

## REVIEW

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## Site-selective modification of peptide backbones

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The site-selective modification of peptide backbones allows an outstanding fine-tuning of peptide conformation, folding ability, and physico-chemical and biological properties. However, to achieve selectivity in the core of these biopolymers is challenging. In the last few years, many advances towards this goal have been developed. This review addresses the selective modification of C $\alpha$ - and N-positions, from the use of "customizable units" to the residue-directed introduction of substituents. The site-selective modification of the peptide bond, *i.e.* the formation of thioamides or heterocycles, which alters backbone rigidity and ability to form hydrogen bonds or recognition by enzymes, is also described. Moreover, not only the modifications in internal backbone positions, but also in the N- and C-termini are discussed. In addition to chemical methodologies, the review addresses some reactions, catalyzed by natural or engineered enzymes, that afford unprecedented regio- and stereoselectivity in backbone modifications.

## 1. Introduction

The modification of peptides and proteins is a hot area in bio-organic, medicinal and synthetic chemistry, allowing the generation of peptide drugs, antibody-drug conjugates, and probes for molecular imaging, among others.<sup>1</sup> However, due to the similar reactivity of amino acids, the selective modification of certain positions has been troublesome until recently. Important advances have been made in the field, as shown by the reviews published recently.<sup>2–4</sup> The ability to introduce resi-

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Alicia Boto

Dr Alicia Boto is Research Scientist at the Spanish Research Council (CSIC) in IPNA-CSIC (Tenerife-Spain). She works on the development of synthetic methodologies and their application to drug synthesis, particularly antimicrobial and antitumoral agents. She has recently participated as an Expert in the preparation of CSIC White Report on the 'Biomedicine and Health' Challenges. She has also been Coordinator of Scientific

Outreach (2007–2019) and Deputy Director of IPNA-CSIC (2013–2020). Her 80 publications include several Featured Articles/Covers in the selective modification of peptides. Part of the results have been transferred to chemical and agrotech companies, and some are commercialized internationally.



Concepción C. González

Dr Concepción González graduated in Chemistry from the University of La Laguna. She carried out her PhD work at IPNA-CSIC, obtaining her PhD in 1997 (University of La Laguna-ULL). From 1998–2001, she worked as a post-doctoral fellow at the University of Strathclyde (Glasgow) under the direction of Prof. John. A. Murphy. Since 2001 she has been a Senior Scientist at IPNA – CSIC. One of her research lines is based on the

study of the different reactivity of O- and N-radicals, obtained from hypervalent iodine reagents, for the selective modification of carbohydrates and peptides.



dues with functional groups (tags) that can be replaced or transformed into other ones (tag-and-modify approach) has made possible the modification of both peptides and proteins.<sup>4</sup> For example, residues with azido or propargyl units in certain positions have allowed click chemistry and the introduction of new chains.<sup>5</sup> Residues with alkenyl side-chains can be modified by olefin metathesis<sup>6</sup> or the addition of different group, such as alkyl thiol chains.<sup>7</sup> Enzymes have been used to transform certain residues or to recognize amino acid sequences to be modified.<sup>8</sup>

The progresses made in this field have been described in different reviews, and an excellent summary up to 2016–17 has been compiled in the review by Baran *et al.*<sup>2</sup>

While most of the modifications have been carried out in the lateral chains, relatively few have been developed for the modification of the peptide backbone,<sup>2</sup> in spite that backbone modifications can deeply affect the conformation, solubility, aggregation properties and therefore, the biological activity of the peptide. This review will address the advances made in the field, including not only the modifications in internal backbone positions, but also in N- and C-termini. In addition, the impact on the secondary structure and the biological activity will be highlighted.

The backbone modifications using “customizable units” will be discussed first. The most common ones use glycine residues, which are transformed into  $\alpha$ -alkyl, aryl- or  $\alpha$ -allyl glycines.<sup>9,10</sup> The advances made in the last years with photo-redox reactions are remarkable, since the use of chiral catalysts has allowed the attachment of lateral-chains to the carbon backbone with promising stereoselectivity and good yields.<sup>10</sup>

Other strategies such as scission reactions of serine, threonine, hydroxyproline, glutamic acid or C-terminal residues, followed *in situ* by the addition of new chains, have also been reported, providing from  $\alpha,\beta$ -  $\alpha,\gamma$ - or  $\alpha,\delta$ -peptide hybrids to peptides with a variety of N-substituents.<sup>11</sup> The oxidation of

proline with iron catalysts affords open-chain counterparts.<sup>12</sup> The transformation of rigid, cyclic residues into acyclic ones or viceversa can have a strong impact on peptide conformation.

Some particular customizable units are dehydroalanine and related dehydroamino acids (dhAAs).<sup>13</sup> The double bond presents both electron-withdrawing and electro-donating substituents, resulting in a captodative effect, which allows the addition of nucleophiles and radicals, cycloadditions and many other reactions.<sup>14</sup> The formation of dehydroamino acids increases the rigidity of the system and favours turns, as will be commented later, while the conversion of dhAAs into acyclic residues increases backbone flexibility and affects peptide conformation.<sup>15</sup>

The formation of heterocyclic rings in the backbone also affects the overall conformation and physico-chemical properties. Nature uses this strategy frequently to create bioactive compounds, such as antimicrobial or cytotoxic oxazole and thiazole peptides, whose increased rigidity favours interaction with the biological targets.<sup>16</sup> This strategy has also been used in the laboratories to generate a variety of heterocycles (triazoles, oxazoles, thiazoles, oxazolidines, *etc.*) with applications as fluorescent probes and drug candidates, among others.<sup>17</sup>

The selective introduction of N-substituents is also commented. This modification can decrease the number of hydrogen bonds available, and thus destabilize some secondary structures such as helices or  $\beta$ -sheets, or affect the formation of turns.<sup>18</sup> These effects on the secondary structure have been used in peptide synthesis to increase their solubility and decrease aggregation, particularly when difficult sequences are involved. Some outstanding achievements in the solubilization of amyloid peptides, polyalanine sequences and other aggregation-prone peptides have been described.<sup>19</sup>

The site-selective introduction of N-substituents in already-formed peptides has been carried out by different methodologies, from the scission of cyclic customizable units<sup>20</sup> to the



**Dácil Hernández**

*Dr Dácil Hernández received her PhD in Chemistry at the University of La Laguna (Spain) in 2007 and was awarded one of the PhD University Prizes for her work on the modification of carbohydrates and amino acids to give nucleoside analogs and alkaloids. After a two-year post-doctoral stay at Aarhus University (Denmark) with Prof. Skrydstrup, working on silyl-amino acids and peptides, she returned to CSIC, where she*

*holds a postdoctoral research contract (Transaludagro project). She has published more than 20 papers and has a licensed patent. She has codirected an industrial PhD and supervised other PhD and master work on peptide modification.*



**Iván Romero-Estudillo**

*Dr Iván Romero carried out his PhD studies on Chemical Sciences at the Universidad de La Laguna (Spain), working under the supervisión of Dr Alicia Boto in IPNA-CSIC. His work on the selective modification of amino acids and peptides (introducing the Hyp ‘customizable unit’) was awarded one of the University PhD Prizes, in the area of Experimental Sciences. He has several Articles and licensed patents on the*

*subject. Currently he is Professor (Profesor-Investigador Cátedra-CONACYT) at the University of Morelos (Mexico), and has directed a Project on the development of antitumoral peptides.*



histidine-directed *N*-alkenylation or arylation of the preceding residues on treatment with boronic acids.<sup>21</sup> In other cases, units with removable *N*-substituents are introduced at key positions during peptide synthesis, and once it is completed, the *N*-substituents are cleaved under appropriate conditions (light, pH changes, *etc.*).<sup>22</sup>

The modification of C- and N-terminal positions will also be commented. The replacement of carboxyl groups by other functions can introduce amino acid analogues in the peptide backbone.<sup>23</sup> The selective modification of N-terminal positions often exploits the differences in  $pK_a$  between the N-terminus and the lateral chains of lysine, ornithine or arginine to perform selective *N*-alkylations or acylations.<sup>24</sup> These chains can also be oxidized to glyoxalate units and further transformed.<sup>25</sup> Their reaction with *in situ* formed electrophiles and residue-selective reagents (for cysteine, serine, proline) will also be summarized.<sup>26</sup>

The review will also describe the exquisite regio- and stereo-selectivity achieved with some enzymes, that recognize particular motifs and transform these positions.<sup>27</sup> Such enzymes can catalyze transamination, oxidation, (macro)cyclization and cycloaddition reactions. Their use for ligation of peptides is beyond the scope of this review,<sup>28</sup> but some particular cases are nevertheless illustrated. Remarkably, although these enzymes are precise in motif recognition, they are however quite promiscuous, accepting different substrates containing the appropriate “key” motif.<sup>29</sup> The rapid advances made in the field of protein engineering will undoubtedly expand the catalog of enzymes for new applications.

In summary, the advances in the site-selective backbone modification is a rapidly expanding area, and each day new methodologies and synthetic tools are incorporated. The fine-tuning of backbone modification would play an important role in the understanding of peptide conformation, physico-chemical properties, and biological action. In turn, this would

allow the design of tailor-made peptides for a certain application in a variety of fields, from biomedicine to materials science.

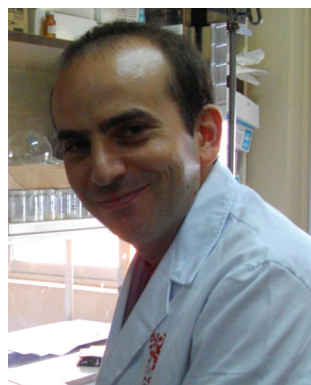
## 2. Backbone modifications at C- $\alpha$ positions

### 2.1. Modifications at C- $\alpha$ using customizable units

The site-selective modification of C- $\alpha$  positions often uses “customizable units” which can be differentiated from the other amino acid residues.<sup>11</sup> Among them, glycine is the most frequently employed. Other units are serine, threonine, glutamic acid, proline and hydroxyproline.<sup>11,12</sup> The special case of dehydroamino acid units will be discussed as well.<sup>13</sup>

The first significative advance was reported by Seebach *et al.*, who generated glycine enolates at low temperatures, and trapped them with electrophiles, while the other amino acids in the peptide remained unaffected. However, the starting units were *N*-protected glycines, which involved extra protection and deprotection steps.<sup>30</sup> Later on, Seebach used an aminomalonate unit as a “customizable” residue to carry out complete lateral chain replacement (conversion **1**  $\rightarrow$  **2**, Scheme 1).<sup>31</sup> The alkylation in basic media was followed by benzyl ester deprotection and *in situ* decarboxylation, to give an aromatic residue. With this method, both D- and L-epimers could be obtained for biological studies.

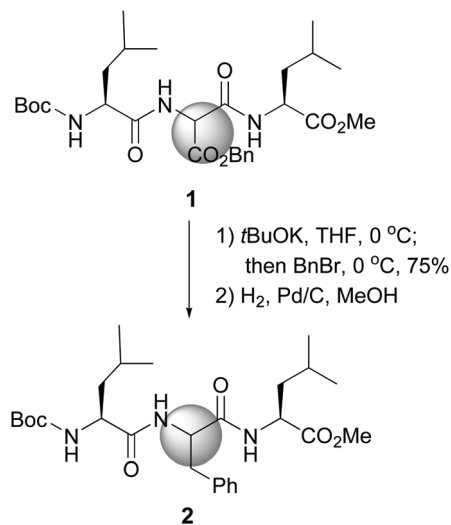
Steglich used glycine units in a different site-selective approach<sup>32</sup> (conversion **3**  $\rightarrow$  **4/5**, Scheme 2). He had shown that derivatives of  $\alpha$ -bromohippuric esters could react with nickelocenes, cyclopentadienylthallium and other organometallics to give 2-cyclopentadienylglycines.<sup>32a</sup> He also reported that a dipeptide **3** with an alkylthio moiety was treated with thionyl chloride and then with cyclopentadienyltrimethylsilane and  $ZnCl_2$  to give the isomeric cyclopentadienyl glycines



Carlos J. Saavedra

Dr Carlos Javier Saavedra obtained his BchD in Chemistry at the University of Sevilla in 2003. He completed his Ph.D. (ULL-2013) working on the selective modification of peptides at IPNA-CSIC. He introduced the glutamic acid unit and worked with serine, threonine and C-terminal units. In 2014, he moved to University of Zaragoza for a postdoctoral contract with Dr Fraile to work on heterogeneous catalysts. Later he got a

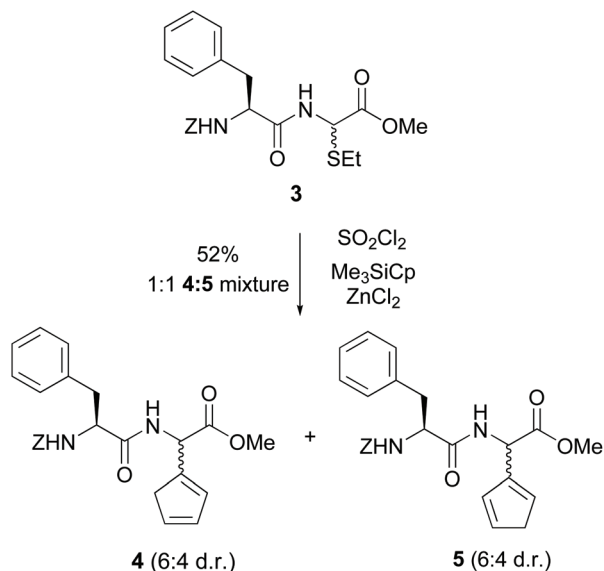
contract “Torres Quevedo” in the company Biosigma S.L. He is currently working on an “Agustín de Betancourt” project (University of La Laguna) for the development and commercialization of new agents for plant protection.



**Scheme 1** Selective modification of an aminomalonate unit by Seebach *et al.*<sup>31</sup>







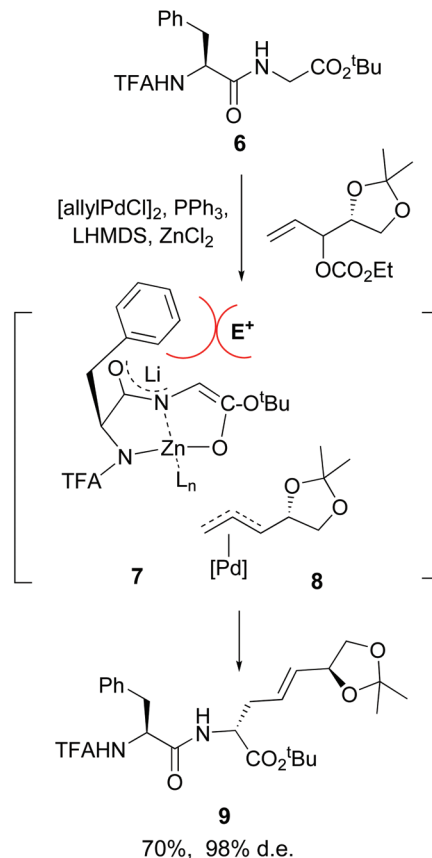
**Scheme 2** Selective modification of glycine units by Steglich *et al.*<sup>32a</sup>

4 and 5.<sup>32a</sup> Since the phenylalanine unit acted as a chiral auxiliary, a moderate diastereoselectivity was observed (about 6 : 4 dr).

Afterwards, Skrydstrup further improved this methodology. He carried out the selective bromination of glycine residues in peptides, and then treated them with  $\text{SmI}_2$ .<sup>33</sup> The resulting enolates reacted with electrophiles to give  $\alpha$ -alkylglycine derivatives. This method did not require protection of the glycine units, but involved several reaction steps.<sup>33a</sup>

Another advance was reported by Kazmaier *et al.* working with small peptides.<sup>34</sup> An efficient conversion of glycine residues 6 into their enolates 7 was achieved, followed by an alkylation or allylation catalyzed by palladium complexes (conversion 7 + 8  $\rightarrow$  9, Scheme 3). Remarkably, an excellent regio- and diastereoselectivity was observed. In the examples studied, an adjacent (*S*)-amino acid resulted in the formation of a C-terminal (*R*)-amino acid, and viceversa. A *N,N,O*-tridentate chelate 7 is probably formed, where the lateral chain of the adjacent amino acid hinders the approach of the electrophile 8 from that face, giving the unusual (*S,R*)-product.

In another promising approach, Li *et al.* reported the site-specific functionalization of glycine residues in peptides using an aryl, alkenyl or alkynyl boronic acid, *tert*-butylhydroperoxide (TBHP) and copper catalysts (conversion 10  $\rightarrow$  13–16, Scheme 4).<sup>35a,b</sup> The CuBr/TBHP system promotes a dehydrogenative oxidation of the substrate 10, probably through a single electron transfer process (SET) which produces a radical cation 11.<sup>35c,d</sup> The latter reacts with Cu(II) species to give the imine 12. A tautomerization follows, and the resulting iminol coordinates with the boronic acid to afford the intermediate 13.<sup>35b</sup> Then the aryl group is transferred to the imine bond, to give the final products. The proposed mechanism was based on two observations: if a tertiary amide was used and the tautomerization was not possible, the reaction did not take place.



