. Anhydrides

Anhydrides are species that readily react with a vast range of nucleophiles such as alcohols, thiols and, of course, amines. This strategy ranges from the use of simple symmetric anhydrides to rather refined mixed anhydrides involving, for example, isoureas or phosphoric acid-derived species.

2.4.1. Symmetric anhydrides. The diversity of commercially available anhydrides is rather limited and, quite often, the desired anhydride has to be prepared beforehand.

Symmetric anhydrides are formed either by heating the corresponding acid or, in milder conditions, by reacting two molecules of acid in the presence of one equivalent of dicyclohexyl carbodiimide (DCC) **22**,⁵⁴ following the mechanism described in Scheme 20. The driving force of this reaction is the formation of the urea by-product.

The anhydride is then reacted in a second step with the selected amine. In theory, no additional base is required as the addition generates a carboxylate anion in situ. This mild and efficient coupling method is compatible with peptide formation. The main limitation is that only half of the acid is effectively coupled and the other half is wasted. This could be a problem if the acid is valuable.

2.4.2. Mixed anhydrides.

2.4.2.1. Mixed carboxylic anhydrides. To overcome this waste problem, mixed anhydride methods have been developed where the second carboxylic moiety is cheap and easy to couple onto the acid. The difficulty is to get regioselectivity in the nucleophilic addition for position **a** over position **b** (Scheme 21). Mixed pivalic anhydrides 23^{55} are one of the rare examples in this series. The desired aminolysis selectivity is believed to be due to the steric hindrance of the *t*Bu group.

2.4.2.2. Mixed carbonic anhydrides. Another strategy is to differentiate both reactive centres by their chemical nature. Excellent selectivity is observed with mixed carbonic anhydrides 24. The carbonate electrophilic centre **a** is more reactive than the carboxylic site **b** as the reactive centre **a** is less stabilised by resonance (Scheme 22).

Ethoxycarbonyl anhydrides **24** can be generated using ethyl chloroformate⁵⁶ (Scheme 22) or 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EDDQ) **25** (Scheme 23).⁵⁷



Scheme 20. Anhydride preparation and consecutive coupling with amines.



Scheme 21. Two-step coupling procedure via pivalic anhydride 23.



Scheme 22. Two-step coupling via ethyl carbonic anhydride 24.



Scheme 23. Ethoxycarbonyl anhydride 24 preparation using EDDQ 25.

Under acidic conditions, ethanol is eliminated from EDDQ **25**, generating a reactive ethyl formate quinolinium salt **26**. This intermediate has a similar reactivity to the pyridinium salts described in the acyl chloride section and readily reacts with the desired carboxylate to form the required ethoxycarbonyl anhydride. The driving force of this reaction is the generation of the aromatic quinoline.

2.4.2.3. *N*-Carboxy anhydrides or Leuch's anhydrides. The anhydride strategy has been explored and expanded further by Leuch in the domain of peptide synthesis. Cyclic anhydrides can be readily prepared from unprotected amino acids and phosgene. An alternative procedure consists of reacting *N*-protected (Boc, Cbz, Fmoc) amino acids with thionyl chloride and DMF (Scheme 24). In this case, the acid chloride reacts with the carbonyl of the neighbouring carbamate to yield the corresponding *N*-carboxy anhydride (NCA) **27**. Such reactivity, once more, illustrates why acyl chlorides are best avoided in peptide synthesis.^{58,59}



Scheme 24. NCA 27 preparation.



Scheme 25. Uncontrolled homopolyamino acid 28 formation.

NCAs 27 can react in different ways. A catalytic amount of a nucleophile (e.g., primary or secondary amine) will initiate a chain reaction that leads to the formation of homopolyamino acids 28. The ring opening followed by decarboxylation yields a new nucleophile that reacts on the next molecule of NCA 27 and so on (Scheme 25).

Under more carefully controlled conditions, however, NCAs 27 can be mono-coupled to the nitrogen of an unprotected amino acid in high optical purity. The NCA 27 has to be added to a basic aqueous solution of the selected amino acid at 0 °C (Scheme 26).

The key factor is the relative stability/instability of the intermediate carbamic acid that prevents the formation of the free aminodipeptide while NCA 27 is still present in the reaction mixture. This process can be repeated several times to form small oligopeptides in solution. The over coupling is the principal limitation of this method. The thio-analogues can also be used. The higher stability of the corresponding thiocarbamic acid (TCA) 29 avoids any over-reaction, but they are prone to racemisation (Scheme 27).

