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A new wave of amide bond formations for peptide synthesis

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This review is dedicated to the scientific career of Prof. Em. Dirk Tourwé.

Abstract The construction of peptidic amide bonds has become a daily laboratory practice by virtue of well-established ‘coupling reagents’. Nonetheless, inherent limitations connected to these classical coupling methods in terms of waste, safety and expense have yet to be conquered. Research efforts have been devoted to synthetic methods able to surpass these limitations. This review focuses on the advances made in these ‘non-classical’ methods for amide bond formation with a specific application in peptide chemistry. It consists of two main sections: i) novel carboxylic activation reagents and ii) carboxylic acid and amine surrogates.

1 Introduction

2 Alternative carboxylic acid activation reagents

3 Carboxylic acid and amine surrogates

4 Conclusion and perspectives

Key words Amide - peptide – amino acid – non-classical peptide couplings – carboxylic acid surrogates – amine surrogates

Introduction

The pharmaceutical industry’s renewed interest in peptides as potential therapeutic agents was brought about by a significant decrease in the number of approved drugs since the start of the 21st century.¹ The increased demand of regulatory authorities for drug efficacy, quality and safety resulted in a decrease in R&D productivity and has required an alternative discovery approach towards new molecular entities.^{1b} In spite of the intrinsic drawbacks of peptides, which seemed to outweigh their strengths, the abovementioned need pushed to overcome limitations encountered during their synthesis and formulation.^{1c} After the introduction of solid-phase peptide synthesis (SPPS), the development of newer and more powerful coupling reagents, as well as reverse-phase chromatography made peptide synthesis at laboratory scale common daily practice.² Innovative tools such as chirality changes, amino acid substitutions, *N*-alkylation’s, conformational constraints and so on,³ granted the translation of native peptides into therapeutics, boosting the profile of peptide drugs.⁴ The renaissance in peptide drug discovery is demonstrated by 30 non-insuline peptides that have been marketed since 2000.^{1c} In 2017 only, a total of 6 peptides were approved by the FDA.^{1a, 2b}

Figure 1. General structures of major classes of coupling reagents (a) and selected examples of Oxyma-based reagents (b)

The main chemical step in the synthesis of peptides is undoubtedly the formation of the amide bond. This reaction, not only of great importance in peptide assembly, is considered to be among one of the most executed transformations in organic chemistry and is typically achieved *via* acylation of an amine by means of an activated carboxylic acid.⁵ Activation of the carboxylic acid can be achieved *in situ* with so-called ‘coupling reagents’.⁶ In peptide synthesis, carbodiimides **1**, uronium- **2** and phosphonium salts **3**, with each their specific co-reagents, such as *N*-hydroxybenzotriazole **4** derivatives, base, etc., are commonly applied. (Figure 1a).

Due to their general application, major advances in these classes of coupling reagents have been pursued over the years, exemplified through the development of OxymaPure **7**,⁷ for the replacement of *N*-hydroxybenzotriazole additive out of safety concerns, and followed by the development of OxymaPure-derived coupling reagents like COMU **5** and PyOxim **6** (Figure 1b).⁸ A set of reviews written in the past decades reveals a wider view on the current status of “classical coupling” in peptide synthesis.^{2, 6, 8a, 9} In contrast, this short review will focus on novel “non-classical” approaches towards amide bond formation, to tackle the drawbacks - such as poor atom economy, price, potential hazardous properties, and the repercussions on chirality^{5b} - still associated with these “classical coupling” reagents, and their application in peptide synthesis. This review is divided in two major approaches: (i) amide bond formation focusing on uncommon carboxylic acid activation and its subsequent reaction with amines, and (ii) amides formed *via* carboxylic acid- and/or amine surrogates.

Alternative carboxylic acid activation reagents

Recent advances in carboxylic acid activation aim to establish more atom-economic and convenient methods by deploying new reagents of which the by-products are often easily separated from the peptide mixture and/or recycled.

Based on the elaboration of OxymaPure, Mandal and co-workers, presented *o*-NosylOxy **9** as a novel recyclable coupling reagent.¹⁰ Couplings towards dipeptides with **9** were carried out in CH_2Cl_2 at room temperature for 2-3 h in the presence of DIPEA (Scheme 1b). These conditions tolerated common protecting groups such as Fmoc and Cbz, and proceeded smoothly for the coupling of sterically hindered amino acids, such as H-Val-OMe and H-Aib-OMe. Important was the preactivation of the carboxylic acid with **9** for 5 min to avoid sulfonamide formation. In addition, the authors demonstrated the utility of their reagent in the synthesis of longer peptides, up to 10 amino acids, *via* SPPS on Rink amide resin. Recycling of the coupling reagent was achieved

with a recovery of 45% by isolating the two by-products, Oxyma **7** and *o*-nitrobenzenesulfonic acid **10** (Scheme 1a, in frame). The latter was easily transformed back into the chloride **8** with SOCl₂.

Scheme 1 Synthesis of *o*-NosylOxy **9** (a) and its application as coupling reagent (b).¹⁰

Later, Mandal and co-workers, developed a modified Yamaguchi reagent based on Oxyma, coined TCBOXY **11**.¹¹ The original Yamaguchi reagent, 2,4,6-trichlorobenzoyl chloride, initially demonstrated in the efficient synthesis of esters *via in situ* formed mixed anhydrides, caused significant epimerization, making it an unsuited method for peptide bond formation.¹²

Scheme 2. Selected examples of coupling with TCBOXY **11** as a modified Yamaguchi reagent.¹¹

The authors demonstrated that **11**, by preactivation of the acid in the presence of DIPEA and DMAP, was applicable to synthesis a set of dipeptides equipped with Fmoc, Boc and Cbz protecting groups, giving rise to isolated yields varying from 70-91% (Scheme 2). The couplings' effect on chirality was examined for two different dipeptides, confirming that no detectable epimerization had occurred. Similar to their previous study, the authors presented their coupling reagent in SPPS assembly up to 10 amino acids (3 examples). The by-products from the coupling, oxyma **7** and 2,4,6-trichlorobenzoic acid, could be recycled back into **11** with an overall yield of 52%.¹¹

a. Synthesis of the 9-silafluorenyl dichloride **13**

b. Dipeptide synthesis with 9-silafluorenyl dichloride **13**

Scheme 3. Synthesis and application of chlorinated 9-silafluorenyl dichloride **13** for peptide bond formation.¹³