**Scheme 3** Selective modification of glycine units by Kazmaier *et al.*<sup>34a</sup>

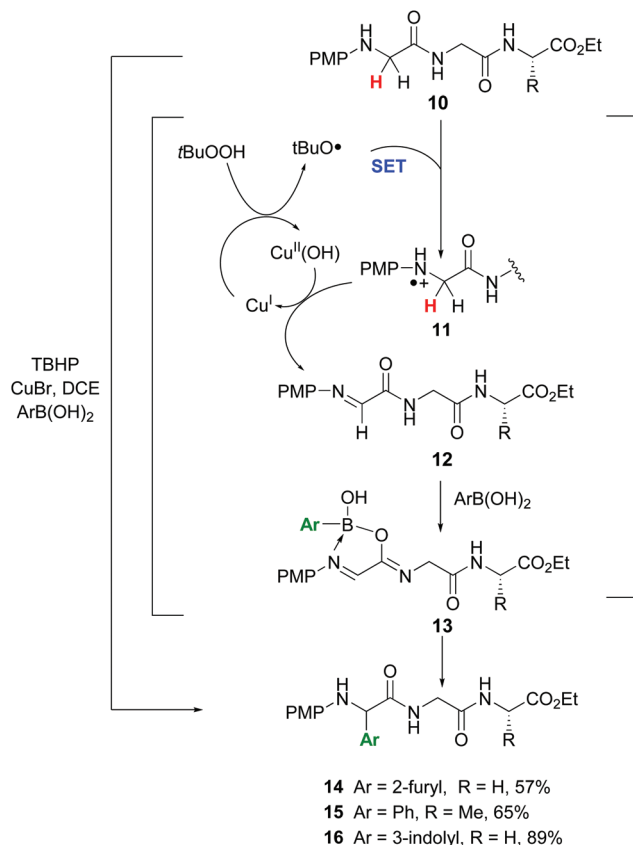
In addition, when the imine 12 was preformed and treated with the boronic acid in the absence of the copper catalyst, the arylation took place in high yield.

The reaction was completely regioselective: the PMP group makes that only the methylene group in the N-terminal position can be functionalized. No racemization of the other stereocenters was observed.

Recently, photocatalytic methods were used to introduce substituents on *N*-aryl glycine residues in peptides (conversion 17  $\rightarrow$  20–24, Scheme 5).<sup>10</sup> The reaction mechanism involves a radical–radical coupling. Thus, the copper(I)-promoted reductive decarboxylation of hydroxyftalimides under blue LED irradiation led to an alkyl radical ( $\text{R}^\bullet$ ). On the other hand, the resulting Cu(II) complex oxidized the N-terminal glycine in substrate 17 to give a cation radical 18. This intermediate underwent a H-shift in the presence of base, affording a glycy radical 19. The coupling of radicals 19 and  $\text{R}^\bullet$  yielded the final products 20–24.<sup>36</sup> The mild conditions allowed good functional group tolerance. Although 1 : 1 mixtures of diastereomers were obtained, probably fine-tuning of the catalyst would improve the ratio. In any case, obtaining both isomers could be interesting for structure–activity relationship (SAR) studies. The authors illustrated the utility of this methodology for drug synthesis with the production of two enkephalin fragments and an analogue of collagen tripeptide.





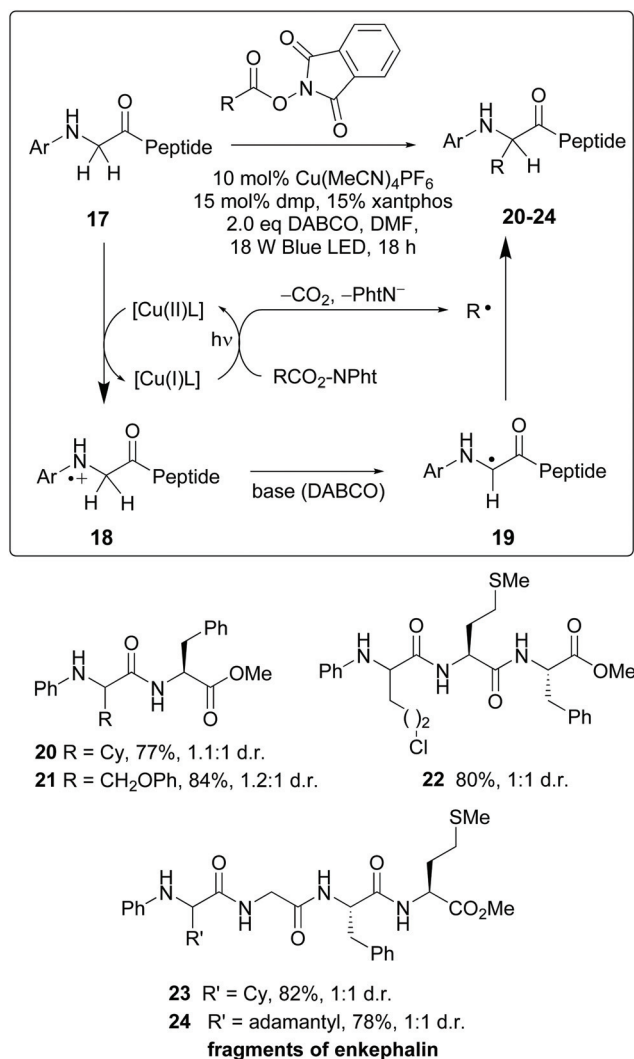


**Scheme 4** Selective modification of glycine units by Li *et al.*<sup>35a</sup>

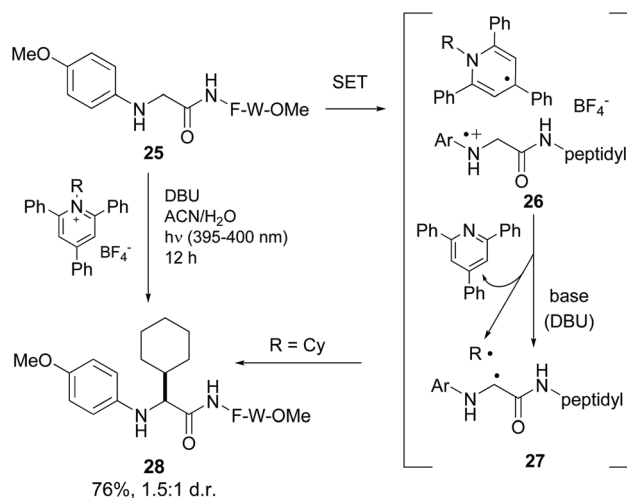
Xu *et al.* reported the alkylation of modified N-terminal N-aryl glycine residues using Katritzky salt.<sup>37</sup> The mechanism is similar to the previous one (conversion 25 → 28, Scheme 6).<sup>10</sup> Upon irradiation, single electron transfer (SET) took place from the glycine amino group to the pyridinium salt, generating the intermediate 26. The dihydropyridine radical underwent rearomatization, releasing a radical (in the example, R' = Cy'), while the glycy radical cation underwent H-shift in the presence of base to give a glycy α-radical (conversion 26 → 27). Both radicals coupled to give the final product (compound 28).

Lately Xu, Chang *et al.* reported the transition-metal free alkylation of N-arylated glycine units in peptides, and its application to the synthesis of new Leu5-enkephalin analogues (conversion 29 → 30, Scheme 7).<sup>38</sup> They used either alkyl chlorides or iodides, under irradiation of about 254 nm. In the case of chlorides, 50 mol% of TBAI was added to facilitate the reaction. Since a variety of alkyl chlorides and iodides are commercially available, this method could be useful to expand the range of α-alkyl glycines for SAR studies.

Other type of “customizable units” use cyclic amino acids that can be opened by the addition of nucleophiles, decreasing backbone rigidity. For instance, Garner coupled amino acids and peptides 31 to derivatives of aziridin carboxylic acids (Azy units) 32 (Scheme 8). The resulting peptides were hydrolyzed

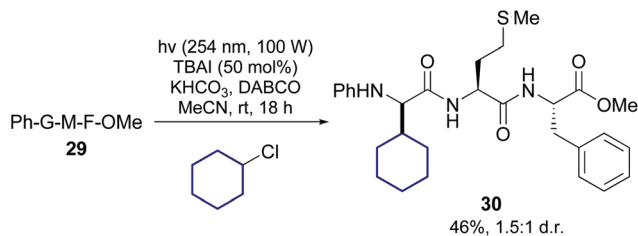


**Scheme 5** Selective modification of glycine units by Wang, Xu *et al.*<sup>36</sup>



**Scheme 6** Selective modification of glycine units by Xu *et al.*<sup>37</sup>



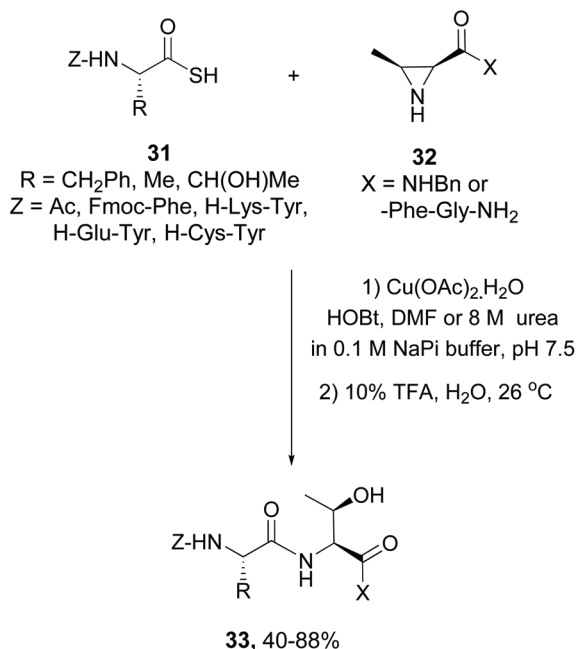


**Scheme 7** Metal-free promotion of peptide modification.<sup>38</sup>

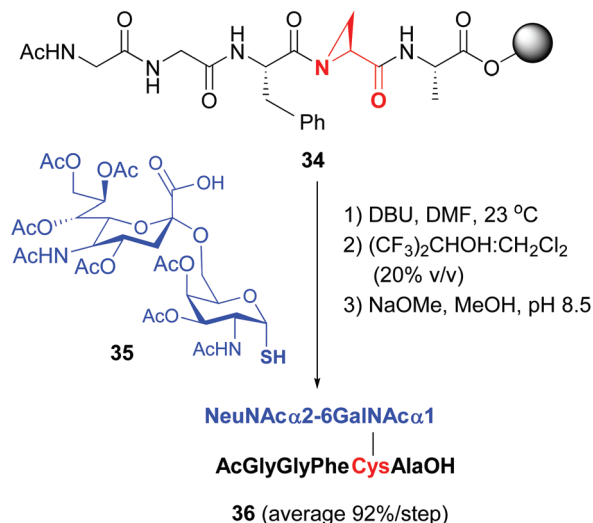
under aqueous acidic conditions to give di- to hexapeptides **33** with a Xaa-Thr moiety.<sup>39</sup> These peptides presented a more flexible backbone, and thus this transformation can be used to modulate ring rigidity and conformation. The aziridine-ring opening reaction can be used with other nucleophiles, as shown in the following examples.

Van der Donk has studied the cleavage of aziridin carboxylic acids in tri- to heptapeptides with *S*-nucleophiles,<sup>40b</sup> to provide valuable glycopeptides in high global yield (conversion **34** → **36**, Scheme 9, 92% average yield per step in the example shown).

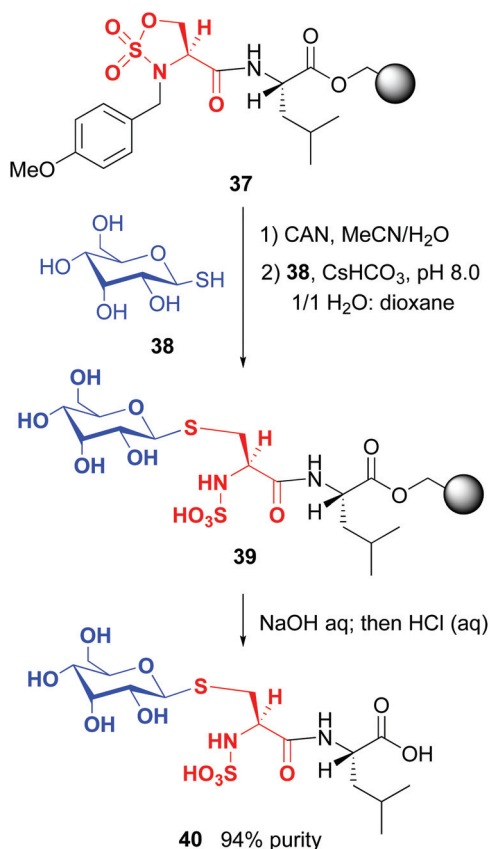
On the other hand, Cohen and Halcomb introduced sulfamidates derived from serine and allo-*L*-threonine for peptide modification (Scheme 10). The customizable units were introduced as their *p*-methoxybenzyl derivatives for solid phase synthesis. Cleavage of the protecting group in peptide **37** and treatment with the 1-thiosugar **38** at pH = 8 resulted in sulfamidate ring opening to afford a glycosyl cysteine derivative **39**. Standard resin cleavage afforded peptide **40** with excellent purity.<sup>41</sup>



**Scheme 8** Modification of aziridin carboxylic acid units by Garner.<sup>39</sup>



**Scheme 9** Modification of Azy units by van der Donk, Gin et al.<sup>40b</sup>



**Scheme 10** Modification of sulfamidates by Cohen and Halcomb.<sup>41</sup>

The sulfamidates were incorporated into peptides for solution and solid-phase synthesis, in both cases affording *S*-linked glycopeptides in good yield and excellent purity. Lubell has developed a related strategy to introduce  $\alpha$ - and  $\beta$ -amino  $\gamma$ -lactams at the N-terminus during solid phase synthesis.<sup>42</sup> The lactams were incorporated into analogues of the

growth hormone secretagogue GHRP-6, restricting their conformational mobility. It was found that the selectivity for two receptors (GHS-R1a and CD36) could be fine-tuned by lactam introduction.<sup>42a</sup>

Busto and Peregrina have reported a related transformation of the peptide backbone, but using a 2-methylisoserine sulfamidate.<sup>43</sup> Upon treatment with nucleophiles, a ring-opening of the cyclic sulfamidate in peptide **41** took place, producing a quaternary center with total inversion of configuration (Scheme 11). Remarkably, when sodium azide was used as a nucleophile, quaternary azides **42** were obtained, which underwent click chemistry to give substituted triazoles at C- $\alpha$  (conversion **42**  $\rightarrow$  **43**).<sup>43b</sup> Moreover, the authors described on-resin sulfamidate ring cleavage with thiocoumarin dyes. Other nucleophiles such as pyridines and thiocarbohydrates were also tried. The latter afforded thioglyco- $\alpha/\beta$ -conjugates, generally in good yields.

In addition to the generation of non proteinogenic  $\alpha$ -amino acids in peptides, the selective transformation can allow the generation of  $\beta$ - and  $\gamma$ -units, in order to provide  $\alpha,\beta$ - or  $\alpha,\gamma$ -peptide hybrids.<sup>44</sup> Many  $\beta$ - or  $\gamma$ -peptides or their hybrids have the ability to form new turns, helices or fibrils, allowing interesting applications in materials science and biomedicine.<sup>45</sup>

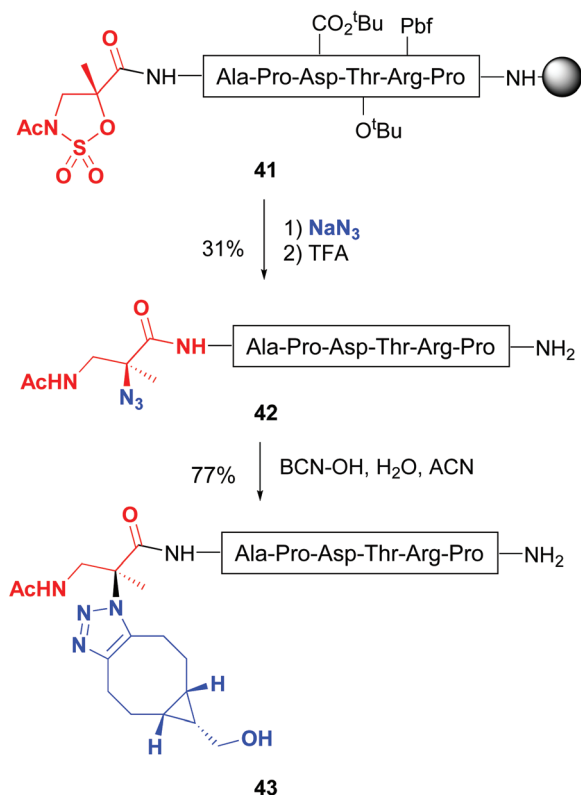
Thus, Boto *et al.* used glutamic acid residues as customizable units, for the generation of a variety of  $\gamma$ -amino acids in the peptide backbone.<sup>46</sup> Since the carboxyl groups of different

glutamic units can have orthogonal protecting groups, it is possible to transform selectively one of the Glu units without affecting the others. An example is shown in the conversions **44**  $\rightarrow$  **49a,b** and **44**  $\rightarrow$  **50a,b** in Scheme 12, where the oxidative radical decarboxylation of substrate **44** afforded a C-radical **45** which reacted with the reagents (iodine or diacetoxyiodobenzene [DIB]) to afford an unstable halogenated intermediate **46**. Substitution of the halo group by acetate from DIB gave a *N,O*-acetal **47**, which was in equilibrium with the iminium ion **48**. In the presence of a Lewis acid, the equilibrium was shifted to the formation of intermediate **48**, which underwent the addition of *C*-nucleophiles, to afford hybrid  $\alpha,\gamma$ -peptides **49a,b** and **50a,b** with good overall yields.