More recently Fuller⁶⁰ has introduced Boc or Cbz N-protected NCAs 30 (also called UNCAs) (Scheme 28). The U stands for urethane (synonym of carbamate) describing the nature of the N-Boc and N-Cbz protection. These compounds are stable crystalline solids (in the absence of water). The obvious advantage of the N-protection is to avoid over-reaction. On the one hand, UNCAs represent an ideal peptide reagent as the only byproduct of the coupling is CO_2 , but, on the other hand, they are obtained from a time-consuming preparation. Their synthesis first requires preparation of the intermediate NCA, followed by the N-protection by acylation in the presence of a non-nucleophilic base such as *N*-methylmorpholine **6**.

2.4.2.4. Broadened concept of mixed anhydrides. The notion of mixed anhydrides can be extended to other activated species resulting from the condensation or addition of the carboxylic acid with phosphoric acid-derived species, boronic acid derivatives, carbodiimides or even ethoxyacetylene. The case of phosphorous-containing coupling reagents is treated separately.

2.4.2.4.1. Ethoxyacetylene. Ethoxyacetylene 31 has been used as a mild dehydrating agent and reported in peptide synthesis.⁶¹ It enables the conversion of sensitive acids into their masked anhydrides 32^{62} The activated acid then undergoes the classical aminolysis (Scheme 29).

2.4.2.4.2. Acyloxyboron intermediates. Acyloxyboron species generated from carboxylic acids and boron reagents often react with amines to give amides. Boron reagents such as BR₃ (R=C₈H₁₇ or OMe),⁶³ ClB(OMe)₂, HB(OR)₂ (R= *i*Pr or *t*Am), BH₃·R₃N (R=Me or Bu),⁶⁴ or BF₃·Et₂O⁶⁵ readily react with carboxylic acids to yield acyloxyboron intermediates, which are coupled to amines in moderate yields. The main drawback of this procedure is the low conversion rate usually observed during the aminolysis step. Mechanistic studies suggest the leaving group ejected in this process fragments to liberate 1 equiv of alkyl alcohol, which competitively destroys the active intermediate by attack at the boron centre.⁶⁶ This difficulty



Scheme 26. Controlled peptide synthesis using NCAs 27.



Scheme 27. Peptide coupling using TCAs 29.



Scheme 28. Peptide coupling using UNCAs 30.



Scheme 29. Two-step amide formation using ethoxyacetylene 31.

has been overcome by using arylboronic species as leaving groups. For example, good yields have been described using catecholborane for the synthesis of lactones.⁶⁷ Once released during the aminolysis, the resulting *o*-phenylene borate **33** is, indeed, less prone to degradation. Furthermore, if any hydrolysis did occur, the leaving group **33** would generate a rather poorly nucleophilic phenolic derivative (Scheme 30).

Arylboronic acids with electron-withdrawing groups such as 3,4,5-trifluorophenylboronic acid, 3,5-bis-(trifluoromethyl)phenylboronic acid⁶⁸ and 3,4,5-trifluorophenylboronic acid⁶⁹ can efficiently act as amidation catalysts. A simplified mechanism is depicted in Scheme 31. The carboxylic acid is activated in the presence of arylboronic acid **34** as monoacyloxyboronic acid **35** with loss of a molecule of water. Then the activated acid undergoes aminolysis, yielding the desired amide and regenerating the arylboronic acid **34**.

2.4.2.4.3. *O*-Acylisourea using carbodiimides as coupling reagents. Dicyclohexyl carbodiimide (DCC) 22, ⁷⁰ diisopropyl carbodiimide (DIC) 36 and 1-ethyl-3-(3'-dimethylamino)carbodiimide HCl salt (EDC or WSC·HCl) 37 (Fig. 2) are frequently used for amide bond formation (this method can also be used to synthesise anhydrides and esters).⁷¹ No additional amine is theoretically required during this one-pot procedure. The carbodiimide reacts with the carboxylic acid to form the *O*-acylisourea mixed anhydride 38 (Scheme 32, see Section 2.4.1). This intermediate can then directly react with the amine to yield the desired amide and the urea by-product 39. The isolation of the symmetric carboxylic anhydride from the reaction mixture, however, suggests that a more complex



Scheme 30. Acylboronate formation and aminolysis.