Likewise, new coupling reagents have been developed. Charette and co-workers presented chlorinated 9-silafluorenyldichlorides such as **13** as reagents. The by-product of the coupling was a polymeric siloxane, which was easily removed by filtration. Unlike traditional methods, no pre-activation of either amino acid units was required and the reaction proceeded in the presence of Et_3N (5 equiv) and DMAP (0.5 equiv). The 9-silafluorenyl dichloride bearing a chlorine at the 3-position (**13**) was easily synthesized *via* a one-step procedure starting from the corresponding chlorinated 2,2'-dibromobiphenyl **12** (Scheme 3a). 9-silafluorenyl dichloride **13** was employed to synthesize a set of 17 dipeptides with yields varying from 61-99% and only limited epimerization was observed for the coupling to Boc-Phg-Gly-OMe (86% ee).¹³ The method is based on a temporary silicon tether **14** (intermediate in Scheme 3b), which rearranges upon heating to produce the desired amide bond by extrusion of an inert siloxane. The driving force behind this transformation is the strength of the newly formed Si-O and amide bonds (Scheme 3b). Elaboration of the mechanistic by Jiang *et al.* indicated that the reaction is most likely to occur *via* a nucleophilic addition, either base-, DMAP- or carboxylic acid assisted (Scheme 4).¹⁴

Scheme 4. Mechanism *via* intermolecular nucleophilic addition of the amine to the 9-silafluorenyldichloride activation with base assistance by Jiang *et al.*¹⁴

Hypervalent iodine reagent have shown to be environmentally friendly recyclable reagents which has led to an increased interest for its application in organic synthesis.¹⁵ Zhang and co-workers developed iodosodilactone **15** (Scheme 5a) for esterification and direct amidation. It was engaged in the epimerization-free synthesis of *N*-Cbz-protected dipeptides including Met, Ile, Pro and Phe residues, in the presence of DMAP and PPh_3 in chloroform at 60°C (Scheme 5a).¹⁶

Scheme 5. Hypervalent iodine compounds as activating reagent for amide formation.¹⁶⁻¹⁷

In an effort to develop milder coupling conditions, the authors later showed *p*-BTfP-iodosodilactone **16** to couple at room temperature within 10 min in absence of the toxic DMAP. Optimisation of the coupling revealed that by using 1.2 equiv of **16** in combination with 1 equiv (4-MeOC₆H₄)₃P and 3 equiv of Et₃N in dichloroethane (Scheme 5b), amide bond formation was achieved for a broad substrate scope with good to excellent yields. Unfortunately, the halogenated solvents used are highly hazardous.¹⁸ It is notable that the by-product **17** of the coupling reagents **16** allows the regeneration of **16** in 90% (Scheme 5b in frame).¹⁷ The tris(4-methoxyphenyl)phosphine oxide as a result of (4-MeOC₆H₄)₃P cannot be recycled. The combination of **16**, in with (4-MeOC₆H₄)₃P, was tested in SPPS and cyclization, as exemplified by the synthesis of pseudostallarin D.¹⁹

Scheme 6. Triazine based activating reagents for amide synthesis.²⁰

To reduce the cost of couplings, Papini and co-workers put the use of triazine-based coupling reagents forth and developed *N*-triazinylammonium tetrafluoroborates **18** for the efficient synthesis of peptides (Scheme 6a). The coupling is achieved by preactivation of the *N*-protected amino acid with **18** and NMM 0°C prior to addition of the amine. The basic properties of the triazine ring

rendered purification of the reaction mixture by acidic extraction highly efficient for the removal of 2-hydroxytriazine by-product and unreacted reagents. The reaction is orthogonal with Cbz, Boc and Fmoc protecting groups, as well as a broad scope of protected side chain functionalities. These conditions showed to be compatible with both solution and solid-phase peptide synthesis. Application in the latter was even demonstrated in an automated system.^{20a}

Similarly, Pattawarapan and co-workers presented 2,4,6-trichloro-1,3,5-triazine **19** (TCT), an inexpensive commercially available reagent, in combination with a catalytic amount PPh₃, for amide bond formation using a solvent-drop grinding to reduce solvent waste (Scheme 6b). Important to note is that this reaction is carried out in inert atmosphere, limiting the applicability in comparison to the previous method. Initially, the *N*-protected α -amino acid is activated *in situ* in the presence of K₂CO₃ for 20 min. Followed by a 20 min reaction with α -amino methyl ester hydrochloride salts. The coupling conditions were applied for the synthesis of dipeptides resulting in an isolated yield varying from 71-92% after column purification. Here, the compatibility with the main *N*-protecting groups was demonstrated as well as the tolerance for sterically hindered side chains.^{20b}

Scheme 7. Carboxylic acid activation by *in situ* formation of carboxyl – sulfinyl mixed anhydride **22**.²¹

One way to reduce waste and improve atom-economy of the reaction is to develop highly efficient coupling reagents which do not require large excesses featuring a low molecular weight. Vogel and co-workers presented a one-pot two-step procedure for the synthesis of amides *via* carboxyl sulfinyl anhydride **22** at room temperature. In the first step, preactivation is performed by reaction of the carboxylic acid with trimethyl((2-methylbut-3-en-1-yl)sulfonyl)silane **20**, 2-methylbut-3-ene-sulfinyl fluoride **21** and Me₃Si at 20°C (Scheme 7). Subsequently, the amine is added alongside AgBF₄. The non-nucleophilic BF₄⁻ anion is required to avoid the *S*-addition of the amine reaction, which was observed in the presence of the iodine anion, leading towards the corresponding sulfinylamide. The by-products of this transformation are all volatile, simplifying the purification. Following their dipeptide synthesis, the authors demonstrated a one-pot *t*Bu-TMS exchange in the presence of **20**, **21** and TMSOTf, which without intermediate isolation could be coupled to a third α -amino ester in the presence of AgBF₄ (Scheme 7).²¹

Scheme 8. Ru-catalyzed activation of carboxylic acid with ethoxyacetylene **23** (top) and selected examples (bottom).²²