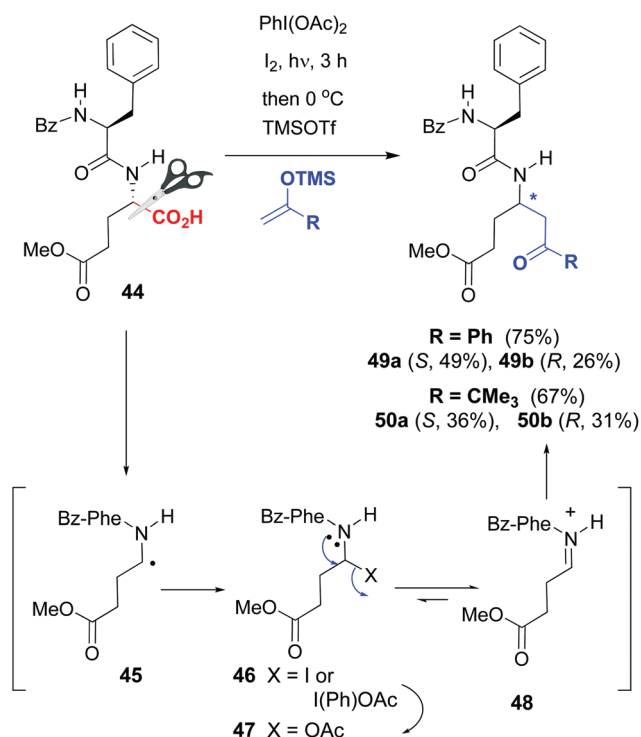
Most transformations commented up to now have a drawback: when several customizable units are introduced in the peptide, it is generally very difficult to differentiate them. Thus, when a transformation is carried out, all the tunable units will undergo the reaction. For instance, if the peptide contains several glycine residues, it would be challenging to achieve the selective enolization of just one of them.

A solution to this problem is to use, as customizable units, amino acids whose lateral chains can be protected with orthogonal groups. In this way, it is possible to carry out the selective transformation of one unprotected residue, while the other (protected) units remain unchanged.

Among the precedents of this strategy, Steglich pioneered the cleavage of serine residues in peptides with lead tetraacetate (LTA) to give  $\alpha$ -hydroxy and acetoxy glycine derivatives.<sup>47</sup> Skrydstrup carried out a variation of this methodology, where



Scheme 11 Modification of sulfamidates by Jiménez-Osés *et al.*<sup>43b</sup>



Scheme 12 Generation and modification of glutamic acid units.<sup>46</sup>

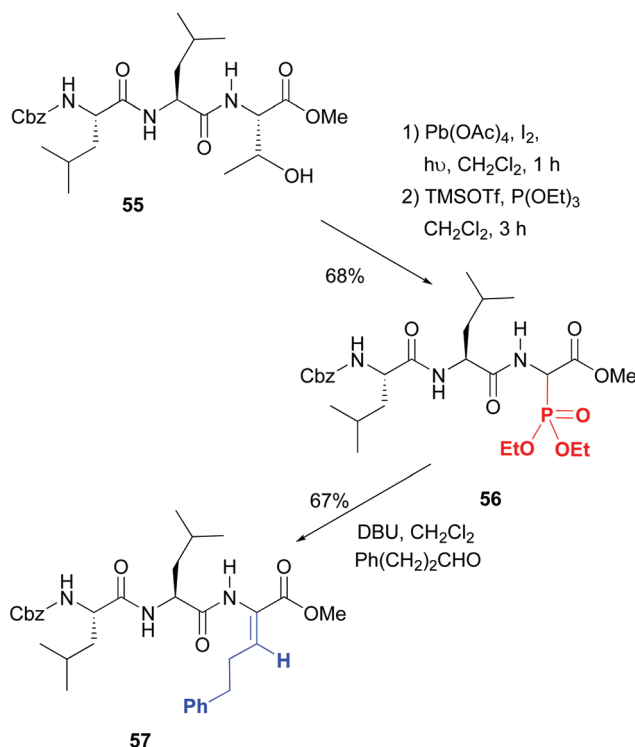




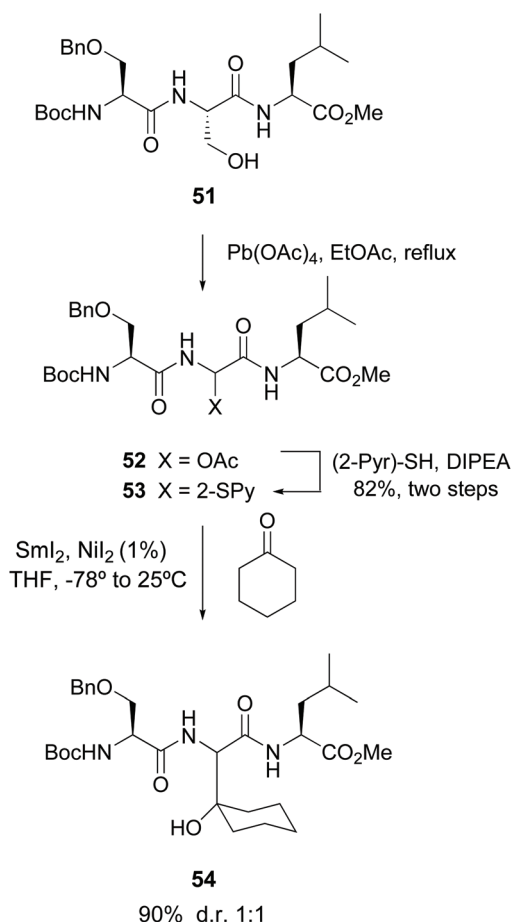
a radical oxidative scission with LTA transformed the serine unit in peptide **51** (Scheme 13) into an  $\alpha$ -acetoxy glycine.<sup>33</sup> The resulting compound **52** was reacted with 2-thiopyridine to afford product **53**. Subsequent treatment with  $\text{SmI}_2$  generated an anion which was trapped by electrophiles, for instance carbonyl compounds, affording unnatural  $\alpha$ -alkylglycines in the peptides (conversion **53**  $\rightarrow$  **54**).<sup>33a</sup>

In an alternative strategy, Boto *et al.* used the oxidative radical scission of serine units to generate an  $\alpha$ -acetoxyglycine which was not isolated, but treated with a Lewis acid to provide an acyliminium intermediate which was trapped by different nucleophiles.<sup>48</sup> The adjacent amino acids served as chiral auxiliaries and low to moderate stereoselectivities were obtained. This one-pot conversion was a shorter route to a variety of  $\alpha$ -alkyl, aryl or allyl glycines.<sup>48b</sup> However, the authors noted that stereoselectivity during nucleophile addition should still be improved, for instance by using chiral catalysts.

On the contrary, excellent stereoselectivities were obtained in the synthesis of  $\beta$ -substituted dehydroamino acids, which are commonly used to increase peptide rigidity, and the modulation of its conformation and biological activity.<sup>49</sup> In the conversion **55**  $\rightarrow$  **57** shown in Scheme 14 the initial step is the radical scission–oxidation of the threonine residue, which was



**Scheme 14** Scission of threonine and generation of dehydroamino acids by Boto *et al.*<sup>49a</sup>



**Scheme 13** Modification of serine units by Skrydstrup *et al.*<sup>33a</sup>

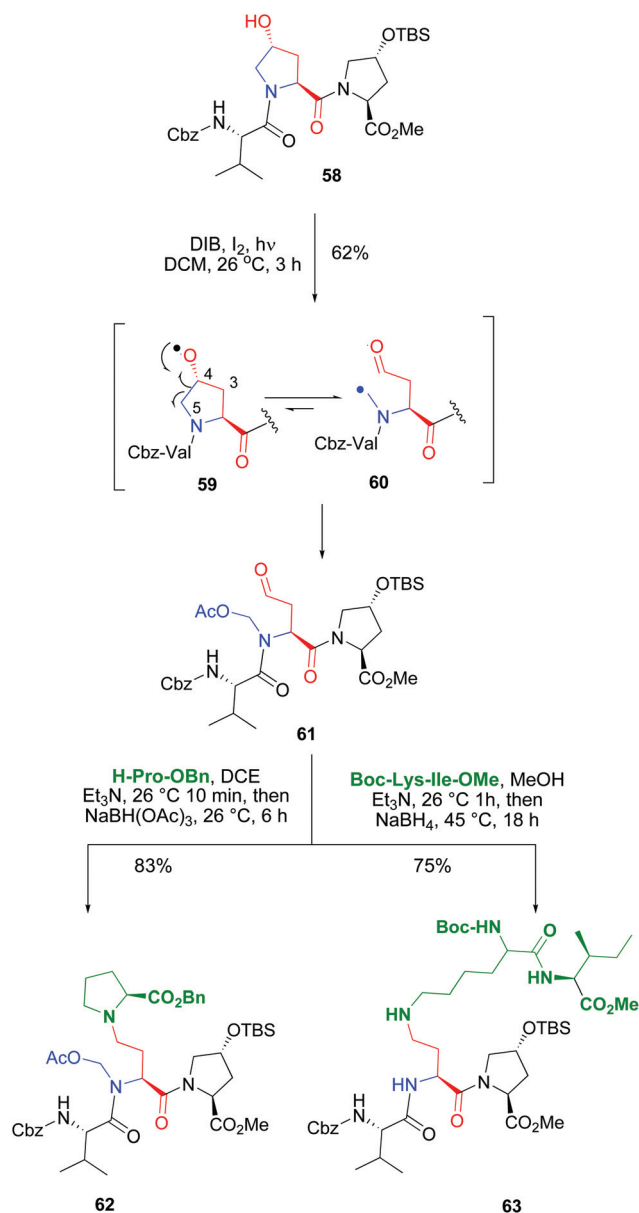
followed by the addition of phosphorous nucleophiles.<sup>49a</sup> The resulting aminophosphonates (*e.g.* compound **56**, 1:1 dr) underwent a Horner–Wadsworth–Emmons reaction to give  $\beta$ -substituted dehydroamino acids such as product **57** with excellent *Z* stereoselectivity. Both hydrophobic and cationic residues can be introduced, which was used for the creation of a library of potential antimicrobials.

The drawback of many of these procedures is the moderate stereocontrol. Therefore, a significative advance was obtained with the development of customizable units, such as hydroxyproline by Boto *et al.*<sup>11,50</sup> and proline by White *et al.*,<sup>12</sup> that allowed the generation of new residues retaining a high optical purity.

The introduction of hydroxyproline units whose pyrrolidine ring could be cleaved at the  $\text{C}_4$ – $\text{C}_5$  bond allowed the conversion of a rigid, cyclic unit into a new acyclic unit with an *N*-substituent (Scheme 15). Both the  $\text{C}_\alpha$  and the *N*-chains could be manipulated independently, which afforded a high diversity in the product libraries. In the example shown (conversion **58**  $\rightarrow$  **61**), an internal Hyp unit was functionalized selectively in the presence of another customizable unit. The terminal Hyp moiety, with a protected reactive group, did not undergo the oxidative radical scission.<sup>11,50a</sup>

The scission of the initially formed *O*-radical **59** was regioselective, involving only the  $\text{C}_4$ – $\text{C}_5$  bond. The alternative  $\text{C}_3$ – $\text{C}_4$  bond scission was not observed. This is due to the different stability of the resulting C-radicals. In both fragmentations a primary radical would be formed, but in the  $\text{C}_4$ – $\text{C}_5$  scission, the C-radical **60** would be stabilized by the adjacent nitrogen.



Scheme 15 Use of Hyp units for selective peptide modification.<sup>50</sup>

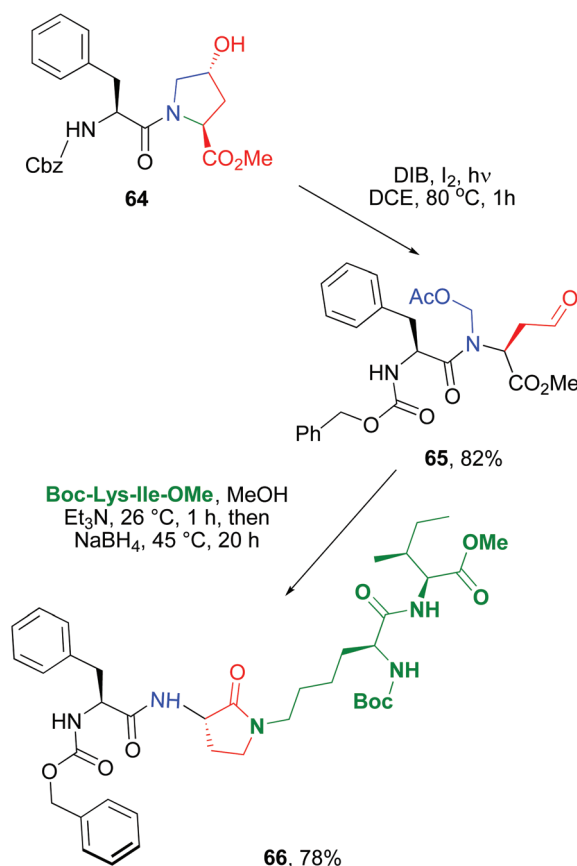
Then the C-radical reacted with iodine or DIB to give an unstable halogenated intermediate (as commented before in Scheme 12), that evolved to give an *N*-acetoxymethyl group. The resulting fragmentation product **61** presented an  $\alpha$ -chain with a terminal carbonyl group, and an *N*-acetoxymethyl substituent. Both chains could be transformed independently as shown in the conversion of compound **61** into the branched peptide **62** using a reductive amination. Interestingly, this reaction can also be used to ligate two peptides, as shown by conversion of tripeptide **61** into the pentapeptide **63**. In this case, using different reductive amination conditions, the *N,O*-acetal was cleaved, and a more flexible backbone was achieved.

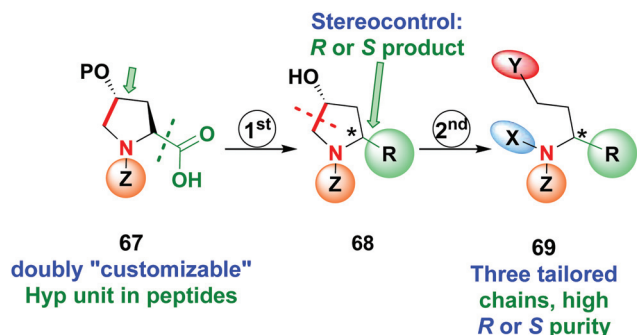
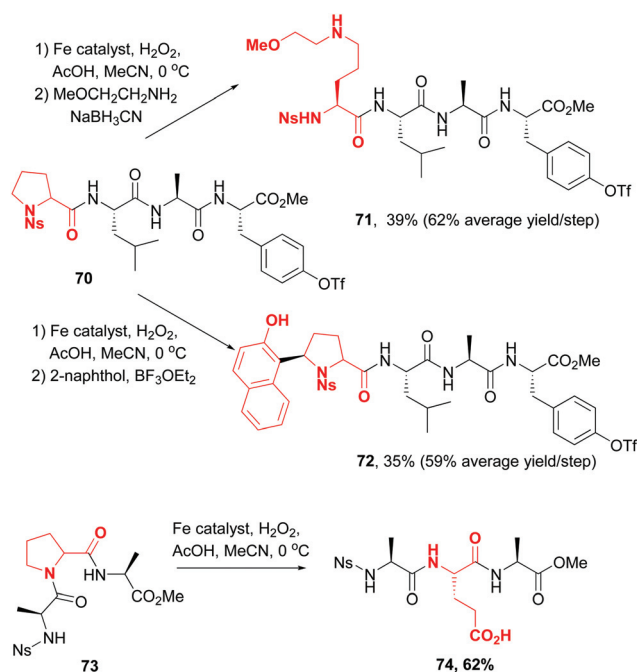
The scission-reductive amination was then used to ligate two peptides, and at the same time, to generate a rigid

$\alpha$ -amino- $\gamma$ -lactam unit to rigidify the backbone (conversion **64**  $\rightarrow$  **66**, Scheme 16).<sup>11,50b</sup> Other applications of this methodology have allowed the preparation of diketopiperazine scaffolds with four different substituents,<sup>51</sup> and the production of a library of cyclic antitumoral peptides, as sansalvamide A analogues.<sup>52</sup>

Recently the use of Hyp as a doubly customizable unit was reported by the same group (Scheme 17).<sup>11</sup> A one-pot radical decarboxylation-oxidation-addition of *C*-nucleophiles allowed the modification of the C-terminal position of the peptide **67** and replacement of the carboxyl group by a variety of other chains, with high stereoselectivity due to the presence of an stereogenic center at C-4. Deprotection of the hydroxyl group activated the unit **68** for C<sub>4</sub>–C<sub>5</sub> cleavage of the pyrrolidine ring, affording a  $\beta$ -amino aldehyde that could be converted into other derivatives as seen before. In this way, products **69** with three tailored chains and high *R* or *S* purity can be obtained.