Scheme 31. Catalytic use of boronic species in amide couplings.



mechanism might co-exist.⁷²⁻⁷⁴ The driving force of this reaction is the formation of the urea by-product **39**.

Often racemisation and acetyl transfer forming the unreactive *N*-acylurea **40** are observed (Scheme 33). This side reaction can be considerably diminished by reacting the acid and the coupling reagent at 0 °C before adding the amine. Furthermore, adding a selected nucleophile that reacts faster than the competing acyl transfer and generates an intermediate still active enough to couple with the amine also prevents the side reactions. Such nucleophiles are DMAP (see Section 2.1.1) and hydroxybenzotriazole (HOBt) **41** (see Section 2.5.2).⁷⁵

Different carbodiimides are commercially available (Fig. 2).

Resulting urea poorly soluble. Eliminated by filtration. Solution-phase chemistry.

Resulting urea soluble in DCM. Eliminated by DCM washes. Solid-phase chemistry.

Resulting urea is water-soluble. Eliminated by washes Solution-phase chemistry.

10837





Scheme 32. One-pot carbodiimide amide coupling.



Scheme 33. Use of HOBt 41 to minimise the formation of unreactive N-acylurea 40.

Caution: all of them are sensitisers and should be handled with care. The difference in solubility of their urea byproducts can be advantageously used during the purification. For example, dicyclohexyl urea DHU is rather insoluble and can be eliminated by filtration. On the contrary, dimethylaminopropyl-3-ethylurea is extremely water soluble and can be eliminated by aqueous workup. When used in solid-phase chemistry the solid DHU is extremely difficult to separate from the resin. Diisopropyl urea **39** is slightly more soluble in dichloromethane than DHU, which therefore, renders it easier to wash off from the solid support.

2.5. Esters

2.5.1. Alkyl esters. Alkyl esters (e.g., methyl, ethyl, benzyl esters) cannot be considered as activated species and are

commonly used as protecting groups in peptide synthesis. Alkyl esters can, however, be displaced occasionally by amines under forcing conditions such as the use of high temperatures or the addition of a Lewis acid (e.g., $TiCl_4$).⁷⁶ Ring formation can also bring about the required assistance. For example, in the synthesis of benzopiperazinone **42** the intramolecular amide is spontaneously formed from the methyl or ethyl ester upon reduction of the nitro group (Scheme 34).⁷⁷ Most of the time, alkyl esters are stable under usual coupling conditions and such examples remain anecdotic.

Another interesting example of condensation between an amine and a methyl ester has been described recently in the chemistry of rhodamines, which are used as standard fluorescent probes (Scheme 35). Fluorescent probes have long been used to study complex biological systems.



Scheme 34. Benzopiperazinone ring formation under reductive conditions.



Scheme 35. Rhodamine methyl ester 42 coupling to amines.

Rhodamines are usually 'conjugated' to the molecule of interest via an amide bond. There are numerous publications describing such coupling reactions between the acid residue of rhodamine and diverse amines, but Adamczyk et al. described a direct coupling of rhodamine methyl ester **43** with amines.⁷⁸ He theorised that the nucleophile could undergo reversible addition at the 9-position of the quinone like structure of the rhodamine. This is followed by intramolecular trapping of the amine by the proximal methyl ester. The final amide is then generated by ring opening of the intermediate spiro-lactam **44**, which also allows the regeneration of the conjugated fluorescent moiety.

2.5.2. Active esters. Activated esters such as aromatic esters are usually easier to hydrolyse than alkyl esters and are prone to react with a wide range of nucleophiles. More importantly, they cleanly react with amines under mild conditions with usually reduced racemisation. Scheme 36 gives a selection of different alcohols that are commonly used. The increased electrophilicity of the carbonyl centre,

compared to alkyl esters, results from the electron-withdrawing character of the selected alcohols.

The choice of the alcohol depends on the type of application. In peptide synthesis, for example, the most commonly used are HOBt **41**, *p*-nitrophenol (PNP) **45**⁷⁹ and the pentafuorophenol moiety (PFP) **46**.⁸⁰ PFP esters have been recommended for the preparation of heterocyclic acids, where DCC **22** or DIC **36** on its own had failed.⁸¹ They are also known to lead to a very rapid coupling with Fmoc-protected amino acids.⁸² 2,4,5-Trichlorophenol **47** derived esters are reported to be more reactive than both PNP esters⁸³ and *N*-hydroxy-5-norbornene-endo-2,3-dicarboxyimide (HONB) esters **48**.^{84,85} 2,4,5-Trichlorophenyl esters are also superior to *N*-hydroxysuccinimide (HOSu) **49** as a racemisation suppressant in peptide synthesis. HOSu esters still, however, offer an interesting alternative, as they are water soluble and therefore, easy to eliminate at the purification stage.