Groößen and co-workers presented a coupling protocol using simple alkynes such as acetylene or ethoxyacetylene **23** with a ruthenium catalyst, i.e. dichloro[(2,6,10-dodecatriene)-1,12-diyl]ruthenium **24**. In the presence of the catalyst, the carboxylic acid reacts with the alkyne to form a vinyl ester intermediate **25**, which subsequently undergoes aminolysis to give the corresponding amide with acetaldehyde or ethyl acetate as the only by-products depending on the alkyne selected. The authors demonstrated that, with a small excess of 1.5 equiv of amine and ethoxyacetylene in a hexane solution, the methodology can be used for the direct amide synthesis of a range of *N*-Cbz protected dipeptides utilizing a catalyst loading of only 1.5 mol% **24** at 80°C for 6h (Scheme 8). Regarding stereoisomeric purity, the epimerization remained below 10%.²²

Scheme 9. Activation of carboxylic acid with **26** (top) and selected examples (bottom).²³

The use of ynamides as coupling reagents had previously been attempted,²⁴ but proved difficult due to their thermal instability and moisture-sensitivity.²⁵ Zhao and co-workers evaluated these reagents further and observed that after introduction of an electron withdrawing substituent on the ynamide, it became synthetically stable and easy to handle. More precisely, terminal ynamide equipped with a tosyl-group, *N*-methyl-*N*-ethynyltoluenesulfonamide **26** showed to be stable for 2 months in a refrigerator and reacted smoothly with the carboxylic acid to form an α -acyloxyenamine **27** that

undergoes aminolysis in the presence of an amine (Scheme 9). The specific properties of the sulfonamide **26** result, upon reaction with carboxylic acid, in α -acyloxyenamines that can be isolated and stored at room temperature. The use of **26** in a one-pot two-step synthesis at 25°C was demonstrated for a set of dipeptides, isolated in excellent yield without any detectable epimerization. In some cases, a slight increase of temperature to 35°C was required to obtain a more efficient coupling. The methodology was compatible with both Boc-, Cbz- and Fmoc-protecting groups and functional groups such as a hydroxyl, thiol, primary amide and indole did not require protection. The side chains of Lys, His, Arg, Tyr, Asp and Glu on the other hand were not compatible.²³

A catalytic process without additional reagents for amide synthesis would be highly valuable in light of atom-economy and waste. The development of such processes in amide bond formation has therefore gained significant interest over the past years. Both enzymes and Lewis acids proved suitable. However, as this review is intended to disclose new chemical methodologies, we would like to refer the reader to recent specialized literature dealing with advances in chemoenzymatic peptide synthesis.²⁶ Regarding Lewis acid catalyzed transformations, two classes are known: (i) metal- and (ii) organoboron-catalyzed processes. Simultaneous removal of water *via* azeotropic distillation (Dean Stark) or (recyclable) molecular sieves is typically required to drive the reactions to completion.

Despite the fact that the metal-catalyzed amidation towards secondary and tertiary amides dates back to the '70s,²⁷ it is only recently that it matured and the scope of the reaction expanded towards amide bond formations in more complex systems such as amino acids.²⁸ However, to the best of our knowledge, examples in peptide synthesis have not been reported.^{26b, 29}

The feasibility of an organoboron-catalyzed amidation was initially demonstrated by Yamamoto and co-workers,³⁰ who combined electron-poor arylboronic acids with molecular sieves. However, these conditions required high temperatures, even for the more efficient catalyst, 3,4,5-trifluorophenylboronic acid (TFPBA) **28**, and remained rather limited in substrate scope.³⁰⁻³¹ One of the first examples of a dipeptide (Cbz-Phe-Ala-O*t*Bu) synthesis *via* such direct amidation was given by Latta *et al.*, yet, analysis indicated that the reaction product fully racemized.³²

Figure 2. Organoboron catalysts used in peptide synthesis.

A first systematic study of arylboronic acids for direct amide bond formation in peptide synthesis was carried out by Whiting and co-workers.³³ Initially, the condensation of *N*-Boc protected amino acids with benzylamine and the coupling of phenyl acetic acid with an α -amino ester HCl salt was studied. Here, the use of TFPBA **28** or *o*-nitrophenylboronic acid (*o*-NPBA) **29** at a loading of 25 mol% in fluorobenzene combined with 3 Å molecular sieves at 65°C showed to preserve chirality in

both cases. When combination of the two partners for dipeptide synthesis, a stoichiometric amount of the arylboronic acid deemed necessary. For Boc-Phe-Phe-OMe, *o*-NPBA **29** showed to be superior to TFPBA **28** (58% vs 31% yield). However, for the coupling with a more sterically hindered amine, H-Val-OMe.HCl, even the superior catalyst deemed insufficient. This, however, could be resolved by using a combination of catalyst *o*-NPBA **29** and 2-methylphenylboronic acid **30** with a loading of 50 mol% each. These mediated conditions proved robust for the synthesis of dipeptides with Boc-, Cbz- and Ac-protection (Scheme 10).

Scheme 10. Organoboronic acid-mediated synthesis of peptides (top) and selected examples, with indication of the amount boronic acid used and the isolated yield.³³

Hall and co-workers pursued their previous work,³⁴ involving 5-methoxy-2-iodophenylboronic acid **31** (MIBA) as a catalyst for the direct amidation at 40 °C, by attempting the synthesis of more elaborate peptides. They observed, however, that mono protected α -amino acid inhibited the catalyst through formation of **37** (Scheme 11). This issue could be circumvented by use of a phthaloyl protecting group (Phth), allowing the direct amidation of two amino acids with **31** (20 mol%). In the case of β -amino acids, complexation with the boronic acid was not observed, hence allowing the use of standard *N*-Boc-protection.³⁵

Scheme 11. MIBA **31** catalyzed amidation for peptide synthesis.³⁵

Based on the observations made by both Whiting³³ and Hall³⁴, regarding the superiority of *o*-substituted arylboronic acids in the dehydrative condensation, Blanchet and co-workers studied the effect of a sulfur containing moiety in this position. Here, 2-(thien-2-ylmethyl)phenylboronic acid **32** worked the best in both fluorobenzene or CH₂Cl₂ with concomitant use of 5 Å molecular sieves. Initial evaluation in the synthesis of Boc-Phe-Val-OMe **38** with a catalyst loading of 25 mol% at

65°C resulted in a 50% isolated yield after 24 h without any detectable epimerization (Scheme 12a).³⁶

a. Coupling with boronic acid 32

b. Coupling with boronic acid 33

Scheme 12. Organoboron-catalyzed peptide synthesis, established by Blanchet and co-workers.³⁶⁻³⁷