In 2016, a remarkable work by White, Osberger, Rogness *et al.* introduced proline as a customizable unit,<sup>12</sup> using an iron-catalyzed oxidation inspired by non-ribosomal peptide synthetases. The cleavage of proline at the C<sub>5</sub>–N<sub>1</sub> positions proceeded with no loss of chirality (Scheme 18, conversions **70**  $\rightarrow$  **71**, **70**  $\rightarrow$  **72** and **73**  $\rightarrow$  **74**). Interestingly, the chemoselectivity could be tuned. Thus, *Ns*-protected *N*-terminal prolines underwent oxidation to an aminol, while those at the C-terminus or

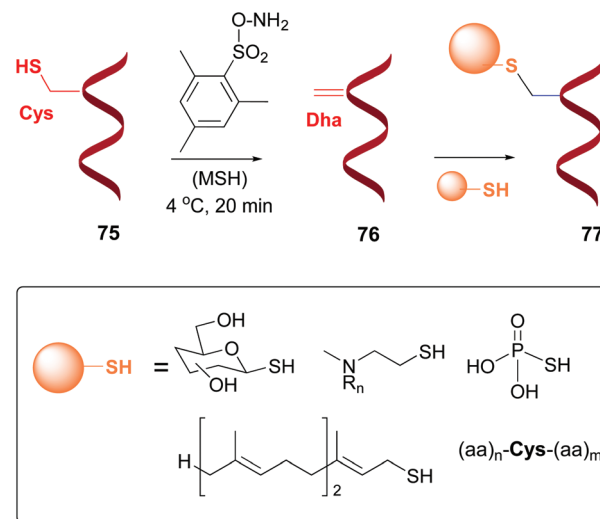
Scheme 16 Use of Hyp units to ligate peptides with rigid Agt units.<sup>50b</sup>

Scheme 17 Use of doubly customizable Hyp units by Boto *et al.*<sup>11</sup>Scheme 18 Use of proline customizable units by White *et al.*<sup>12</sup>

internal position underwent oxidation either to glutaraldehyde or to glutamic acid. The intermediate amins or aldehydes can be further modified, for instance by reductive amination (compound 71), the addition of C-nucleophiles (compound 72) or oxidation (compound 74).

## 2.2. Modifications at C-α using dehydroamino acids

Dehydroalanine (Dha) and other dehydroamino acids (dhAAs) are special cases of "customizable units". The transformation of alicyclic amino acids into dehydroamino acids and viceversa can have a profound impact on the backbone and general peptide conformation, and thus on its biological activity.<sup>13</sup> The dhAAs present a planar conformation with restricted conformational flexibility, and a strong preference for *trans* peptide bonds. Therefore, they are valuable turn inducers and increase peptide rigidity, in many cases favoring interactions with the target.<sup>15</sup>

Scheme 19 Generation and modification of Dha units.<sup>4a,55b</sup>

Dha can be formed from serine or cysteine, by using an elimination reaction (conversion 75 → 76, Scheme 19).<sup>13,14</sup> The advantage of using cysteine is its relative scarcity, which in many cases allows the creation of just one Dha residue (or one surface-exposed unit).<sup>53</sup> There are different methods to prepare Dha residues from cysteine, but the most used is oxidative elimination,<sup>54–56</sup> by treatment with *o*-mesitylenesulfonyl hydroxylamine (MSH)<sup>6b,54,55</sup> or with dibromobisamides.<sup>56</sup> For instance, mutant protein S156C SBL underwent oxidative elimination with MSH of its single surface-exposed cysteine (Cys156) to afford a derivative with one Dha residue.<sup>54</sup> In a similar way, histone mutant H3 was treated with a dibromobisamide, affording a derivative with a single Dha unit (Dha-9).<sup>56</sup> Other examples can be found for GFP mutants, AurA kinase domain, and antibodies, among others.<sup>3f,55a,57</sup>

The resulting Dha residue presents an "umpolung" reactivity with respect to the parent cysteine. Since the double bond has electron-withdrawing and electron-donor substituents, which create a push-pull effect, Dha easily undergoes the addition of different nucleophiles, as well as radical additions, cycloadditions and metal-promoted cross-coupling reactions.<sup>14</sup> A comprehensive review by Bowers *et al.* was recently published<sup>13</sup> but herein we will summarize the transformations affecting the peptidic backbone.

Davis *et al.* reported the transformation of cysteine into dehydroalanine residues as shown in Scheme 19, and the subsequent addition of sulfur nucleophiles to give peptides 77 (conversion 76 → 77, Scheme 19).<sup>54a,55b,56</sup> The peptides were thus glycosidated, aminoalkylated, phosphorylated, or prenylated, among many conversions.<sup>56,58</sup>

Davis applied this method for the functionalization of subtilisin, giving more than 95% conversion in 90 min in a phosphate buffer.<sup>54a</sup> Van der Donk used a similar strategy for peptide functionalization.<sup>59</sup>

Brik *et al.* have reported the use of dibromobisamides to transform cysteine into Dha in ubiquitin probes.<sup>60</sup> These



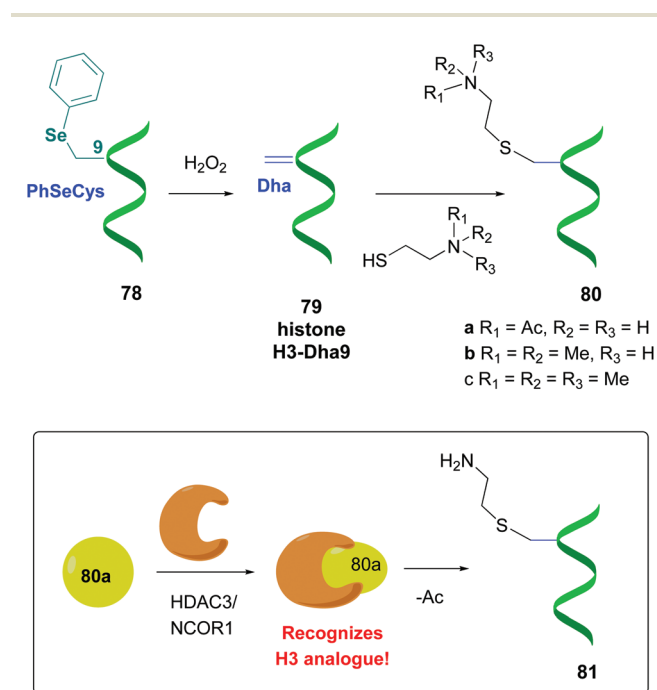


probes were constituted by an ubiquitin fragment (Ub 1–75) attached to a TAMRA fluorophore (carboxytetramethylrhodamine) through a PEG linker. The dehydroamino acid “trap” units were used to capture different enzymes by their catalytic cysteines, for instance USP2 (belonging to the universal stress protein family), forming adducts with an intense fluorescence.

In a similar way, Pearson created a library of mutant *N*-acetylneuraminic acid lyases (NAL) by using precursors with dhAA units and treating them with different thiol nucleophiles. One NAL mutant was able to process erythrose, that was poorly accepted by the native enzyme, with high efficiency.<sup>61</sup>

Davis *et al.* prepared a library of pantothenate synthetases with histidine-like residues at the active site, using precursors bearing dhAAs at the active site, by treating them with azoles.<sup>62</sup> These transformations could be expanded in a future, when reactions developed for dehydroamino acids are optimized for peptides, from the addition of different carbon nucleophiles to Friedel–Crafts reactions.<sup>63–67</sup>

Other promising strategies use selenocysteine derivatives to generate the dehydroalanine unit. For instance, Schultz *et al.* performed genetic incorporation of phenylselenocysteine into histone 78 (Scheme 20).<sup>58a</sup> Treatment with hydrogen peroxide afforded the H3-Dha9 derivative 79. Thiols added to the Dha-residue in a Michael reaction, giving methyl and acetyllysine analogues (compounds 80a–c). Similarly to the natural proteins, compound 80a was recognized by the enzymatic HDAC3/NCOR1 complex, which performed a selective deacetylation. Moreover, the resultant product 81 was accepted by Aurora B kinase, which phosphorylated the adjacent serine residue (S10).



**Scheme 20** Protein modification using selenocysteine derivatives by Schultz *et al.*, in order to study histone function.<sup>58a</sup>

Davis described a reversible protein labeling method using allylselenocysteine analogues (Seac).<sup>54d,68</sup> They used as substrate a histone H3 with a Dha residue at position 9 instead of the usual lysine (K9). When the substrate was treated with allylselenide, a conjugate addition to Dha took place, providing the allylated selenocysteine.<sup>68</sup> Olefin metathesis then gave a mimic of K9Ac side-chain (write step). The resultant histone could be “read” (recognized) by anti-LysAc antibodies. The label could then be removed (“erased”) *via* selenoxide elimination to regenerate the substrate H3-9Dha. In this way, many different labels can be installed in order to study their impact on protein function.

The addition to Dha usually produces an epimeric mixture, but as Davis pointed out, the resulting products are still valuable for biological studies. For instance, Davis *et al.* prepared different protein substrates in order to study their interaction with endoglycosidase A (Endo A) in the synthesis of glycoproteins. Dha residues were incorporated into proteins and then GlcNAc cysteine residues were created by addition of sugar thiols.<sup>54e</sup> Apparently, both epimers were accepted by the enzyme. In another example, Davis prepared modified histones (H3) with several K9 mimic units, which were recognized by antibodies for the original histone.<sup>54a</sup> Besides, a related phosphoserine mimic which presented diastereomeric mixtures was recognized by a chromatin reader protein. In fact, even if the activities were different for epimers, comparison of both epimers and their more rigid Dha-containing precursor could be valuable in structure–activity relationship (SAR) studies.

Although the method is useful even when stereocontrol is not achieved, some promising reports on stereoselective conjugate additions to dehydroamino acids have appeared, using organocatalysts<sup>69</sup> or chiral auxiliaries.<sup>70</sup> Similar methods will likely be optimized for peptides.

Interestingly, Naidu *et al.* reported that the conjugate addition of thiols and amines to dehydroamino acids can be accelerated if the reaction was carried out in water.<sup>71</sup> Therefore, this chemistry is quite useful for protein and peptide modification.<sup>72</sup>

Moreover, C–C bonds can be created by addition of organic radicals to the  $\beta$ -position of the Dha double bond, using modifications of the Luche methodology.<sup>73</sup> Thus, these radicals are formed by treatment of organic halides with Cu(II) and Zn(0) usually under acidic conditions (pH 4.5).<sup>74,75</sup> Baran and Blackmond have described that the zinc-catalyzed radical cleavage of an activated ester provided radicals that added to dhAAs.<sup>75</sup> Park applied this reaction to generate C-radicals from alkyl iodides, and thus introduced different chains onto histone H3, ubiquitin, and green fluorescent protein with high site-specificity. Remarkably, in histone H3, methylated analogues of Lys79 were thus produced. Since methylated histones stimulate transcription, this methodology allowed a better understanding of this key biological process.<sup>76</sup>

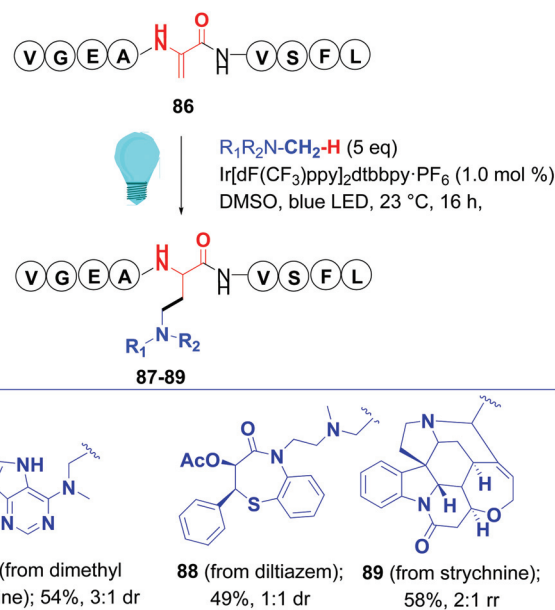
In another seminal work, Davis *et al.* found that radicals could be generated from alkyl iodide and sodium borohydride in aqueous solutions.<sup>77a</sup> The generation of radicals was



observed by electron paramagnetic resonance (EPR); besides, the reaction was inhibited by TEMPO or acrylamides. With  $\text{NaBH}_4$ , less alkyl iodide equivalents were needed for reaction completion, and high-quality products were obtained. Many functionalized chains were tolerated, such as phosphate, phosphonate, ammonium, guanidino groups and sugar residues. It also served to create methylated Lys analogues. Davis functionalized many proteins, including annexin V, Np $\beta$ , SBL, histone H3, H4, p38 $\alpha$ , cAbLys, and AcrA. In Scheme 21 the functionalization of Np $\beta$ -Dha61 (conversion 82  $\rightarrow$  83) and H3-Dha9 (conversion 84  $\rightarrow$  85) is shown.

Dixon and Jui have generated radicals at room temperature using iridium photoredox catalysts and blue light. Ruthenium and copper catalysts can also be used in such reactions, and the use of different ligands can modulate the redox potential of the metal complex.<sup>78</sup> Thus, Jui used an iridium catalyst and alkyl amines or 3-bromopyridines to generate either  $\alpha$ -amino or pyridyl radicals.<sup>78,79</sup> In this way, dimethylaniline, dimethyladenosine, and complex molecules such as strychnine and the drug diltiazem, and a cholic acid derivative, were attached to peptides (Scheme 22, conversion 86  $\rightarrow$  87–89). Using chiral auxiliaries with amino acid substrates provided d.e. up to 95%.<sup>79</sup> Later, Dixon generated  $\alpha$ -aminoradicals from electrophilic imines, and their addition to dhAAs generated 1,3-diamines.<sup>80</sup>

In a remarkable work, Roelfes achieved the functionalization of complex antimicrobial peptides such as thioestrepton and nisin by addition of C-radicals to dehydroalanine (Dha) and dehydrobutyrine (Dhb), using a photocatalytic activation of organoborates under irradiation with visible light.<sup>81</sup> The reaction used the iridium complex  $\text{Ir}(\text{dF}(\text{CF}_3)\text{ppy})_2(\text{dtbbpy})\text{PF}_6$



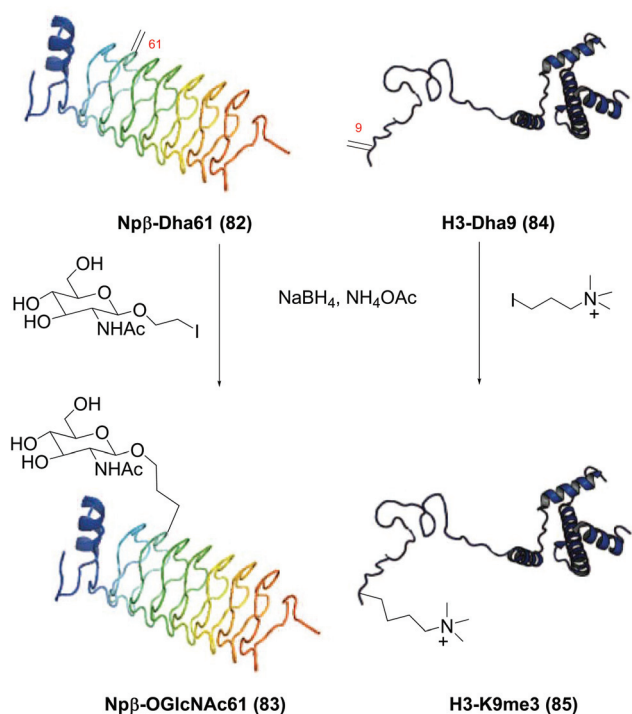
Scheme 22 Peptide functionalization with C-radicals by Jui *et al.*<sup>79</sup>

as photoredox catalyst. Scheme 23 illustrates the conversion of thioestrepton **90** into the addition derivatives **91**. The ratio of mono-, di-, tri- or tetrasubstituted products depended on reaction time and the substrate itself. Thus, thioestrepton needed irradiation with blue LEDs for several hours to give tri- or tetra-substituted products, but with the more hydrophylic nisin, almost complete addition to the three double bonds had been achieved after 1 h. Although the “customizable units” were not differentiated among them, the reaction was selective for the dehydroamino acids; the other residues were not affected.