Hydroxy-7-azabenzotriazole (HOAt) 50 has been reported



Scheme 36. Commonly used alcohols in amide coupling.

to be more efficient than HOBt **41** in some difficult cases such as coupling with hindered bases. The increased efficiency might be due to the additional chelation or to the neighbouring effect provided by the pyridine nitrogen during the aminolysis step (Scheme 37).⁸⁶



Scheme 37. Additional chelation with HOAt 50.



Scheme 38. Succinimidyl ester preparation.

2.5.2.1. Multistep procedures. Active esters can be prepared in advance, purified and stored over time. Some amino acids are even commercially available as their benzotriazole (Bt) esters. Activated esters are usually

synthesised using standard ester-formation methods such as DCC **22** (see Section 2.4.1 and Section 2.4.2.4.3), but some other more exotic procedures can be found in the literature.

2.5.2.1.1. Succinimidyl esters. Succinimidyl esters **51** can be generated by reacting the corresponding acid and N,N'-disuccinimidyl carbonate (DSC) **52**.⁸⁷ The mechanism is similar to the CDI **18** mechanism (Scheme 38).

2.5.2.1.2. Use of 1,2,2,2-tetrachloroethyl chloroformate as intermediate. Activated esters have been prepared using 1,2,2,2-tetrachloroethyl chloroformate **53**, which is readily synthesised from chloral and phosgene. This reactive species can advantageously be reacted with an alcohol (e.g., HOBt **41** and HOSu **49**) to yield the corresponding 1,2,2,2-tetrachloroethyl carbonate **54**, which can be further reacted with an acid under basic conditions to generate the activated ester (Scheme 39).⁸⁸

2.5.2.1.3. Isoxazolium salts. In the 1960s, Woodward developed a method using *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, also called Woodward's reagent K or NEPIS **55** (Scheme 40).⁸⁹ This zwitterion reacts with *N*-protected amino acids in the presence of triethylamine to generate an enol ester **56**. This activated ester does not require purification and readily reacts and undergoes aminolysis to yield the peptide derivative, accompanied by the enol that tautomerises to the ketone **57**. The sulfonate by-product can easily be eliminated by aqueous washing. Unfortunately, the ionic nature of this reagent makes it difficult to use on solid-phase.



Scheme 39. Multistep preparation of activated esters using 1,2,2,2-tetrachloroethyl chloroformate 53.



Scheme 40. Coupling procedure using reagent K 55.



Scheme 41. Two-step coupling procedure using *N*-ethylbenzisoxazolium tetrafluoroborate 58.

Another example is *N*-ethylbenzisoxazolium tetrafluoroborate **58** (Scheme 41). Mechanistic studies show that the *N*-ethylbenzisoxazolium cation readily undergoes basecatalysed ring opening to form the transitory *N*-ethylbenzoketoketenimine **59**.⁹⁰ In the presence of carboxylic acids, the addition product rapidly rearranges to form the active phenol ester. The major limitation of this method is the slowness of the aminolysis step (four times slower than with *p*-nitrophenol) that enables side reactions to take place and therefore make this reagent less popular.

2.5.2.2. One-pot solutions. One-pot coupling conditions have been developed for peptide synthesis where the active ester is prepared in situ as an intermediate and subsequently reacts with the desired amine. As described in Section 2.4.2.4.3, this can be simply achieved by adding catalytic or stoichiometric amounts of HOBt **41** to standard DCC **22** coupling conditions.

More recently, efficient catalysts, which already incorporate the phenol have been proposed as elegant solutions for peptide couplings. Most coupling reagents are nowadays commercially available. They can be sorted according to their nature, that is, uronium, phosphonium and immonium salts. Among them, HOBt- and HOAt-based onium salts and halogenated coupling reagents have been designed. Some other examples of one-pot amide formation will be discussed at the end of this Section.