In an effort to improve the catalytic properties of their organoboron reagent, the authors hypothesized that borinic acids, possessing an amplified Lewis acidity,³⁸ potentially can improve the catalytic system and would allow a versatile peptide coupling. Here, *o*-chloro-substituted phenyl borinic acid **33** performed best, suggesting a specific electronic requirement in the *o*-position for an optimal catalytic cycle. With a catalyst loading of 25 mol% in fluorobenzene and 5 Å molecular sieves at 65°C (Scheme 12b), dipeptide synthesis was made accessible for *N*-Boc and *N*-Cbz amino acids with *C*-Me, *C*-Et and *C*-Bn amino esters. Even the coupling of more sterically hindered amines, such as H-Val-OMe, showed no decrease in yield.³⁷

Scheme 13. Borate ester **34** catalyzed amidation for peptide synthesis (top) with solid-phase work up (middle) and selected examples (bottom).³⁹

Sheppard and co-workers had previously shown a direct amidation utilizing stoichiometric amounts of borate ester, B(OCH₂CF₃)₃ **34**, during the synthesis of two dipeptides.⁴⁰ Consecutively, they evaluated the use of this commercially available borate ester in a catalytic process in 2-methoxy-2-methylbutane (TAME). It was crucial to prevent the partial hydrolysis of B(OCH₂CF₃)₃ **34** resulting in a poorly reactive oligomeric boron oxide. This condition was fulfilled by the use of a Dean-Stark apparatus (for water removal), avoiding the need for molecular sieves. The absence of any additives or dehydrating agents makes this process very interesting. Moreover, the reaction set-up could be combined with solid-phase workup by means of commercial resins (Scheme 13, resins depicted in the frame). This allowed the isolation of a set of dipeptides in excellent yields. Notable is that even threonine was used without side chain protection (Scheme 13).³⁹

Scheme 14. Coupling of *N*-trifluoroacetyl protected Met with H-Phe-OBn.HCl using **35** as catalyst and subsequent deprotection in dry acidic conditions.⁴¹

More recently, Ishihara and co-workers reported the use of 2,4-bis(trifluoromethyl)phenylboronic acid **35** for the synthesis of dipeptides. However, similarly to the work of Hall,³⁴ the standard carbamate protecting groups inhibited the catalyst. The authors utilized a more electron-deficient *N*-protecting group, thereby weakening the nucleophilicity of the amine moiety. Through aid of the *N*-trifluoroacetyl protection, the catalytic coupling of a set of dipeptides with variable sterical hindrance in the lateral chain and good yields up to 96% could be realized. Unfortunately, hazardous (CHCl₃)₂ was used as solvent.¹⁸ The deprotection of the *N*-trifluoroacetyl protecting group was accomplished in dry acidic conditions in MeOH at 60°C (Scheme 14).⁴¹

Scheme 15. Catalytic amidation using DATB **36** as catalyst (top) and selected examples (bottom) illustrating a substantial functional group compatibility.⁴²

Despite the progress being made towards greener and milder reaction conditions and their application in peptide synthesis, the side chain scope in the abovementioned studies remained mainly limited to aliphatic and aromatic side chains. Even more importantly, they did not always seem orthogonal with the standard *N*-Fmoc-, *N*-Boc- and *N*-Cbz-protecting groups. In an effort to further extend the scope of catalytic amidations, Kumagai and co-workers performed a systematic study on amino acid substrates involving 1,3-dioxo-5-aza-2,4,6-triborinane **36** as a catalyst. It was shown that by means of 5 mol% **36** in toluene and 4 Å molecular sieves at 80°C, a catalytic amidation protocol could be used for a wide range of functionalized and unfunctionalized amino acids with both standard *N*-Boc and *N*-Fmoc protections. The applicability of the developed methodology was nicely established through a convergent strategy, providing the protected form of pentapeptide OGP(10-14) **39** (Scheme 15).⁴²

Carboxylic acid and amine surrogates

Carboxylic acid or amine surrogates added to the well-established synthetic routes towards amide formation based on the coupling of carboxylic acid and amine with activating reagents. A review focusing on non-classical methods towards amides by de Figueirido *et al.* gives a comprehensive view on the developed chemistries.²⁹ Not all of these methodologies were applicable to or have

been evaluated yet in peptide chemistry. The implementation of surrogates in this transformation has been driven by the poor atom-economy associated with the classical coupling reagents and to access new chemoselective methods with high functional group compatibility. These methods preferentially lower the need for protecting groups use and open synthetic possibilities for larger peptides *via* a ligation of segments.^{5b} The golden standard in segment condensation is by far the native chemical ligation (NCL) technique pioneered by Kent and co-workers.⁴³ This very well-established technique is among the most famous methods of amide bond formation through a thioester intermediate. Notwithstanding, this review focuses on recent advances and the reader is referred to literature for a broader overview on developments in NCL.⁴⁴

Scheme 16. Amidation of methyl α -amino ester HCl salts **40** with amino acid ionic liquids **41**.⁴⁵

Unactivated esters are interesting carboxylic acid surrogates, since they make the carbonyl more electrophilic. However, alkyl esters are often used in peptide synthesis as side or main chain protecting groups. The development of a catalytic method using esters as educts, and especially methyl, would be of profound interest in view of minimizing waste production of the transformation. Oshima and co-workers, inspired by the methods that mediate amidation *in vivo*, presented NaOMe, as an efficient catalyst for the unprecedented amidation of amino acidmethyl esters.⁴⁶ The authors recognized that an acidic alcohol additive was required to balance the pH and prevent epimerization during the coupling. The best result, based on both yield and enantiomeric excess, was obtained with 4-trifluoromethylphenol (30 mol%) in combination with 3 Å MS, allowing lower catalyst loadings. Unfortunately, for the reaction of Boc-Phe-OMe with 4 equivalents of H-Ala-*O**t*Bu an isolated yield of 53% was obtained after 5 days at 80°C, with a recovered 39% of starting material.⁴⁶ Furukawa *et al.* developed a direct amide synthesis between methyl α -amino ester HCl salts **40** and amino acid ionic liquids (AAIL) **41**, tetrabutylphosphonium salts of zwitterionic amino acids.⁴⁵ Here, coupling was achieved at 60°C for 6 to 48 h and the condensation product was purified by means of filtration and acidification, resulting in an unprotected peptide fragment **42** (Scheme 16). Notable is the large excess of AAIL **41** used, which seemed necessary to prevent the formation of oligopeptide and diketopiperazine. The optimized protocol consists of the simply mixing of the two reagents under argon followed by the acidification with AcOH. It was utilized for the synthesis of a set of dipeptides, encompassing various sterically hindered amino acids such as Val, Pro and Phe, and showed compatible with unprotected Tyr in the synthesis of H-Tyr-Gly-Gly-OH.⁴⁵