Other recent methods reported for dehydroamino acids will likely find applicability in peptide chemistry. Thus, Molander reported that alkyltrifluoroborate derivatives added to Dha derivatives to provide  $\alpha$ -fluoro- $\alpha$ -amino acids.<sup>82a</sup> Fluoro groups have been used as bioisosters of H and other moieties and also to tune the biological properties of peptides and drugs.<sup>82a</sup> In another example, Leonori has reported that alkyl halides, in particular iodides, can be suitable radical precursors.<sup>82b</sup> Due to the commercial availability of alkyl halides, this reaction could be quite interesting to obtain peptide diversity.

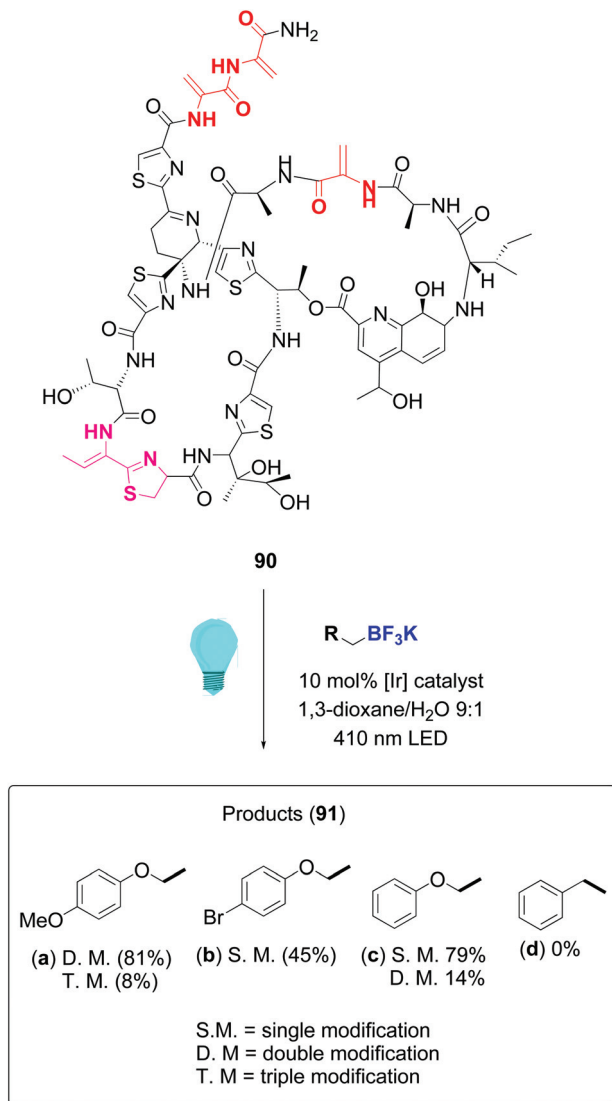
Cycloaddition reactions have been less studied, but Raines reported that the lantibiotic nisin was successfully labeled in a crude protein mixture, by treatment with electron-rich diazoamides in water/acetonitrile.<sup>83a</sup> Roelfes has recently reported the Diels–Alder reaction in peptides such as thioestrepton, nisin and nosiheptide. In the case of thiopeptide nosiheptide, only one dhAA unit reacted, in 75% yield (Scheme 24, conversion 92  $\rightarrow$  93).<sup>83b</sup>

Sun and Wang have reported 1,3-dipolar cycloaddition (DC) between Dha units and *C,N*-cyclic azomethine imines, where the protecting group (phthalimido or amido) determined predominance of normal- and inverse-electron demand 1,3-DC (conversions 94  $\rightarrow$  95 and 96  $\rightarrow$  97, Scheme 25).<sup>83c</sup>



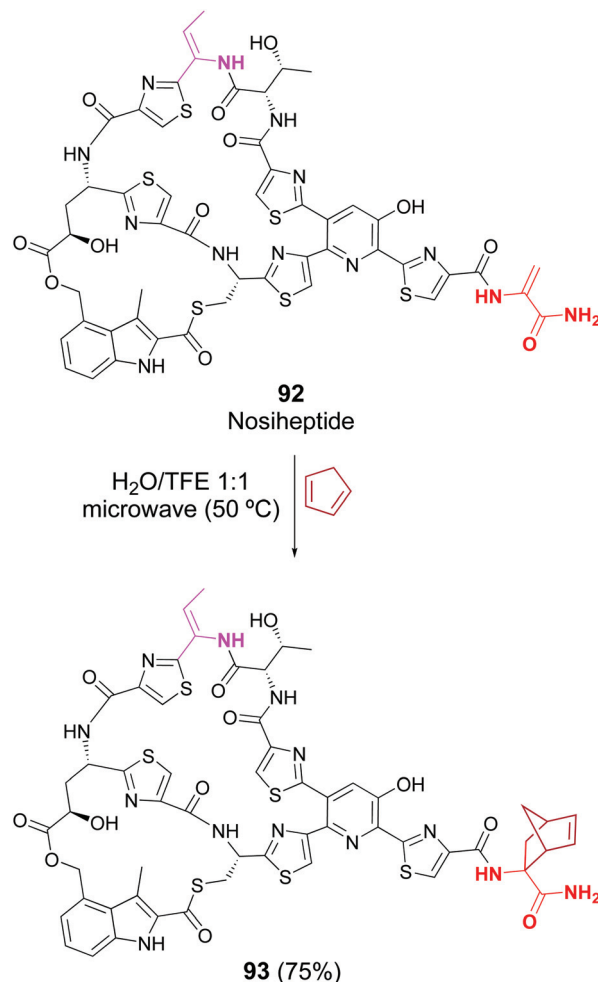
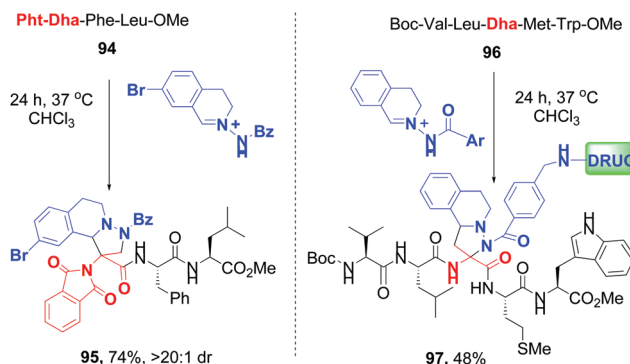
Scheme 21 Protein functionalization with C-radicals by Davis *et al.*<sup>74a</sup>



Scheme 23 Thiostrepton functionalization by Roelfes *et al.*<sup>81</sup>

Cycloadditions can also be catalyzed by enzymes. Different pyridine synthases from the thiopeptide biosynthesis catalyze aza-[4 + 2] cycloaddition to give a substituted pyridine.<sup>84</sup> Other enzymes were engineered to carry out the cyclopropanation of dehydroamino acids in thiopeptides with high regio- and stereoselectivity. The cycloadditions introduce a rigid ring in the peptide backbone that restricts its conformational flexibility.

With respect to transition-metal catalyzed reactions, different from the cited above, a classical example is the reduction of dehydroamino acids using rhodium-catalyzed hydrogenation.<sup>85</sup> Dong *et al.* have applied hydrogenation to the stereoselective reduction of four dehydrophenylalanines in a cyclopentapeptide in protic solvent.<sup>86</sup> The dpp ligand gave alternating D- and L-centers, but other ligands could provide different stereochemistries. It must be said that, although the last example was not really site-selective, in other cases the

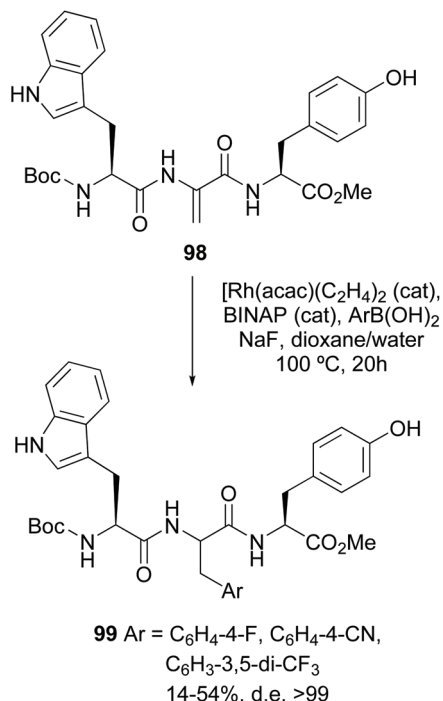
Scheme 24 Selective [2 + 4]-cycloaddition in nosiheptide by Roelfes *et al.*<sup>83b</sup>Scheme 25 Selective cycloaddition reactions by Sun, Wang *et al.*<sup>83c</sup>

method could serve as such, due to the scarcity of dhAAs in complex peptides.

In another seminal example, the stereoselective creation of C-C bonds using Dha-containing small peptides was carried out by Frost *et al.*<sup>87</sup> Thus, the Dha unit was arylated using a rhodium catalyst, with moderate to good yields (30–86%). For







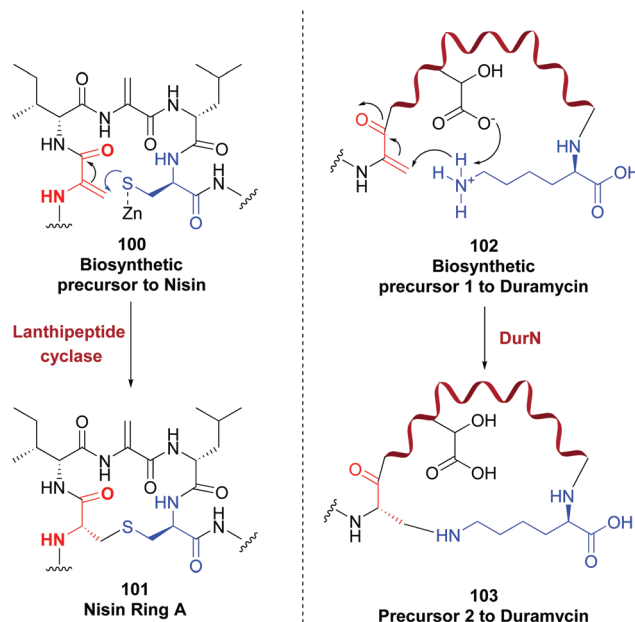
**Scheme 26** Arylation of Dha units by Frost *et al.*<sup>87</sup>

some substrates, excellent d.e. were obtained (conversion **98** → **99**, Scheme 26). In other cases, the use of a chiral ligand allowed some stereocontrol. The reaction was applied to the synthesis of urotensin analogues.<sup>87</sup> Other examples by Willis and Frost have shown the potential of this methodology.<sup>88</sup>

Miller reported milder conditions (room temperature, by increasing catalyst load) for the site-selective modification of thiostrepton. Although thiostrepton possesses four dhAAs, only Dha16 was modified, and with good stereoselectivity.<sup>89</sup> In a related approach, Roelfes reported the use of EDTA-Pd complexes to couple arylboronic acids with dhAAs in peptides and proteins, such as nisin and a small version of SUMO protein. Remarkably, a good tolerance of an aqueous buffer was demonstrated.<sup>90</sup>

Enzymes can also modify dehydroamino acids with exquisite site- and stereoselectivity. An example for their use in cycloadditions has been commented before. Other well known example is the role of lanthipeptide cyclase to create the lanthionine link by addition of a cysteine to a Dha (conversion **100** → **101**, Scheme 27), and the labionin unit.<sup>55b,91</sup>

NisC, a lanthipeptide cyclase, catalyzes the creation of five thioether bridges in nisin with total regio- and stereocontrol.<sup>92</sup> But these enzymes show some substrate promiscuity, that makes them useful for providing modified proteins and peptides with high stereoselectivity, as shown for the cyclase ProcM.<sup>93</sup> In a similar example, the enzyme DurN creates a lysinoalanine in duramycin biosynthesis, by addition of the ε-amine in lysine to a Dha unit (conversion **102** → **103**, Scheme 27).<sup>94</sup> The stereoselective reduction of dhAAs (in many cases to D-amino acids) is also catalyzed by enzymes such as



**Scheme 27** Generation and modification of Dha units.<sup>55b,94</sup>

SacJ, LtnJ, PenN, LasJ, Bsj and CrnJ, among others.<sup>95</sup> No doubt that with the ongoing discovery of new enzymes, the range of possible reactions will increase in the next years.

### 3. Selective formation of heterocyclic units and other modifications of the peptidic bond

Many peptides present oxazole and oxazolidine or thiazole and thiazolidine units in their backbones. These heterocyclic units confer a high rigidity to the biomolecule, thus enhancing interaction with their biological targets.<sup>96</sup> While oxazole and oxazolidine rings derive from serine units, by intramolecular dehydrocyclization, the thiazole and thiazolines usually derive from cysteine. There are many methodologies reported for these cyclizations.<sup>97</sup> Since the cysteine units are relatively scarce in proteins, their selective functionalization with respect to other amino acid units is in many cases feasible.<sup>53</sup> In case that other Cys units are present, the selectivity can be achieved by protecting the different residues with orthogonal groups, and deprotecting each Cys unit selectively before transforming that position.<sup>97</sup> Other than this, enzymes can provide exquisite selectivity, but their substrate range is not usually very large.<sup>27</sup>

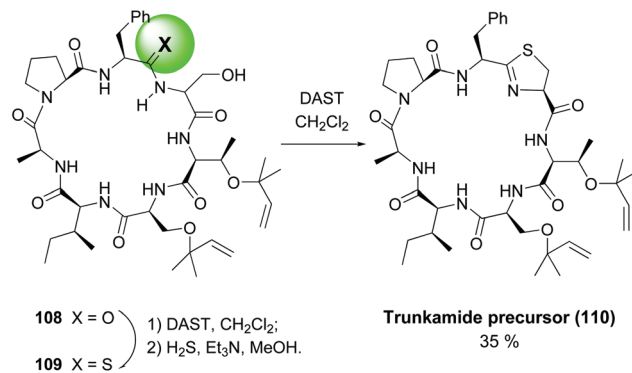
For the lector interested in the formation of different heterocyclic rings in peptides, it is recommended the comprehensive review by Balalaie *et al.*<sup>17a</sup> In most cases, the heterocycles are formed before the unit is inserted into the peptide, and thus these syntheses escape the scope of this review. However, some site-selective examples are highlighted below.

An example of the use of orthogonal protecting groups to differentiate tunable units is shown in the synthesis of telo-



mestatin by Pattenden *et al.* (Scheme 28), in which an oxazole and a thiazolidine ring were formed sequentially.<sup>98</sup> The precursor for the oxazole ring, a serine unit, was protected as an isopropylidene acetal before macrocyclization (substrate **104**). Deprotection of serine and the adjacent acid and subsequent cyclization provided the macrocycle **105**. The formation of the oxazole ring then took place by dehydration to a Dha unit and intramolecular halocyclization. The resulting *N,O*-acetal underwent methanol elimination under acid treatment to provide the oxazole ring in product **106**. Then the cysteine residue was deprotected and transformed into the thiazolidine moiety, thus completing the synthesis of telomestatin (**107**) in good overall yield.