2.5.2.2.1. Phosphonium salts. Benzotriazol-1-yl-oxy-

tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) **60**, also called Castro's reagent,⁹¹ is the first published example of these HOBt-based onium salt reagents. The one-pot coupling is performed mixing the desired acid and amine in the presence of BOP **60** and triethylamine or Hünig's base. The deprotonated acid first reacts with BOP **60** to generate both an activated acylphosphonium species and HOBt **41**. HOBt **41** readily reacts with the activated acid to produce a reactive Bt ester, which finally undergoes aminolysis. The driving force of this phosphonium-based reaction is to generate the corresponding oxide **61** (Scheme 42).⁹²

Caution: Castro's reagent is very effective, but generates hexamethylphosphoric triamide (HMPA) **61**, which is extremely toxic. Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop[®]) **62** has therefore been developed.⁹³ It is equally efficient, but generates a less toxic by-product, 1,1',1''-phosphoryltripyrrolidine **63** (Fig. 3).

N-Methyl- α -amino acids are very difficult to couple even with PyBop[®] **62** or BOP **60**. The couplings are slow, low yielding and racemisation starts to take place. One explanation could be that the HOBt ester intermediate (that is so effective with primary amines) is too bulky to readily react with the secondary amine, hence enabling degradation to take place. Some effective reagents have been developed where HOBt **41** has been banned. For example, bromotri(pyrrolidino)phosphonium hexafluorophosphate



Scheme 42. One-pot coupling procedure using BOP 60.



Figure 3. PyBop[®] 62 and its phosphoric amide by-product 63.

(PyBrop) **64** (Scheme 43) is an efficient peptide coupling reagent for *N*-methylated amino esters.⁹⁴ The deterrent effect of HOBt **41** can be further confirmed by the fact that the addition of HOBt **41** to the coupling mixture enhances degradation and racemisation.

Different mechanistic pathways have been suggested (Scheme 43), one of which speculates the in situ formation of the acyl bromide (see TFFH **13** activation in the Section 2.1.2).



Scheme 43. One-pot coupling procedure using PyBrop 64.

Table 1. Phosphonium-based coupling reagents





Scheme 44. One-pot coupling procedure using HBTU 65 or TBTU 66.



Scheme 45. Equilibrium between uronium and guanidinium species.



Scheme 46. Potential guanidine by-product formation when using HBTU 65.

2.5.2.2. Uronium salts. Another family of reagents has been developed around uronium species such as O-(1*H*-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexa-fluorophosphate (HBTU) **65**¹⁰⁵ or its tetrafluoroborate equivalent TBTU **66**.¹⁰⁶ The counterion has no influence on the reactivity. The coupling is performed in a similar way to that using the phosphonium species. In this case, the driving force is the generation of the urea by-product (Scheme 44).

In solution, benzotriazole uronium species (*O*-form) are in equilibrium with the guanidinium species (*N*-form). The guanidinium *N*-form is usually reported as the crystalline form (Scheme 45).¹⁰⁷ HATU **67** has been proven to be very efficient in difficult sterically hindered couplings and usually gives a minimal level of racemisation.^{108,109} It involves the formation of 7-azabenzotriazol-1-yl esters, very highly reactive species towards amines, probably because of intramolecular general base catalysis.

Uronium species are also known to be guanidylation agents (Scheme 46). This side reaction can be diminished by adding HOBt **41** to the reaction (a similar concept to the use of DCC **22** and HOBt **41**).



Scheme 47. One-pot coupling procedure using TOTU 68.

Table 1 gives a non exhaustive list of other literature phosphonium-based coupling agents, $^{95-104}$ and their mechanisms can be easily deduced by comparison with the reagents previously described.

As explained for the phosphonium salts (see PyBrop 64), one of the limitations of the activated esters (HOBt etc.) is the steric hindrance during the aminolysis step (e.g., α , α -dialkyl-amino acids or *N*-alkyl-amino acids). Some novel







coupling reagents have been designed to offer alternative activation intermediates. For example, O-((ethoxycarbonyl)cyanomethylene amino)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TOTU) **68** generates an activated acyl oxime **69** and low-racemisation peptide couplings have been described (Scheme 47).¹¹⁰

Tables 2 and 3 give a non-exhaustive list of, respectively, other literature uronium- and iminium-based coupling agents.^{35,36,39,111–118} Iminium-based reagents can be considered as an extension of their uronium-based counterparts and analogous mechanisms can be applied. These reagents are used either on their own or in combination with HOBt **41**.