Scheme 17. Two step approach for dipeptide synthesis *via* amidation of methyl *N*-hydroxyimino esters **43** with *tert*-butyl α -amino ester followed by diastereoselective hydrogenation.⁴⁷

A metal-catalyzed approach using a hydroxyl-directed amidation was established by Yamamoto and co-workers. Herein, a methyl or phenyl β -hydroxy carboxylic ester allowed a bidentate chelation of the catalyst, Ta(OEt)₅, hereby activating the ester for nucleophilic attack. Nonetheless, the need for this specifically positioned hydroxyl group, limits the *C*-terminal coupling partner to methyl esters of Ser and Thr.⁴⁸ To broaden the application scope, the authors developed a two step procedure.⁴⁷ In the first step, a *N*-hydroxyimino ester derived from an α -ketoester **43** reacted with *tert*-butyl α -amino ester in the presence of a Nb(OEt)₅ catalyst, resulting in *N*-hydroxyimino dipeptides **44**. Subsequently, the hydroxyimino group was transformed in an amine **45** by a diastereoselective hydrogenation in acetic acid (Scheme 17). The final step was highly diastereoselective and associated with high yield. However, in the case of Phe or Phg, a D/L mixture was observed, but the isolated diastereomeric ratio could be increased to 99:1 by purification with flash chromatography.⁴⁷

a. Introduction of the 4-(methylsulfonyl)phenyl ester 47

b. Direct peptide arylester aminolysis

Scheme 18. (a) Introduction of the 4-(methylsulfonyl)phenyl ester **47** by Chan-Lam reaction and subsequent oxidation, (b) direct aminolysis of peptidic arylester.⁴⁹

Van Maarseveen and co-workers presented 4-(methylsulfonyl)phenyl esters **47** as substrates for amide formation and NCL.⁴⁹ Initially, a 4-(methylthio)phenyl ester **46** was introduced onto the peptide *C*-terminus *via* a copper(II)-mediated Chan-Lam reaction and subsequently oxidized to its corresponding sulfone **47** (Scheme 18). This ester reacted spontaneously with an α -amino ester HCl salt in THF under dropwise addition of DIPEA to liberate free amine. The method showed no epimerization, by exception of Phg (81 %de).⁴⁹ Important to note is that aromatic esters have also found application in amide bond formation in NCL and *via* a serine/threonine mediated ligation.⁵⁰

a. Activation with Sanger 48 or Mukaiyama reagent 49

b. Cu(II)-catalyzed coupling

Scheme 19. Amidation *via* thioacid activation with Sanger **48** or Mukaiyama **49** reagent (a)⁵¹ and Cu(II)-catalysis (b).⁵²

Thioacids are of profound interest as substitutes for their oxygen counterparts, due to their enhanced nucleophilicity. This has transcended into significant applications in the fields of, but not limited to, peptides and peptidomimetics.⁵³ Notwithstanding, thioacids remain challenging to synthesize and they are not as stable as their corresponding carboxylic acids.^{29, 53} A recent comprehensive review

by Narendra *et al.* details their synthesis in the amino acid and peptide context.⁵³ We will give a short overview of the recent progress that has been described, specifically focusing on those that led to peptides. Of note, these methods are often used in combination with the release of the thioacid from its fluorenylmethyl (Fm) ester by deprotection with piperidine in DMF (40%).^{51, 54} Similar to carboxylic acids, thioacids do not spontaneously react with amines to form amides, but the desired conversion also requires activation *via* coupling reagents.²⁹ Crich and co-workers established the coupling by preactivation with the Sanger **48** or Mukaiyama reagent **49** in the presence of Cs₂CO₃ in DMF (Scheme 19a).⁵¹ Couplings making use of the Mukaiyama reagent **49** were shown to be superior to the classical couplings in segment condensations, as it completely suppressed epimerization.⁵¹ Thioacid activation can also be metal mediate, as was already established in 1981.⁵⁵ Latterly, Gopi and co-workers presented a Cu(II)-catalyzed peptide synthesis in methanol. This methodology was compatible with Fmoc-, Boc- and Cbz- protection and led to 2+2 segment condensations with isolated yields up to 77% (Scheme 19b).⁵² An alternative activation approach based on an *in situ* formed *S*-silylthiol was described by Liebeskind and co-workers.^{54b} The thermodynamically driven sulfur-to-oxygen tautomerization from **51** to **52** and its subsequent aminolysis smoothly led towards the formation of amide bonds (Scheme 20). The direct coupling was demonstrated for mono- and dipeptidic thioacids with various α -amino ester HCl salts with bis(trimethylsilyl)acetamide **50** (BSA) as silylating agent at ambient temperature.

Scheme 20. Amide formation *via* thioacid activation involving silatropic switch.^{54b}

Xian and co-workers presented an activation mode *via in situ* thiol *S*-nitrosation **54** at the *C*-terminus without any need for preactivation.⁵⁶ This protocol was performed with isopentyl nitrite **53** at 0°C in THF and presented no issues upon use of Boc, Fmoc or Cbz protecting groups. Interestingly, serine could be coupled under these conditions without side chain protection. This method of activation was later practiced by Khaybullin *et al.* for the coupling of arginine onto peptides.⁵⁷

Scheme 21. Amide bond formation by *in situ* *S*-nitrosothioacid **54** formation.⁵⁶

Danishefsky and co-workers described an oxidative dimerization of thioacids that served in turn as an acylating agent for amines (Scheme 22). The dimerization did not require any preactivation and was initially achieved in the presence of HOBT (2 equiv) in DMSO with 4 Å MS.⁵⁸ Even though the method was demonstrated for ligation of peptides (up to 32 amino acids) in good yields and without epimerization, the reaction time remained long in comparison to the previous methods (ca. 12 h). The authors circumvented this issue by addition of an oxidant, I₂, reducing the reaction time to 30

min. Gopi and co-workers later described a two-step synthetic approach towards these *N*-protected amino thioacid dimers allowing isolation for both Fmoc- and Boc-amino acids in moderate yields (25-40%).⁵⁹ The loss of product was associated with the hydrolysis/dissociation of the dimer during aqueous workup and column chromatography.