In another example, the synthesis of Trunkamide A by the same group (Scheme 29), a remarkable selective conversion of just one amide of precursor **108** into a thioamide **109** was achieved, by treating the peptide with DAST and then hydrogen

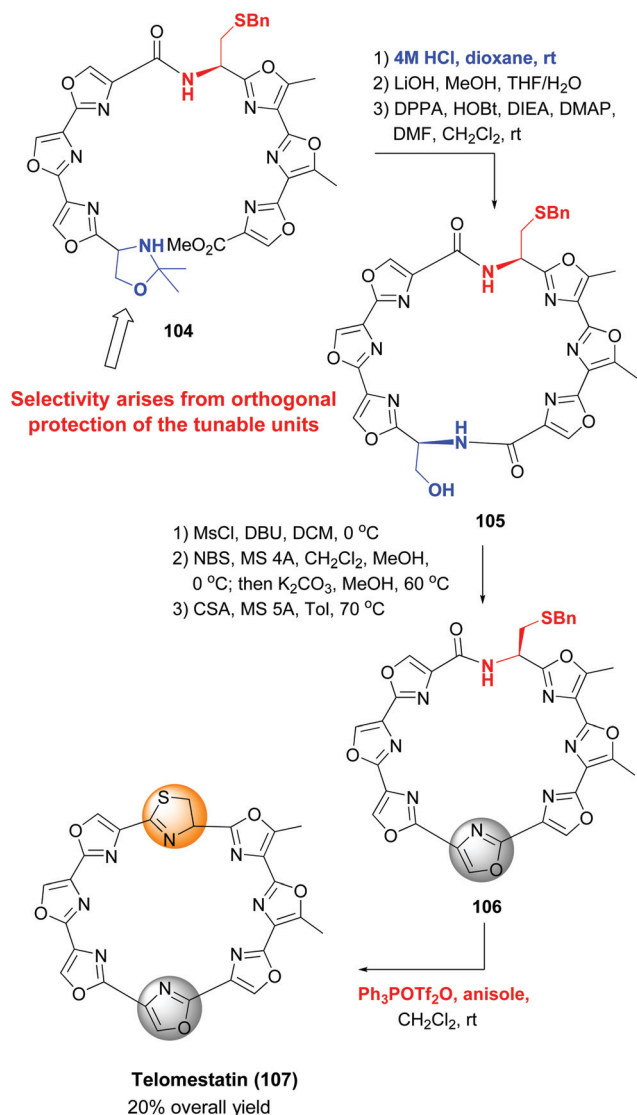


Scheme 29 Generation of thioamides as thiazolidine precursors.<sup>99</sup>

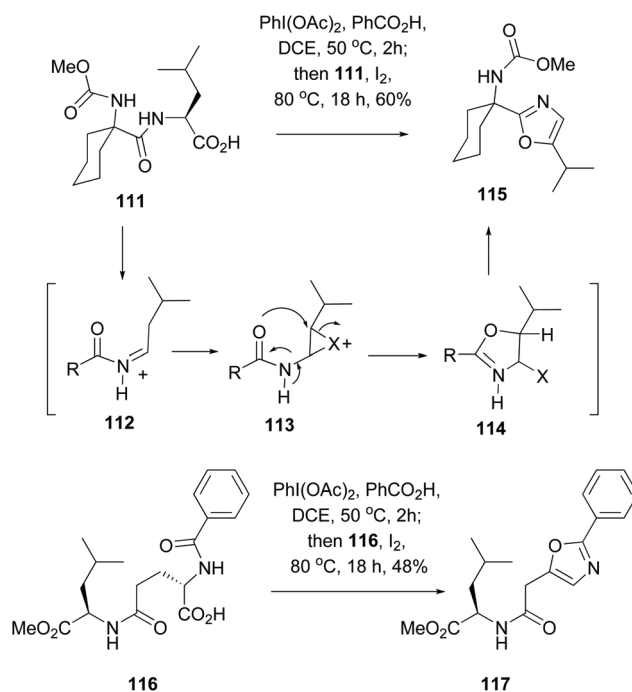
sulfide.<sup>99</sup> Once installed, the thioamide underwent an intramolecular cyclization with the adjacent serine residue on treatment with DAST, to afford the final thiazolidine ring (conversion **109** → **110**).

It must be said that thioamides are another useful modification of the peptide backbone, and although they are considered bioisosters of the carbonyl group, their reactivity could differ notably. Their different ability to form hydrogen bonds and stabilize/destabilize certain conformations, can translate into different biological activities, as shown in the review by Mitchell *et al.*<sup>100</sup>

A method for the formation of oxazole rings that does not involve serine or hydroxylated units, and that can be applied to any residue in a terminal C-position, is shown in Scheme 30 (conversions **111** → **115** and **116** → **117**).<sup>101</sup> Thus, decarboxylation of substrate **111** on treatment with a (diacylodo)benzene



Scheme 28 Formation of backbone heterocycles, Pattenden *et al.*<sup>98</sup>



Scheme 30 One-pot generation of oxazole units.<sup>101</sup>



and iodine generated an iminium ion **112** which isomerized to an enamide. The latter underwent an intramolecular halocyclization involving an adjacent amide carbonyl group (**113** → **114**). Aromatization of the system, by elimination of the leaving group X, provided the oxazole **115**. This one pot oxidative radical decarboxylation–isomerization–iodination–cyclization took place in good global yields, and proceeded with different acyl substituents.

The previous methodology was only reported for small-size peptides, but recently Qvortrup *et al.* described another site-selective oxazole synthesis that used tryptophan as a “tunable” unit, and that could be applied to large-size peptides.<sup>102</sup> As shown in Scheme 31, treatment of a tryptophan-containing peptide **118** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) produced the oxidation of the lateral chain to give an  $\alpha$ -indolylketone **119**. Cyclodehydration followed, to provide a conjugated indolyl-oxazole moiety **120** that could serve as a promising fluorescent label, with an intense emission at 425 nm. Interestingly, this band does not overlap with those produced by natural residues. Moreover, since tryptophan is a low-frequency amino acid in natural proteins (1.1%), many proteins of interest will present only one or a few units, facilitating the modification of selected positions.<sup>103</sup> This was shown in the selective fluorescent labeling of GLP-1 hormone (**121**), which took place in 85% yield.<sup>102</sup>

Other tunable residues are pseudoprolines, which are cyclic, protected derivatives of serine and other hydroxyamino

acids.<sup>104</sup> The conformation and bioactivity of the peptide containing this unit can be different from the acyclic counterparts. Most pseudoprolines are acetal derivatives that can be deprotected using acid treatment, or other standard treatment. The promising sulfamidate derivatives have been previously commented in section 2.1.<sup>41–43</sup>

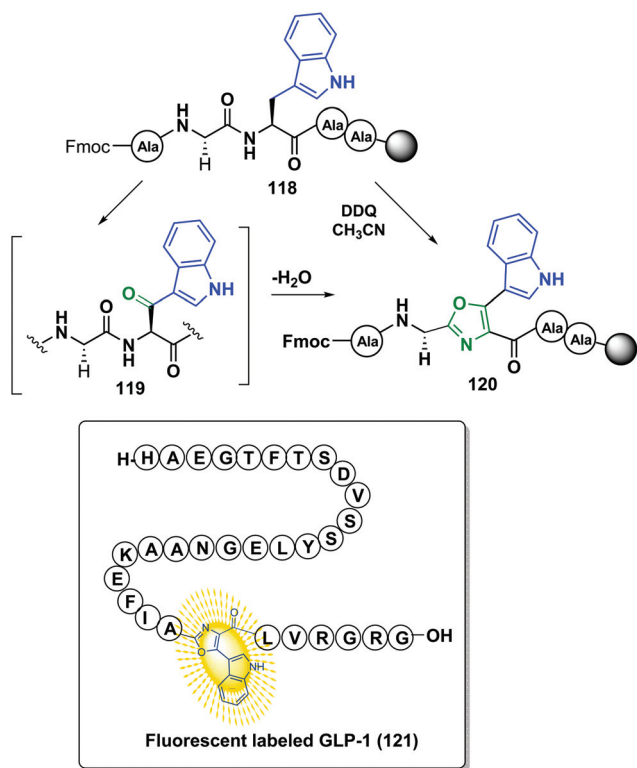
The N-terminal position in peptides offers more possibilities to form heterocyclic units. In a recent paper, van der Eycken reported the modification of a N-terminal benzamide with formation of a isoquinolone. The reaction was regioselective and racemization-free. It provided fluorescent peptides, peptides conjugated to drugs (carriers) and other bioactive products.<sup>105</sup> No doubt that the selective formation of different heterocycles in peptides will offer many applications for biomedicine and materials science in a future.

## 4. Backbone conversions: modification of the N-substituents

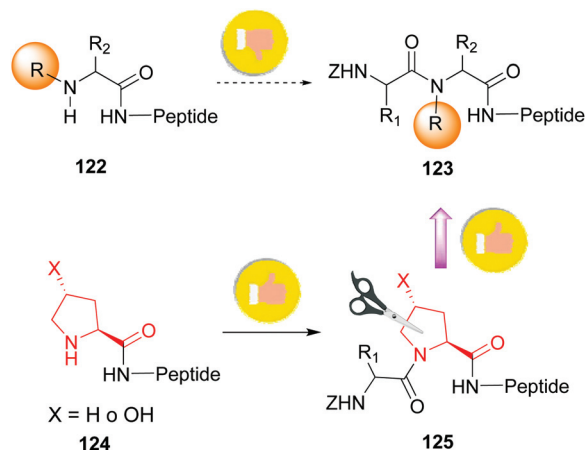
### 4.1. N-Modifications using customizable units

The introduction and variation of N-substituents is another way to create diversity in the peptide library. However, the selective modification of these substituents is difficult. The traditional way to introduce N-alkylated amino acids in a peptide is troublesome, with poor reaction rates and many side-reactions (Scheme 32, conversion **122** → **123**).<sup>106–108</sup> An exception is the coupling of cyclic amino acids, such as proline and hydroxyproline. Therefore, Boto and all have suggested an alternative route (Scheme 32, conversions **124** → **125** → **123**) where hydroxyproline is introduced first in the desired position, and then its scission and functionalization would readily give the desired N-alkylated peptides.<sup>20</sup>

According to this, the addition of different nucleophiles was studied (Scheme 33, conversions **126** → **127** → **128–131**), and the N,O-acetals were transformed in good yields in a N-methyl group (by reduction of the N,O-acetal, compound **128**) and other N-alkyl groups (by addition of C-nucleophiles,



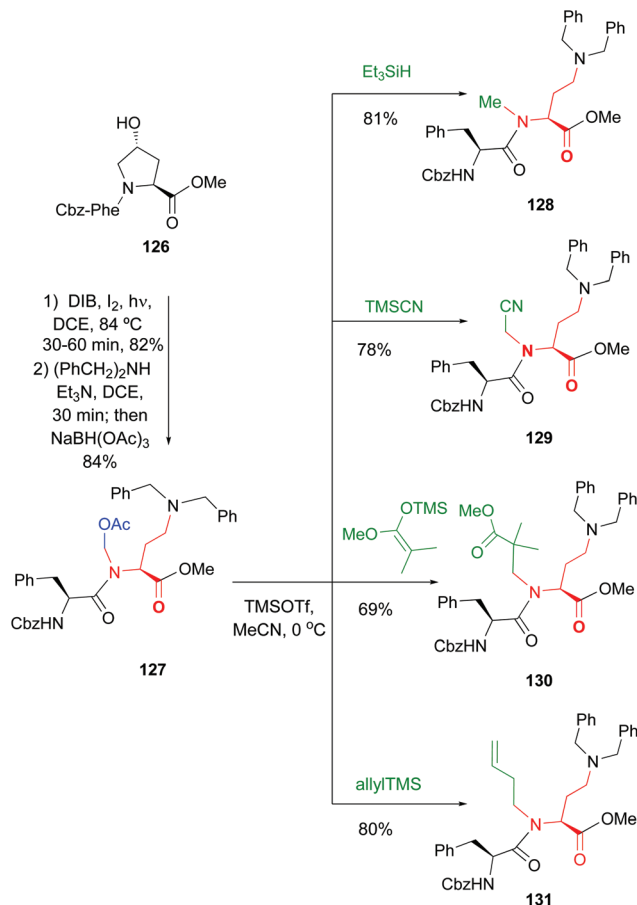
**Scheme 31** Site-selective formation of oxazole-based fluorophores by Qvortrup *et al.*<sup>102</sup>



**Scheme 32** Generation of N-substituted peptides.







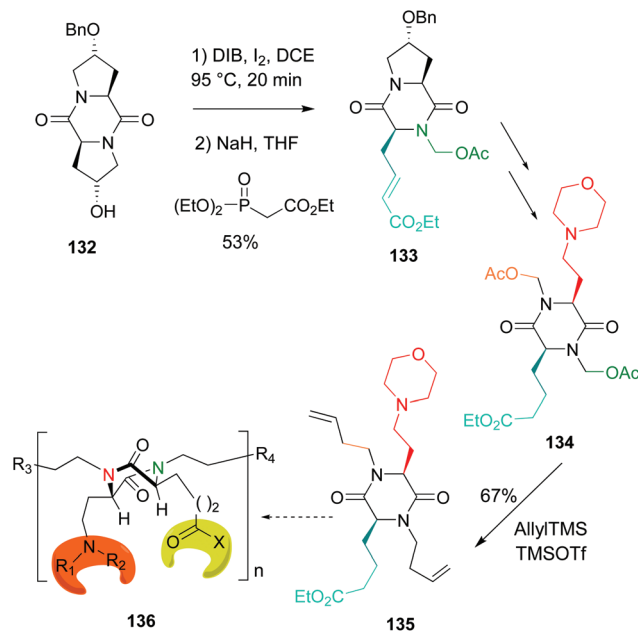
**Scheme 33** Conversion of *N,O*-acetals into *N*-substituents.<sup>20</sup>

compounds **129–131**).<sup>20</sup> Encouragingly, even very bulky *N*-substituents (as in **130** and **131**) could be introduced in good yields, and mild conditions. No side reactions were observed. The introduction of the homoallyl chain in **131** is remarkable, since even larger substituents could be now generated by olefin metathesis.

An application of this methodology to the preparation of tetrasubstituted diketopiperazine scaffolds was also reported (Scheme 34, conversion **132** → **136**).<sup>51</sup> This conversion is usually carried out by alkylation of unsubstituted *N*-diketopiperazines with strong bases and very reactive electrophiles, and usually heating or other activation source is required.<sup>109</sup> These harsh conditions were avoided using the methodology shown in Scheme 34. In this case, the first scission process was followed by a Horner–Wadsworth–Emmons reaction to create a hydrophobic residue (conversion **132** → **133**). In the final step (conversion **134** → **135**, the two *N,O*-acetals were allylated, to give a diolefinic compound **135** that could be the base of dimers or oligomers **136**.<sup>51</sup>

#### 4.2. *N*-Modifications using removable protecting groups

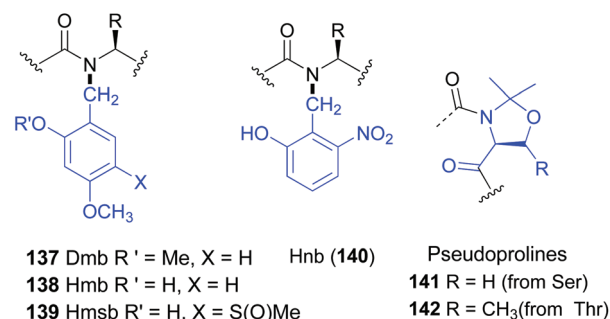
The introduction of amino acids with removable *N*-substituents can be useful to obtain peptides with improved solubility, less aggregation, or other useful properties.<sup>110</sup>



**Scheme 34** DKPs as scaffolds for oligomeric materials.<sup>51</sup>

These *N*-substituted residues can be considered as “units for tunable physicochemical properties”.

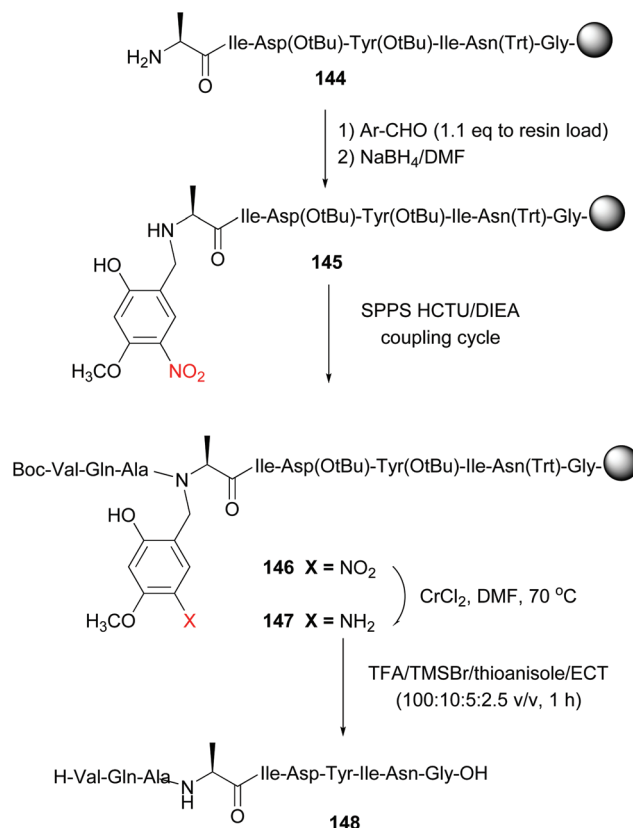
*N*-Protected glycine and alanine residues are often used as such “tunable units”. Other residues are less frequent, since the steric hindrance created by both the lateral chain and the *N*-substituents hinder peptide coupling.<sup>106,107</sup> The use of backbone protecting groups to enhance solubility was originally put forward with the dimethoxybenzyl (Dmb) group (Fig. 1, structure **137**).<sup>111</sup> The introduction of Dmb disrupted the hydrogen bonding that generated scarcely-soluble secondary structures, such as  $\beta$ -sheets, and inhibited interchain aggregation. Even polyalanine chains could be solubilized with Dmb.<sup>111</sup> Other protecting groups followed, such as Hmb, Hnb, Hmsb and pseudoprolines (**138–142**, Fig. 1).<sup>112</sup> All of them favour open-chain, disordered and hence more soluble structures. They are widely used to deal with difficult sequences in solid phase peptide synthesis (SPPS), avoid aggregation and prevent aspartimide formation during Fmoc SPPS.<sup>112c</sup>



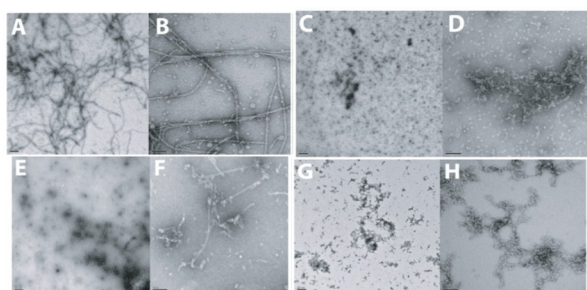
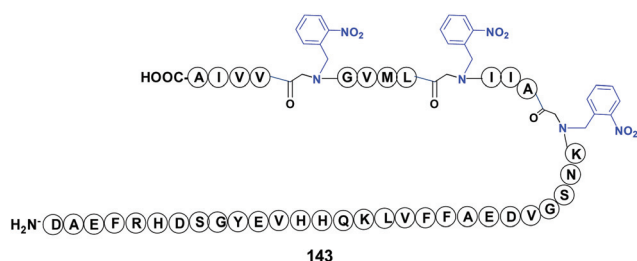
**Fig. 1** *N*-Removable protecting groups.