2.5.2.2.3. Ammonium salts.

2.5.2.2.3.1. Triazinyl esters. Recently, Kunishima and co-workers^{119,120} have described the use of 4-(4,6-dimethoxy-(1,3,5)triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) **70** as an effective activating agent not only for ester coupling, but also for amide bond formation and peptide synthesis (Scheme 48).¹²¹ This reagent initially undergoes similar S_NAr reactions as in the case of cyanuric fluoride (see Section 2.1.2). The activated ester **71** is then displaced by the amine. The advantage of this one-pot

procedure is that no additional base is required as *N*-methylmorpholine is liberated during the first step. The triazinone by-product **72** is easily eliminated by aqueous washing.

2-Chloro-4,6-dimethoxy-1,3,5-triazine has also been utilised. It is a commercially available and very cheap reagent. As mentioned previously for DMTMM **70**, a similar mechanism occurs. The insoluble hydroxytriazine by-product is formed and can be removed by filtration.¹²²

2.5.2.2.3.2. Mukaiyama's reagent. Mukaiyama's reagent, 2-chloro-1-methylpyridinium iodide **73**, gives, in the presence of a carboxylic acid and a tertiary amine, an activated pyridinium ester **74** that reacts with a range of nucleophiles. Some of the applications include the conversion of β -amino acids into β -lactams, the formation of esters (e.g., activated Bt ester) and amides (Scheme 49).¹²³ This reagent is not often used in peptide synthesis and, due to the poor solubility of the pyridinium iodides in conventional solvents, the reaction has to be performed under reflux in methylene chloride.

Recently, Xu et al. have published alternatives to Mukaiyama's reagent **73**. In order to improve the solubility of the pyridinium compounds, the tetrafluoroborate and hexachloroantimonate counterions were adopted (Fig. 4). 2-Bromo-3-ethyl-4-methylthiazolium tetrafluoroborate (BEMT) **75** was successfully applied to the synthesis of oligopeptides containing *N*-alkyl or α -*C*-dialkyl amino acids,¹²⁴ and, later, they developed other 2-halopyridinium salts such as 2-bromo-1-ethylpyridinium tetrafluoroborate (BEP) **76**, 2-fluoro-1-ethylpyridinium tetrafluoroborate (FEP) **77**, 2-bromo-1-ethylpyridinium hexachloroantimonate (BEPH) **78**, and 2-fluoro-1-ethylpyridinium hexachloroantimonate (FEPH) **79**.¹²⁵ These α -halopyridiniumtype coupling reagents were also used in solid-phase peptide



Scheme 48. One-pot coupling procedure using DMTMM 70.



Scheme 49. One-pot coupling procedure using Mukaiyama's reagent 73.



Figure 4. Alternative Mukaiyama-type reagent with improved solubility.

synthesis, especially for the synthesis of peptides containing *N*-methyl amino acid residues.

2.6. Other coupling methods

This section will review some original strategies and alternative methods to generate an amide bond, as well as providing a brief overview on the use of solid-supported strategies for the formation of amides.

2.6.1. Staudinger ligation. The Staudinger ligation¹²⁶ of peptides with a *C*-terminal phosphinothioester **80** and an *N*-terminal azide **81** is an emerging method in protein synthesis from peptide fragments (Scheme 50).

The first stage is to prepare the two peptides. The *C*-terminal part of the first peptide is coupled with the phosphinomethyl thiol **82** and the *N*-terminal part of the second peptide is converted into the corresponding azide **81**. The two fragments are then reacted together and undergo a Staudinger type of reaction, detailed in Scheme 51, to lead to the iminophosphorane **83**. This intermediate readily undergoes an *S*- to *N*-acyl transfer to produce the

corresponding amidophosphonium salt **84**. Hydrolysis generates the desired amide **85**.

2.6.2. Using proteases and amidases. Although proteases and amidases naturally hydrolyse amide bonds, there are examples in the literature, which show their use to form amide bonds. Two different methods are used, namely thermodynamic and kinetic control.

Under thermodynamic control, the reaction conditions are modified to drive the equilibrium to the synthesis of the amides, instead of their hydrolysis. For example, replacing the water by organic solvents to suppress the ionisation of the starting material, or increasing the concentration of the starting materials, or choosing protective groups to promote precipitation of the product, can tilt the equilibrium towards amide bond formation.¹²⁷

Under kinetic control, the carboxylic compound is usually activated as an ester, which forms with the enzyme an acyl enzyme intermediate, which subsequently reacts with the amine to give the desired amide. Kinetically controlled syntheses are more common and generally faster than those which are thermodynamically controlled.