Scheme 22. Amide formation *via* oxidative dimerization of thioacids.⁵⁸

The combination of thioacids with amine surrogates bestowed a different niche in peptide synthesis. Chen *et al.* introduced dithiocarbamic acids **55**, *in situ* formed from an amino acid or α -amino ester and CS₂, which upon reaction with thioacids gave amide. The by-product CS₂ is required for amine activation and can therefore be used in a catalytic amount. The procedure is tolerant towards unprotected side chain functional groups.⁶⁰ For the introduction of the dithiocarbamic acid entity CS₂ was used in combination with pyridine as a base. The latter deemed crucial to prevent the formation of side products 2-thioxoimidazolidin-4-ones **56** and thioureas **57** (Scheme 23). The activated amine reacts spontaneously to the desired amide upon addition of a thioacid. The protocol, originally designed in CH₂Cl₂, proved compatible with *N*-Boc- and *N*-Fmoc-protected thioacids. The *C*-termini of the amine partners could be equipped with Me, Et and Bn esters. To expand this method, the authors examined a mixed organic - aqueous buffered medium, which showed that the methodology was compatible with carboxylic acids, primary amides, as well as unprotected Arg, Tyr and Ser side chains. These conditions were demonstrated for sequences up to 7 amino acid residues.

Scheme 23. Amide formation by *in situ* formation of *N*-terminal dithiocarbamic acid **55** and subsequent coupling with thioacids.⁶⁰

Elaborating on work by Tomkinson dealing with the coupling between 2,4-dinitrobenzenesulfonamides and thioacids,⁶¹ Crich *et al.* translated the protocol to peptides, departing from a *N*-Boc protected amino acid 9-fluorenylmethyl (Fm) thioester **58**.^{54a} In the first step, the thioacid was deprotected using a mixture of piperidine in DMF and reacted with a *N*-2,4-dinitrobenzenesulfonyl activated amino acid methyl ester **59** in the presence of Cs₂CO₃ (Scheme 24a). This methodology was demonstrated for a set of 14 *N*-Boc protected methyl ester dipeptides with yields varying between 51 and 82%. The authors expanded this protocol further to enable ligation of three building blocks by developing sulfonamides with a different reactivity.⁶² Replacement of one nitro group with a methyl ester (ENS) **60**, nitrile (CNS) **61** or CF₃ (FNS) **62** group (Scheme 24b) showed to significantly reduce the reactivity compared to the DNS, but they still provided the desired amides. Unfortunately, the reduced reactivity limited the coupling to *N*-protected glycine thioacids, and non-electron-deficient or sterically hindered thioacids. To expand the scope of their method, the authors established an alternative approach to overcome the fact that

the deprotection conditions for the Fm thioester are similar to those of the Fmoc protecting group. Herein, they demonstrated that a Tmob thioester **63**, which can be removed under acidic conditions, can serve as an orthogonal protection during the synthesis of *N*-Fmoc protected peptides. Finally, Sucheck and co-workers advanced this method by demonstrating the introduction of the sulphonamide on a solid bound peptide. The subsequent cleavage from the resin allowed the coupling with a thioacid in solution.⁶³

a. Thioacid deprotection and coupling with a DNS sulfonamide

b. Alternative sulfonamides and thioester presented by Crich *et al.*

Scheme 24. (a) Thioacid sulfonamide coupling^{54a} and (b) alternative sulfonamides and thioester protective group.⁶²

Selenoesters are well-known alternatives to thioesters in NCL.⁶⁴ Siciliano and co-workers presented phenyl selenoesters **64** as carboxylic acid surrogates to be used in peptide synthesis.⁶⁵ The authors established a mild procedure for the introduction where diphenyl diselenide reacted with carboxylic acid anhydrides. For the *N*-Fmoc protected amino acids, the required anhydride was obtained using *iso*-butyl chloroformate in combination with NMM. Coupling of the selenoesters with amines occurred in the presence of DIPEA at room temperature and was shown to be orthogonal with unprotected carboxylic acids (Scheme 25).⁶⁵

Scheme 25. Phenyl selenoesters as activated C-termini (top) for amidation, with selected examples (bottom).⁶⁵

Expanding on previous work,⁶⁶ Danishefsky and co-workers established isonitriles as amine surrogates for amide bond formation.⁶⁷ The authors proposed that a reaction between a carboxylic acid and an isonitrile would lead to an *O*-acyl formimidate **65** that could adopt an anti- or syn conformation (Scheme 26). In case of the anti-conformation, **65** can undergo a thermally induced

1,3-*O*→*N* acyl transfer to give *N*-formylamide **66** (Scheme 26, path a). Nonetheless, in the presence of an amine or oxygen nucleophile (Scheme 26, path b), the intermediate could be converted to an amide/ester by the release of a formamide. In this case it catalogues as an alternative carboxylic acid activation. Protocols to achieve both pathways were developed. In the absence of a nucleophile, a selective deformylation of the *N*-formylamide **66** was established leading to an *N*-alkyl amide.⁶⁸ However, the high temperature (150°C) required for both cases would limit their application on sensitive biological targets.