In a representative example, Meredith *et al.* used an *N*-(*o*-nitrobenzyl) glycine to modify the bend region of amyloid- $\beta$  (Ab), a peptide involved in Alzheimer's disease (Fig. 2, compound **143**).<sup>113</sup> The replacement of Gly29 at the bend region of the amyloid by the "physicochemical tunable unit" gave a peptide that was able to form oligomers but not fibrils at physiological pH and temperature. The reasons for this were not clear, but the authors presented three possible explanations. In the first, the introduction of a protecting group removed hydrogen bond/s that usually stabilize the bend. In the second, the steric bulk of the protecting group would alter the folding of the bend section, and/or approximation of the N- and C-terminal  $\beta$ -sheets prior to fibril formation. In the third, the *cis/trans* isomerization of the glycine and thus the bend conformation would change. Such variations would prevent the structural requirements for fibril formation, but oligomerization would still be possible through hydrophobic collapse of the hydrophobic sequences in the peptide. Cleavage of the protecting group by photolysis could restore the original amyloid properties.

Offer *et al.* used an alanine residue with an Hmnb group for the synthesis of difficult sequences, including aggregation-prone polyaniline sequences.<sup>114a</sup> The automated synthesis of ACP (65–74) with Hmnb-alanine is shown in Scheme 35. The



**Scheme 35** Alanine unit with a removable *N*-Hmnb protecting group in the synthesis of ACP (65–74) by Offer *et al.*<sup>114a</sup>



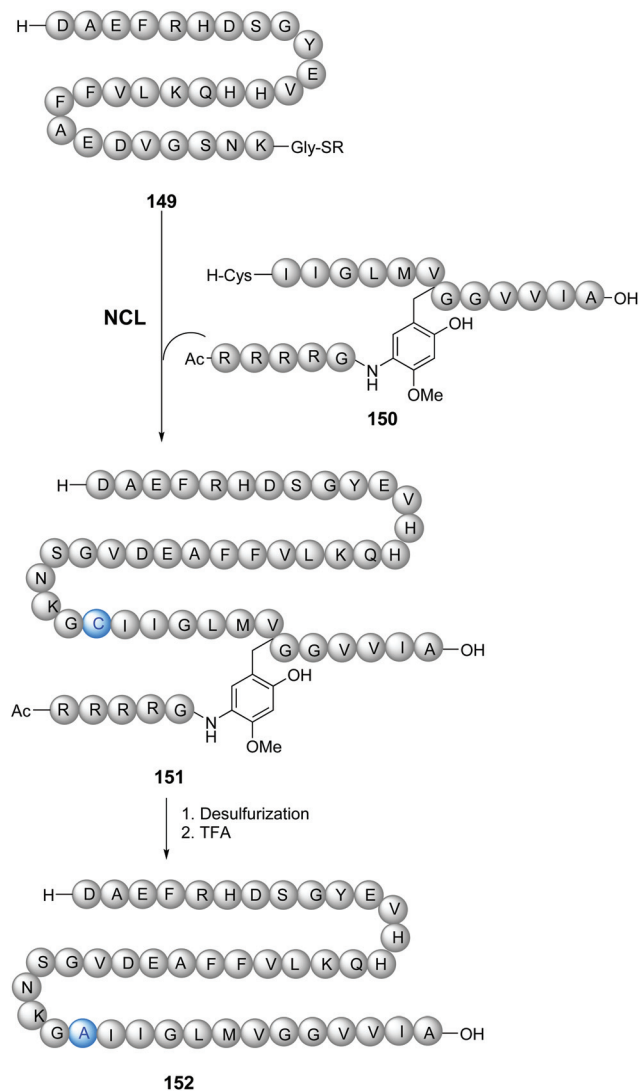
**Fig. 2** *N*-Removable protecting groups in amyloid synthesis. Photolabile (2-nitrobenzyl)glycine units are used in key peptide positions. (A and B) Transmission electron microscopy (TEM) of non-protected A $\beta$ 1–42 at 24 h showed formation of long fibrils. (C and D) TEM at 24 h of A $\beta$ 1–42 with backbone modification at Gly29 using the 2-nitrobenzyl group (NB). The introduction of NB inhibited fibril generation, but smaller oligomer aggregation could be observed. (E and F) TEM at 24 h of A $\beta$ 1–42 with protecting groups in Gly29, Gly33 and Gly38. The hydrophobicity increased and also the aggregation of oligomers. (G and H) In A $\beta$ 1–42 modified at Gly33 and Gly38, fibrils formed but were shorter and thinner than those in the native A $\beta$ 1–42 sequence. TEM photos are originals from Meredith *et al.*, reproduced by license of Wiley-VCH.<sup>113</sup>

tunable unit is formed during SPPS, by reaction of peptide **144** with the Hmnb-aldehyde, followed by reduction of the imine intermediate with sodium borohydride, to give Ala(Hmnb)-containing peptide **145**. The synthesis of the peptide continued without incidents using standard SPPS. It must be said that if the protecting group had not been introduced at this point, the synthesis would probably have failed, due to peptide solubility and aggregation problems.

Once the total sequence was ready (compound **146**), the protecting group was removed. In a first step, the phenylnitro group was reduced under mild conditions (CrCl<sub>2</sub>/DMF system). The resulting aniline **147** was cleaved with TFA and TMSBr/thioanisole mixtures, conditions that also allowed cleavage of the remaining protecting groups and the resin linker. The free peptide **148** was obtained with high purity. Recently, Chiba has reported the use of a *N*-(substituted benzyl)glycine unit in the central position of peptides to favour a conformation that facilitates head to tail cyclization on solid support (a "molecular claw").<sup>114b</sup>

The tunable units can be quite complex and can be attached through the removable link to peptidic chains (Scheme 36). Thus, Zheng *et al.* synthesized longer amyloid beta-peptides (Ab, from 43 to 49 amino acids) to study their role in Alzheimer's disease.<sup>115</sup> These peptides are usually quite difficult to prepare, due to solubility and self-assembly pro-

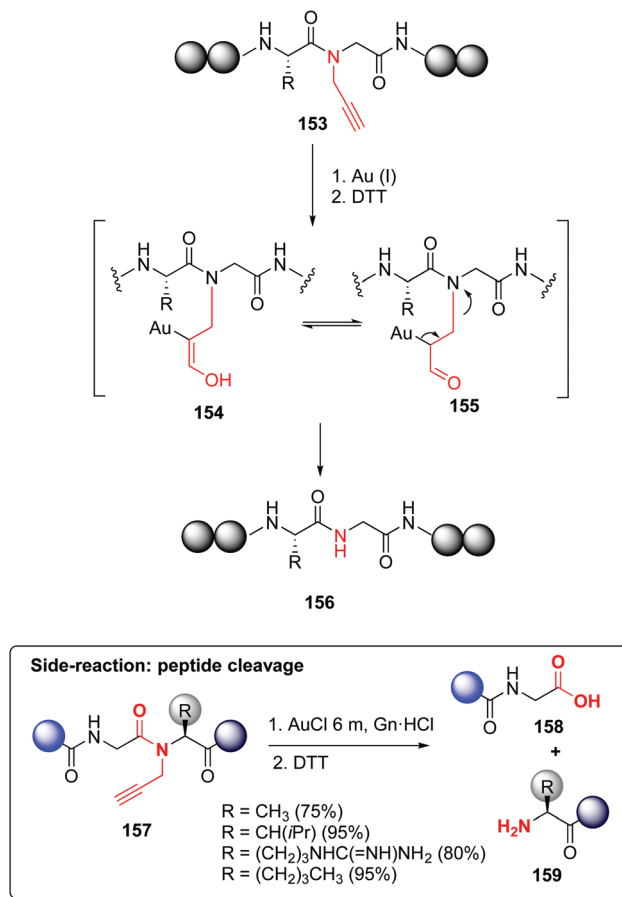




**Scheme 36** N-Removable protecting groups to avoid aggregation in the synthesis of amyloid beta-peptides by Zheng *et al.*<sup>115</sup>

blems. The authors introduced a glycine unit with a removable RMB group, consisting of a substituted benzyl derivative attached to a Arg4-Gly peptide chain (Scheme 36). This RMB motif solubilized the developing peptide, facilitated the ligation step (conversion **149/150** → **151**), and allowed the synthesis of the 42-amino acid Aβ-peptide to proceed successfully. In the final step, a TFA cocktail cleaved the benzylic bond and thus removed the Arg4Gly tag, releasing the desired peptide **152** for biological studies.

In a similar way, Brik reported the backbone modification of synthetic ubiquitin(Ub)-aldehyde, using a Thz linker to attach a cell-penetrating peptide (CPP).<sup>116,117</sup> The CPP allowed the cell internalization of ubiquitin, and once inside, the linker was cleaved by an external stimulus (addition of palladium). The released ubiquitin aldehyde was a potent inhibitor of cellular deubiquitinating enzyme USP2a.<sup>117</sup> In another example (Scheme 37, conversion **153** → **156**), Brik incorporated



**Scheme 37** N-Removable protecting groups by Brik *et al.*<sup>118</sup>

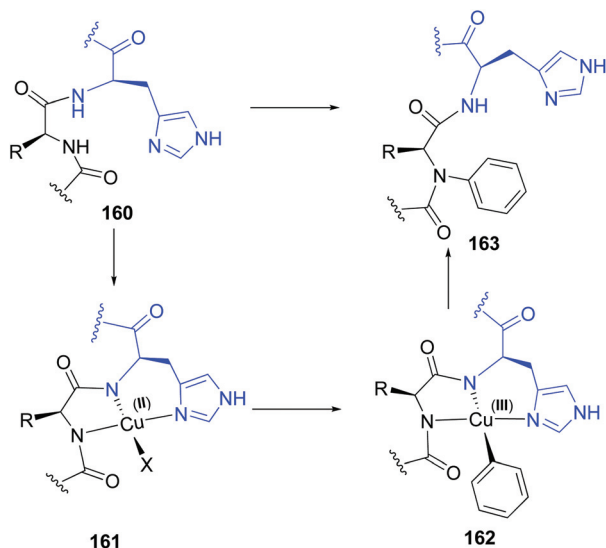
propargylated amides during the synthesis of the NEDD8 protein. This backbone modification improved solubility and avoided aggregation during SPPS. The propargyl group can be readily removed using Au(I) catalysts.<sup>118</sup> The removal probably involves hydration of the alkyne through Au(I) activation, to give intermediate **154**. Tautomerization to the carbonyl compound **155** followed by β-N elimination would give the unprotected peptide **156**.

The authors added a warning note: for certain Gly-Aa couples, cleavage of the peptidic backbone occurred (conversion **157** → **158/159**), so the propargyl-Gly unit should be avoided in these junctions. Palladium can also be used for propargyl group removal, with even superior yields.<sup>118</sup>

#### 4.3. Residue-directed selective N-modifications

Based on the Cham-Lam coupling (a metal-catalyzed oxidative reaction where X-H bonds are coupled with an organoboron reagent),<sup>119</sup> Ball and others have developed a selective N-alkenylation and N-arylation of the peptide backbone.<sup>21a,b,120</sup> The transformation takes place in the amino acid preceding a histidine residue. The histidine directs the incorporation of an N-substituent into the adjacent residue after treatment with an aryl or alkenyl boronic acid and a Cu(II) salt. A copper complex is formed involving the histidine NH and imidazole groups,





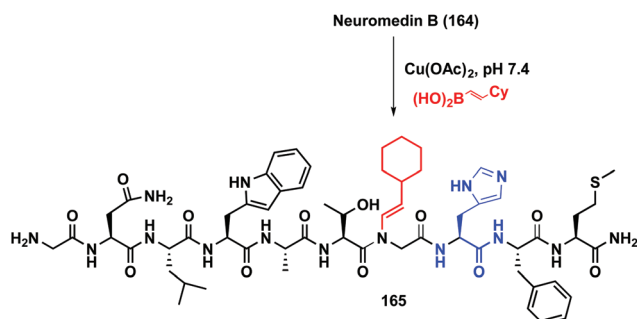
**Scheme 38** Histidin-directed backbone arylation by Ball *et al.*<sup>21a,b</sup>

and the NH group of the preceding amino acid (conversion **160** → **163**, Scheme 38). The intermediate **161** resembles natural ATCUN motifs (amino-terminal Cu<sup>2+</sup> and Ni<sup>2+</sup> binding motifs) that occur in proteins.<sup>121,122</sup> However, while ATCUN motifs are tetradentate complexes, the non-terminal sequences form a tridentated complex A. The free site allows the Cu(II) complex to react with the boronic acid in a transmetalation reaction, generating the Cu(III) intermediate **162**, which transfers the aryl or alkenyl residue to the backbone nitrogen to give the *N*-arylated product **163**.<sup>121</sup>

The reaction is sensitive to steric hindrance, and while *trans*-alkenylboronic acids give usually good yields, the *ortho*-substituted arylboronic acids are unreactive.

The reaction has been applied to different peptides and proteins. As shown in Scheme 39, neuromedin B **164** is only alkenylated on the glycine residue preceding histidine, to give peptide **165**.<sup>21a</sup> Other residues such as tryptophan or asparagine are not involved in the transformation.

It must be said that histidine is a relatively rare amino acid in peptides and proteins, and thus this reaction can be very useful to incorporate backbone *N*-substituents at just one (or a



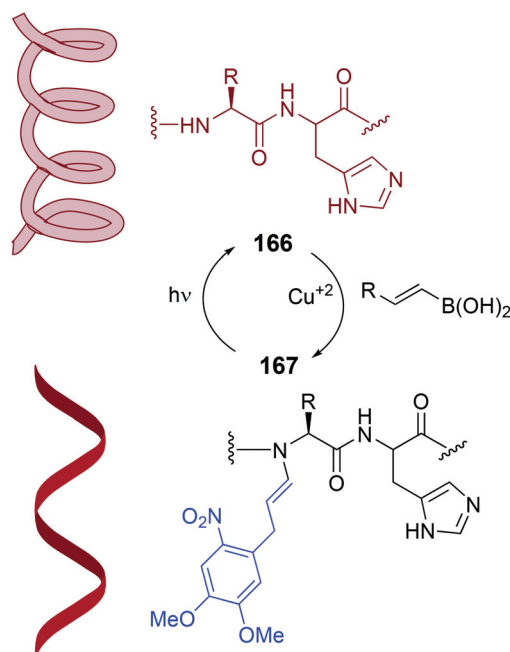
**Scheme 39** Histidin-directed neuromedin arylation by Ball *et al.*<sup>21a</sup>

few) positions.<sup>21b,120</sup> Lysozyme (PDB:1HEL) was modified at Arg14 preceding the only histidine residue.<sup>3c,123</sup>

This reaction can be very interesting to control protein and peptide structure and function. The N-H substitution disrupts the hydrogen bonding that allow the formation of helices,  $\beta$ -sheets and other secondary structures.<sup>110</sup> Conversely, the *N*-dealkylation would restore the folding ability of the peptide. In the photocaging system shown in Scheme 40 (conversion **166**/167), the alkenylation of the protein or the removal of the alkenyl group under an external stimuli determine the protein structure.<sup>120</sup> Thus, a collagen-resembling triple helix peptide was *N*-alkylated, and the resulting peptide could no longer fold in solution. However, when the modified peptide was irradiated with UV light, the *N*-substituent was removed, and the peptide folded again into a triple helix. The authors also showed that backbone N-H modification could also prevent protease degradation. When uncaging took place by photoirradiation, the peptide became sensitive to protease action.