Enzymes belonging to the following families are routinely used as catalysts: proteases,¹²⁸ subtilisin,¹²⁹ acylases, amidases and lipases.¹³⁰ One of the main disadvantages of biocatalysis is that enzymes only feature limited substrate compatibility. Therefore, the enzymatic approach is often neglected at the discovery stages, because it frequently requires a time-consuming screening process, even to establish the feasibility, whereas it is strongly considered



Scheme 50. Staudinger ligation.



Scheme 51. Mechanism of Staudinger reaction.

at the process development stage where the optimisation efforts are worthwhile, considering the following potential synthetic and economic advantages of biocatalysis:

- reaction temperatures are significantly reduced to nearambient conditions and, as a result, its applications can be extended to thermally labile compounds.
- reactions are frequently performed in aqueous media, which considerably reduces the problem of production waste management and enables the implementation of environmentally friendly chemistry (Green Chemistry).
- use of immobilised enzyme reactors or enzyme membrane reactors facilitates the purification and allows an easy recycling of the enzymatic catalyst.
- with enzymes, enantioselectivity of over 99% ee can be achieved routinely (although by no means in every case), allowing the manufacture of enantiomerically pure drugs or advanced pharmaceutical intermediates.

Kyotorphin (Tyr-Arg),¹³¹ a potent analgesic, was produced on a kilogram scale using α -chymotrypsin, a peptidase isolated from bovine pancreas, as catalyst. Unprotected tyrosine and arginine were selectively coupled to form only one out of the two possible dipeptides. Another industrial example is the 100 tons-per-year production of ampicillin **86** (penicillin-derived antibiotic) from 6-aminopenicillanic acid (6-APA) **87**. 6-APA is subjected to an enzymatic acylation reaction in the presence of immobilised penicillin acylase with phenylglycine methyl ester or amide (Scheme 52).¹³²

Gotor et al. reported *Candida antartica* lipase (CAL)- and *Pseudomonas cepacia* lipase-catalysed amidation reactions

of β -hydroxyesters,¹³³ β -aryl esters,¹³⁴ α , β -unsaturated esters,¹³⁵ α -haloesters¹³⁶ or diesters using different types of methyl and ethyl esters.

2.6.3. Microwave activation. In several cases, microwave irradiation has been a successful alternative to conventional high temperatures to perform direct condensation of amines to carboxylic acids without prior activation. The use of direct microwave heating is reported to reduce the chemical reaction time, reduce side reactions, increase yields and improve reproducibility.¹³⁷ The microwave irradiation may be run with or without catalyst.¹³⁸ Different kinds of catalysts such as K-10 montmorillonite,¹³⁹ imidazole,¹⁴⁰ zeolite-HY,¹⁴¹ polyphosphoric acid,¹⁴² *p*-toluenesulfonic acid,¹⁴³ TaCl₅-silica gel,¹⁴⁴ KF-alumina and -silica gel¹⁴⁵ have been used.

2.6.4. Solid-phase strategy. No attempt is made here to provide an exhaustive catalogue of solid-phase coupling conditions, but merely to demonstrate that solid-supported strategies can advantageously be used to synthesise amides. Methods involving solid-phase have been initially developed to facilitate the synthesis of peptides.¹⁴⁶ This process is now performed on automated synthesisers that can assemble sequences of up to 50 amino acids in a few days. During the last two decades, solid-supported chemistry has yielded numerous applications, especially in the field of parallel and combinatorial chemistry.¹⁴⁷ Amides are a convenient way of introducing a point of diversity on a template as numerous amines and acids are commercially available. Using such parallel techniques, libraries of more than 20,000 compounds have been produced. Three different strategies are used in solid-supported synthesis:



Scheme 53. Solid-supported peptide synthesis.

- classical polymer-supported synthesis
- polymer-supported reagents
- catch and release strategy.