Scheme 26. Activation of a carboxylic acid with an isonitrile yielding **65** followed by (a) 1,3-*O*→*N* acyl transfer in the absence of nucleophile and (b) acylation in the presence of a nucleophile.⁶⁷

Accordingly, the same authors developed an approach where a thioacid reacted with isocyanides at ambient temperature.⁶⁸ This methodology in combination with the previously mentioned deformylation was used for the synthesis of cyclosporine.⁶⁹ Activation of the *N*-Boc or Fmoc protected amino thioacid and *tert*-butyl isocyanide followed by reaction with an α -amino ester proved to be an extremely useful protocol for the formation of secondary amides. It was exemplified for both solid-phase and fragment based couplings in solution,⁷⁰ and used for the synthesis of oxytocin,⁷¹ and the human granulocyte colony-stimulating factor.⁷²

Scheme 27. First coupling step for the synthesis of thymopentin **70**.⁷³

Progressing on the mechanism presented by Danishefsky and co-workers,⁶⁷ Hutton contemplated the direct formation of *N*-acetyl amide **69** from a thioamide **67**, used here as an amine surrogate, and a carboxylic acid in the presence of a thiophilic metal, Ag_2CO_3 (Scheme 27b) *via* imide **68**.⁷³ The

thioamides of the amino acid were obtained by acetylation, followed by a treatment with Lawesson's reagent or the direct thioacetylation with Rapoport's reagent or ethyldithioacetate. Following the coupling reaction, the *N*-Ac is smoothly removed simultaneously with the saponification of the ester. This methodology allowed an *N*→*C* directed peptide synthesis, which was demonstrated through the synthesis of a pentapeptide thymopentin **70** (Scheme 27b).⁷³

Scheme 28. Peptide synthesis *via* CDI activation of amine (top) with selected examples (bottom).⁷⁴

N,N'-carbonyldiimidazole (CDI) is typically acclaimed for *C*-terminal activations,⁶ but Campagne and co-workers exploited CDI for the activation of amines **71** with an imidazolylcarbonyl moiety. Here, the activated amine **72** was obtained from the reaction of its HCl salt with CDI in the presence of Et₃N, and isolated *via* filtration. The activated form could be coupled to an *N*-protected amino acid in the presence of CuBr₂ (10 mol%) and HOBt (10 mol%) at room temperature. The conditions were compatible with all protecting groups standardly used in peptide synthesis and resulted in isolated yields varying from 52 to 90% (Scheme 28). A comparative study of classical activation methods with the CDI method on the preservation of chirality in dipeptide Boc-Cys(Bn)-Ala-OMe **73**, confirmed that the absence of *C*-terminal activation prevented the epimerization of the Cys residue. Finally, the applicability of the method was further demonstrated through the synthesis of a tetramer **74** (Scheme 28, depicted in the frame, synthesized in a *N*→*C* fashion).⁷⁴

Broadening on the traceless Staudinger ligation⁷⁶ where an azide **75** acts as amine surrogate, Ashfeld and co-workers developed a chemoselective *O*- to *N*-acyl migration using chlorophosphites as a dual activation agent.⁷⁵ The reaction proceeds *via* an intermediate ester azaylide **76** formed from the reaction of a preactivated carboxylic acid with the bisfunctional chlorophosphite and an azide. The protocol showed compatible with Boc, Fmoc and Cbz protected amide for the synthesis of various dipeptides (Scheme 29).

Scheme 29. Direct traceless Staudinger ligation of carboxylic acid and azide.⁷⁵

The α -ketoacid-hydroxylamine amide ligation (KAHA) initially established by Bode and co-workers in 2006 demonstrated a sustainable method to amide bond formation.⁷⁷ After all, the decarboxylative addition that occurred between the α -ketocarboxylic acid C-terminus **77** and the hydroxyl amine at the N-terminus **78** proceeded without any additional reagents, resulting in H₂O and CO₂ as the only by-products (Scheme 30).

Scheme 30. α -Ketoacid-hydroxylamine amide ligation (KAHA).⁷⁷

The reaction originally required polar solvents such as DMF or DMSO at 40°C and was orthogonal with a vast range of protected and unprotected functional groups (e.g., carboxylic acids, amines, alcohols). Introduction of the α -ketoacid on the C-terminus was achieved in solution. Hereto, a sulfur ylide was coupled to the N-protected amino acid, yielding a cyanosulfur ylide **79** which was preserved during peptide synthesis as a protecting group. Prior to ligation, the C-terminus **80** was oxidized with oxone under aqueous conditions yielding the corresponding α -ketoacid **81** (Scheme 31a).⁷⁸ The methodology was subsequently translated towards solid phase, resulting in a cyanosulfur ylide peptide upon cleavage.⁸¹ Later, Fmoc protected α -ketoacid building blocks **82** were developed for SPPS (Scheme 31b).⁷⁹ Herein, the ketone was protected as an acetal and deprotected while cleaving the peptide from the resin. With regard to the hydroxylamine component, the authors developed a solid phase approach to transform the N-terminal peptide **84**. Herein, the amine reacted with (*S*)-**83** to form a nitron that subsequently is hydrolyzed toward the hydroxylamine in the presence of NH₂OH.HCl and imidazole (Scheme 31c). Note that the peptidic hydroxylamine was stable against epimerization, yet the latter could occur at the nitron stage.⁸⁰ To prevent any loss of chirality, the authors developed a N-benzylidene nitron protecting group that was introduced on an *tert*-butyl α -amino ester and remained stable during saponification. The obtained N-protected amino acid can be coupled under standard peptide coupling conditions. Afterwards, the deprotection of the N-benzylidene nitron was achieved by an initial reaction with NH₂OH.HCl and imidazole, followed by the addition of oxalic acid to form a more stable and easy-to-handle peptidic hydroxylamine salt.⁸² These breakthroughs opened the KAHA ligation gateway to larger unprotected peptides⁸³ and macrocyclization.⁸⁴

Scheme 31. Synthetic route to the α -ketocarboxylic acid – (a⁷⁸ and b⁷⁹) and hydroxylamine peptide from amino acid residues (c).⁸⁰

Notwithstanding, the KAHA ligation did not perform well in water, the medium typically used for peptide solubilization. This problem was caused by the free hydroxylamine, but alkylation of the oxygen would allow to surpass this issue, and hence stable 5-oxoproline residues were used as the *N*-terminus (**86** in Scheme 32b).^{86a}

Scheme 32. (a) *N*-Boc protected 5-OxaPro synthesis⁸⁵ and (b) KAHA type II⁸⁶

The preparation of both the *N*-Boc or *N*-Fmoc protected building blocks was achieved by a high-pressure cycloaddition with ethylene.^{86a, 87} To obtain a more scalable synthesis, the authors prepared *N*-Boc protected analogue **85** from the corresponding β -amino aldehyde (Scheme 32a).⁸⁵ Upon ligation of the oxoproline with a α -ketoacid in water, a depsipeptide intermediate **87** was formed, which subsequently rearranged to give the amide, providing a homoserine residue at the

ligation site **88** (Scheme 32b).⁸⁶ This improved form has taken KAHA ligation to the next level and showed applicable for protein synthesis.⁸⁸

Scheme 33. Oxidative coupling of *N*-protected α -amino hydroxamic acid **89** with amines (top) with selected examples (bottom).⁸⁹

Coupling of hydroxamic acids with amines was demonstrated in 1964 by Hoffmann *et al*, but the long reaction times and poor yields turned it into a less attractive method for peptide synthesis.⁹⁰ Sureshbabu and co-workers tackled this issue by establishing an oxidative coupling protocol of *N*-protected α -amino hydroxamic acids **89** with amines (Scheme 33).⁸⁹ After synthesis of the hydroxamic acids from their corresponding acids,⁹¹ amide bond formation was achieved in a one-pot two-step fashion. The oxidation occurred in the presence of I_2 in DMSO (30 min, rt), to give an acyl nitroso intermediate **90**, which was trapped by the amine with loss of nitrous oxide. These conditions were applied to deliver a set of dipeptides with different sterical hindrance. Preservation of chirality was demonstrated *via* the synthesis of Fmoc-Phg-Phe-OMe, and, moreover, the method was compatible with the Fmoc, Cbz and Boc protections.⁸⁹

Johnston and co-workers disclosed α -halo nitroalkanes as carboxylic acid surrogates. In contrast to classical couplings wherein nucleophilic amines reacts with an electrophilic activated ester, the α -halo nitroalkane acts as the nucleophilic component by deprotonation.⁹² The amine is activated by *N*-iodosuccinimide (NIS) to convert it into a electrophilic partner, in the presence of K_2CO_3 to maintain a mildly alkaline environment. A carbon-nitrogen bond was formed by nucleophilic attack of the nitronate onto the *N*-iodoamine. The tetrahedral intermediate obtained was finally hydrolysed into the corresponding amide (Scheme 33).⁹³ For this, the reaction was realized in a THF:H₂O (3:1) mixture at 0°C. The optimized conditions were initially tested for the coupling of a Boc-Phg(3-Cl) analogue **91** with a set of α -amino esters (Scheme 34).⁹² The authors expanded their methodology to aerobic conditions and ascertained in this case that the coupling only required a catalytic amount of NIS.⁹³⁻⁹⁴

Scheme 34. α -Bromo nitro alkane as carboxylic acid surrogate in dipeptide synthesis (top) with selected example (bottom).⁹²

The *N*-Boc protected building blocks were initially prepared from an *N*-Boc imine and bromo nitromethane using a chiral proton catalyst.⁹² To broaden the scope, the authors described an alternative synthesis using *N*-(trimethylsilyl)imines in an enantioselective aza-Henry addition of bromo nitromethane. The obtained silyl amine intermediate was subsequently functionalized by a range of protecting groups including the Fmoc, Cbz and Alloc groups.⁹⁶ Unfortunately, these methods remained limited to α -aryl side chains. Interestingly, a homogenous organocatalyst **93** that displayed compatibility with bromo nitromethane granted an enantioselective aza-Henry addition to *N*-Boc protected amino acid surrogates with aliphatic side chains in *S*-configuration **92** (Scheme 35).⁹⁵ Also the *R*-enantiomer are accessible using (*R,R*)-**93**. Led by these advancements, Johnston and co-workers further developed their methodology to a one-pot tandem bromination/umpolung amidation of primary nitroalkanes encompassing aliphatic and aromatic side chains.⁹⁷

Scheme 35. Enantioselective aza-Henry addition on an α -amido sulfone derivative.⁹⁵

In analogy, Hayashi and co-workers developed a oxidative coupling of amines with primary nitroalkanes in the presence of I_2 , K_2CO_3 and O_2 (Scheme 36).⁹⁸ Of note, in this case the authors only presented the coupling of *N*-Boc protected 2-nitro-1-phenylethylamine **94** with various α -amino ester. Mechanistically, nitronate is α -iodinated first. With base and O_2 a peroxy radical is presumably formed leading to a intermediate **95**.⁹⁹

Scheme 36. Oxidative coupling of nitroalkane **94** with α -amino esters with proposed mechanism (top) and selected examples (bottom).⁹⁸

Conclusion and perspectives

In summary, this review highlights the recent breakthroughs in new and ‘uncommon’ amide bond formation, specifically devoted to peptide assembly. As illustrated, the new methodologies try to maximally address the inherent drawbacks associated with ‘classical’ peptide couplings. A first approach starts from carboxylic acids and amines and looks into new activating reagents development for the former designed for the acylation of amines. Herein, the focus lays mainly on reducing waste production in the transformation by developing lower molecular weight coupling reagents with preferentially reduced toxicity, allowing recycling featuring easy applicability (ideally without preactivation and necessitating protecting groups) and showing a broad scope. Though not yet with a very broad scope even catalytical amidations are now available and major advances are still to be expected here. Noteworthy is that all these coupling protocols not only target an improved coupling, but also pay attention to the efficient separation of its by-products and the possibility to recycle them, whenever relevant and feasible. A second approach has been the implementation of carboxylic acid and/or amine surrogates for the chemoselective formation of the amide bond, mainly to increase the functional group compatibility of the ‘classical couplings’. These alternative functional groups are typically accessed from their corresponding carboxylic acid or amine counterpart. This requires additional synthetic steps toward the desired peptide consequently influencing the overall green metrics. Important to note here is that green metrics should never be applied on isolated transformations but should always be seen in the context of the overall synthetic sequence as results might be different than one intuitively expects.¹⁰⁰ The major impact of these methodologies based on surrogates is revealed in the ligation of segments towards large peptides/proteins and not in the coupling of a single amino acid. These alternative procedures are designed to safeguard the chiral purity of the peptide and minimize the use of protecting groups. Regularly, the methods were even compatible with sterically hindered amino acids. The presented methodologies add to the chemical toolbox of peptide medicinal chemists and they represent a basis towards more efficient and sustainable peptide synthesis.¹⁰¹ In this respect the search for suitable alternative (co-)solvents is an important point of future attention, as halogenated and dipolar aprotic solvents are not preferred with respect to safety, toxicity and environment but still often used.¹⁸ After all, solvents accounts for the largest amount of waste produced in a reaction. *n*-Butyl pyrrolidone (TamiSolve™ NxG) and 2-MeTHF are interesting and promising alternatives.^{101b} What remains to be seen, however, is which new synthetic methodologies will finally be implemented on a daily basis in peptide discovery, to boost their profile as promising therapeutics, and manufacturing.

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