2.6.4.1. Classical polymer-supported synthesis. One of the best examples of amide bond formation on solid-phase is encountered in peptide synthesis.¹⁴⁸ The principle of polymer-supported peptide synthesis is illustrated in Scheme 53. The first *N*-protected amino acid **87** is loaded onto the resin **88** using standard coupling reagents. After removal of the protecting group, the second *N*-protected amino acid **89** is coupled to the first. This sequence is repeated until all desired amino acids are loaded. The last step is the cleavage of the final peptide **90** from the polymer support. The choice of the resin and the *N*-protecting group is crucial as the conditions of the repeated coupling/deprotection sequences should not trigger a premature cleavage of the unfinished peptide or induce alteration of the polymer-supported peptide. For example, hydroxybenzyl-

based resins such as Wang resin are stable under basic conditions and are advantageously used in conjunction with fluorenemethyloxycarbonyl (Fmoc) *N*-protected amino acids. The peptide is cleaved off the resin under acidic conditions using trifluoroacetic acid (TFA). Fmoc is readily removed under basic conditions with a piperidine solution wash and is stable in the presence of standard uronium/ phosphonium coupling reagents such as PyBop[®] **62**, PyBrop **64**, TBTU **66** etc.

The amide functionality is often used as a convenient way of introducing diversity in parallel synthesis as numerous acids/acyl chlorides and amides are commercially available. Both the amine or the acid can be polymer-supported, as illustrated in Scheme 54.¹⁴⁹ In this example an α -amino methyl ester **91** is loaded via reductive amination on an aldehyde resin **92**.¹⁵⁰ The resulting secondary amine **93** is then coupled to a selection of acids **94**. After saponification of the polymer-supported amino acid, a functionalised amine **95** is coupled. Both coupling were performed using



Figure 5. Resins for solid-phase-assisted amide synthesis.

3 equivalents of EDC **37** and an excess of acid or amine. The final release of the final compound **96** is performed under acidic cleavage conditions.

This method presents the usual advantages of solid-phase chemistry. The reagents can be used in a large excess to push the reaction to completion and the final excess of reagent and by-products can be washed off. The full range of coupling reagents and methods are potentially applicable and the choice has to be case based.

2.6.4.2. Polymer-supported reagents. The second strategy consists of using polymer-supported activating agents such as PS-DCC 97 (Fig. 5). The main advantage is that the coupling reagent-related by-products are polymer bound and easy to eliminate by filtration.

2.6.4.3. Catch and release strategy. The final strategy entails immobilising the acid on a polymer-support as an active ester. When reacted with a nucleophile such as an amine, the amide is cleaved off the resin. The main difference between this and the previous strategy is that this latter method usually tolerates the performance of some chemistry on the polymer-supported ester, provided that strong nucleophiles are avoided. During the cleavage, a limiting amount of amine can be used to avoid the presence of excess amine in the final mixture. The acid is loaded onto the resin using classic ester condensations methods for tetrafluorophenol (TFP) resin **98**,¹⁵¹ HOBt resin **99**¹⁵² and oxime resin **100**.¹⁵³ The triazine resin **101** is loaded via an aromatic nucleophilic substitution in the presence of the acid (Fig. 5).¹⁵⁴

3. Conclusions

Methodologies to form an amide bond have been described since the beginning of organic chemistry, but, in the past two decades, the design and the synthesis of innovating coupling reagents has been an area of intense investigation. Most of these new developments were originally aimed towards the highly demanding and specialised field of peptide synthesis. Indeed, many of these reagents have been developed on purpose, to enable the coupling of specific amino acids, or to work in conjunction with a precise protecting group (e.g., Fmoc, Boc etc.). The main difficulties to overcome were to synthesise hindered peptides, to avoid racemisation or to be robust enough for solid-phase synthesis. Today, peptides are routinely synthesised on solid support using automated systems. Furthermore, a significant number of the coupling reagents described in this review are commercially available and have significantly expanded the arsenal of the synthetic chemist for the formation of any type of amide bonds.

The predominance of carbodiimide and active ester techniques has been gradually replaced with the so-called 'onium salts'. Among these reagents, HOBt- and HOAtbased uronium, phosphonium and immonium salts are proving to be very efficient. Many other reagents, however, could be more adapted to a specific case, since they might be cheaper or facilitate the final purification. For example, robust and classical methods involving acyl halides, anhydrides, acylimidazoles and enzymes are still largely used and should be considered.

Therefore, depending on the demands of the specific synthesis, the chemist will have a choice between many different conditions and strategies, as widely enumerated in this review.

Acknowledgements

We thank Dr Bob Marmon, Dr Herve Deboves, Dr Tom Coulter and Dr Manuel Cases for helpful discussions.

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