A particularly reactive sequence is pyroglutamate-histidine, which can be labeled selectively even in the complex cell lysate.<sup>21a</sup> While most backbone N-H modification took hours, Pyr-His sequences reacted in an average 5 minutes. Pyroglutamate can be formed from glutamine using the enzyme glutaminyl cyclase (conversion **168** → **169**, Scheme 41). Once formed, the Pyr-His motif can undergo selective labeling (conversion **169** → **170**). Since the Pyr-His unit is not frequent in natural proteins, monolabeled peptides can be obtained selectively.

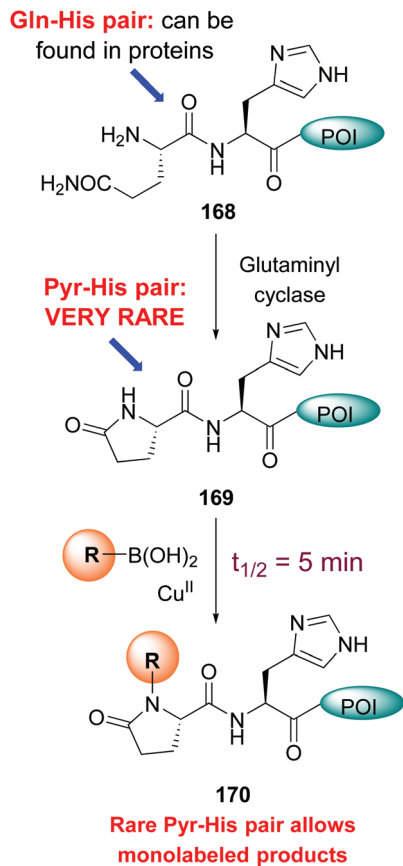
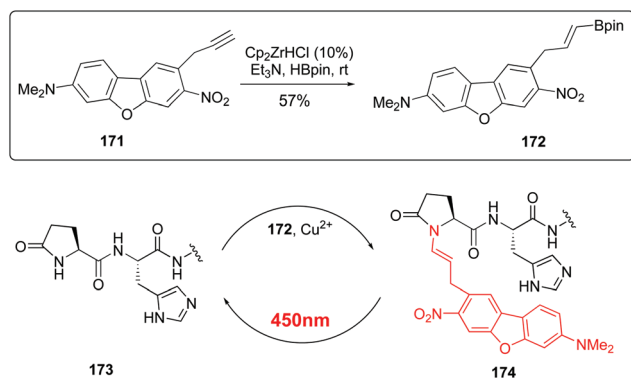
Recently, Ball *et al.* have reported red-shifted backbone N-H photocaging systems (Scheme 42).<sup>124</sup> The use of near-IR light has elicited much interest due to its superior tissue pene-



**Scheme 40** Photocaging systems by Ball *et al.*<sup>120</sup>

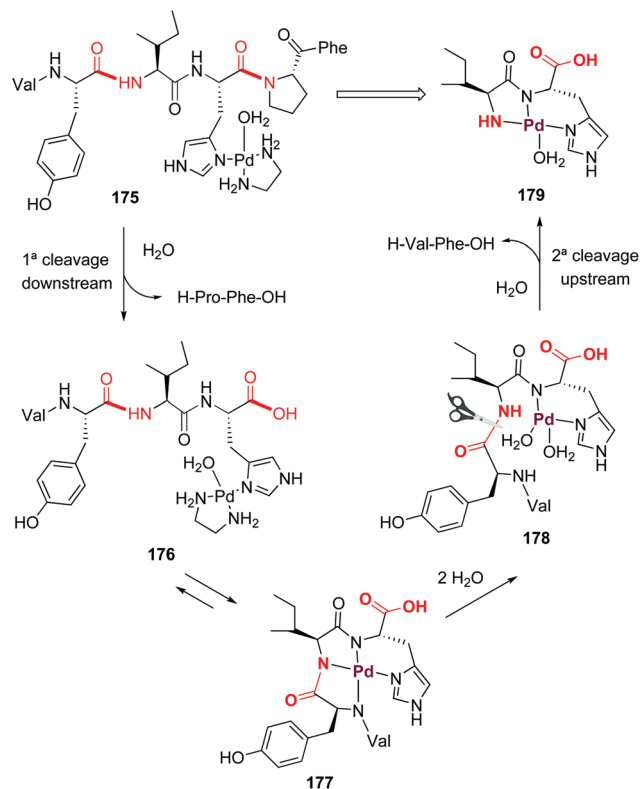




Scheme 41 Pyr-His pair for monolabeling of proteins.<sup>21a</sup>Scheme 42 Red-shift backbone photocaging systems.<sup>124</sup>

tration and since it does not overlap with the absorption of natural biomolecules, thus allowing improved precision for uncaging. The Scheme shows the preparation of a boronic acid with the cromophore (conversion **171** → **172**) and its His-directed introduction at the terminal Pyr position (conversion **173** → **174**). The peptide is released under uncaging conditions (irradiation at 450 nm).

Palladium can also form ATCUN-like complexes (Scheme 43) but the reaction outcome can be quite different.

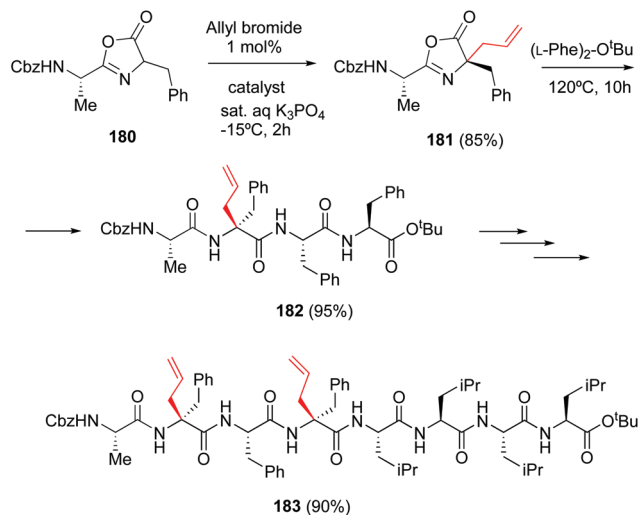
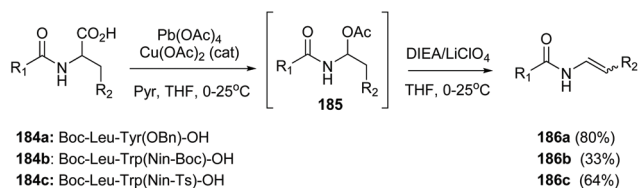
Scheme 43 Palladium complexes promote peptide cleavage (Kostić *et al.*).<sup>125</sup>

In 2002, Kostić *et al.* reported that on treatment of the peptide with  $[\text{Pd}(\text{en})(\text{H}_2\text{O})_2]^{2+}$ , the histidines in interior positions directed the formation of palladium complexes which resulted in regioselective cleavage of the second peptide bond upstream and the first peptide bond downstream (as shown for angiotensin complex **175**, Scheme 43).<sup>125</sup> A first cleavage downstream produces the tetrapeptide **176**, which is in equilibrium with the ATCUN-like complex **177**. Ligand exchange with water produced the intermediate **178**, where the second cleavage (upstream) releases a H-Val-Phe-OH dipeptide and the complex **179**.

## 5. Selective conversion of C-terminal units

The particular reactivity of the C-terminal position allows its site-selective modification. In a classical example, Ooi *et al.* reported the site-directed asymmetric quaternization of a peptide backbone at the C-terminal position by formation of an azlactone, alkylation of its  $\alpha$ -position, and opening of the azlactone with a peptide (Scheme 44, conversion **180** → **182**).<sup>126</sup> Remarkably, using the chiral catalyst PTC (tetraamino-phosphonium chloride), the alkylation proceeded with high diastereoselectivity and good to excellent yields, using a variety of alkyl halides. This methodology was used to create a tetra-

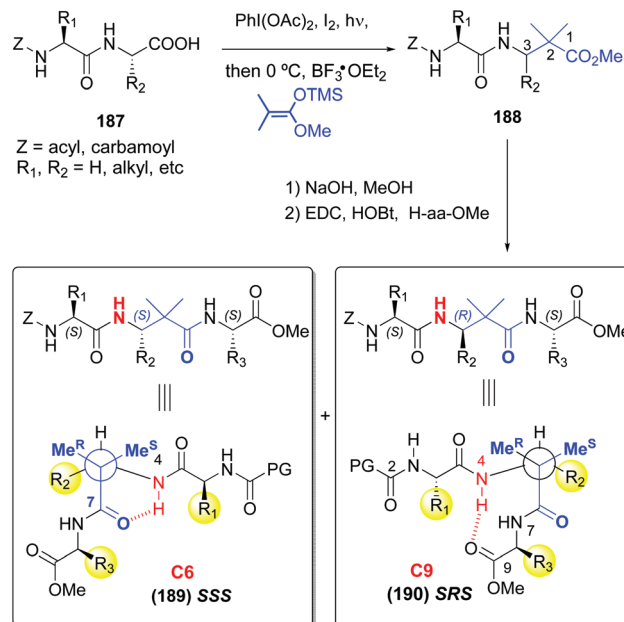


Scheme 44 Selective C-terminal modification by Ooi *et al.*<sup>126</sup>Scheme 45 Formation of C-terminal enamides by Porco *et al.*<sup>127</sup>

peptide **182** that was coupled to a tetra-leucine residue, affording the final octapeptide **183**.

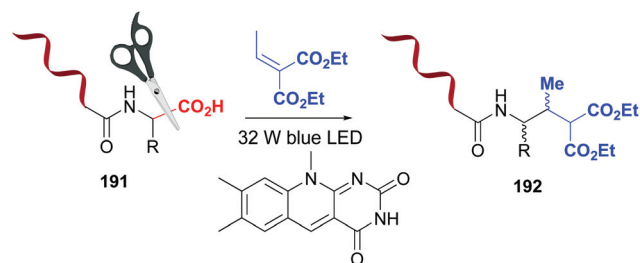
In another example, Porco carried out an oxidative radical decarboxylation of the C-terminal residue in several peptides **184a–c** (Scheme 45) generating an intermediate  $\alpha$ -acetoxyamine **185**, that under base treatment yielded a peptide enamide **186a–c**. The methodology was later used to synthesize chondriamides A and C.<sup>127</sup>

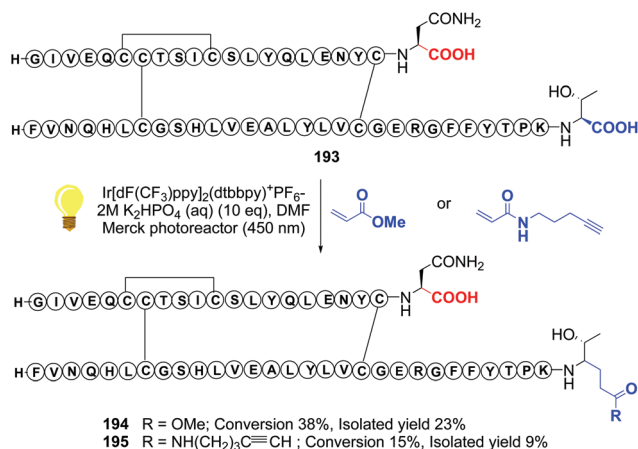
A related decarboxylation strategy was used to obtain peptide hybrids, such as  $\alpha,\beta$ -peptides, which can present new folding patterns, depending on the  $\alpha:\beta$  ratio and distribution.<sup>128,129</sup> In addition, they usually display superior resistance to proteases and hence, *in vivo* stability. Boto *et al.* described the synthesis of  $\alpha,\beta,\alpha$ -tripeptides where the central unit was a  $\alpha,\alpha$ -disubstituted  $\beta$ -amino acid (conversion **187**  $\rightarrow$  **189/190**, Scheme 46).<sup>130</sup> These hybrids formed unusual turns such as deltaturns or extended beta-turns in solid phase. The tripeptides were formed from  $\alpha,\beta$ -dipeptides **188**, generated by modification of the C-terminal residue of  $\alpha$ -peptides **187**. In the conversion **187**  $\rightarrow$  **188** the major diastereomer presented the “natural” (*S*) configuration, since the N-terminal residue acted as a chiral auxiliary. Moreover, in this work a procedure was developed which used catalytic amounts of a Lewis acid (0.1 equivalents of  $\text{Cu}(\text{OTf})_2$ ), affording moderate to good yields of the final products (40–70%), which could allow in a future an increase of the stereoselectivity by using chiral catalysts.

Scheme 46 Generation and modification of C-terminal positions.<sup>131</sup>

Interestingly, in a subsequent work by Aizpurua *et al.*,<sup>131</sup> the SSS and SRS tripeptides showed different conformations in  $\text{CDCl}_3$  or DMSO solution (Scheme 46). Thus, while the SSS peptides **189** displayed a turn by hydrogen bonding of the carbonyl and NH groups of the central  $\beta$ -residue, the SRS isomers **190** presented an extended delta-turn. This duality can be of interest to design drugs or peptide catalysts.

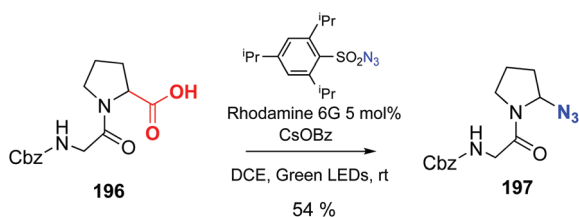
Photocatalytic methods also allow selective C-terminal modifications of short peptides, by replacing the carboxyl group by other functions.<sup>10</sup> These reactions are regioselective, due to the difference in reduction/oxidation potentials between the C-terminal carboxyl group and those present in the lateral chains of aspartic and glutamic acid residues.<sup>10,132,133</sup> MacMillan *et al.* used this difference in oxidation potentials to carry out a flavin-catalyzed decarboxylation of the C-terminus in proteins (Scheme 47, conversion **191**  $\rightarrow$  **192**).<sup>133</sup> The resulting  $\alpha$ -amino radical underwent a Michael addition to give the final product **192**. Using a variant of this reaction, the alkylation on the C-terminus of insulin A chain was achieved in 40% yield.<sup>132</sup>

Scheme 47 Photocatalytic modification of C-terminal positions by MacMillan *et al.*<sup>133</sup>

Scheme 48 Photocatalytic modification of insulin by Shi *et al.*<sup>134</sup>

Recently, Shi reported a photocatalytic decarboxylation-conjugate addition to acrylates or acrylamides, using iridium complexes, and applied it to the C-terminal modification of insulin (conversions **193** → **194** and **193** → **195**, Scheme 48).<sup>134</sup> The authors warned that this decarboxylation was only useful when tryptophan was not present in the molecule, since in its lateral chain reacts preferentially. In proteins such as insulin, however, the reaction can be very useful to install C-terminal functionalities and create  $\gamma$ -amino acid units.<sup>134,135</sup>

A related decarboxylation-alkynylation was developed by Waser *et al.*<sup>136a</sup> The method used a DACA (donor-acceptor cyanoarene) catalyst and EBX (ethynyl benzidioxolone) to achieve the modification of di- to hexapeptides, in low to excellent

Scheme 49 Photocatalytic decarboxylation-azidation by Leonori *et al.*<sup>137</sup>

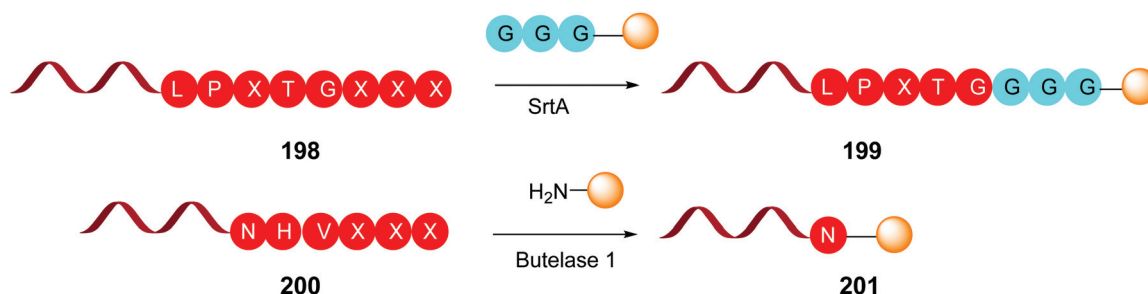
yields, depending on the substrate. However, aromatic amino acids such as tyrosine, histidine or tryptophan can give side reactions that decrease the yields. Recently, Waser has reported a promising decarboxylation to give *N,O*-acetals that on treatment with aromatic nucleophiles (indoles, phenols) provided C-aryl substituted peptides.<sup>136b</sup>

Leonori has reported the decarboxylation-azidation reaction of peptides to afford C-terminal azides, catalyzed by Rhodamine 6G (conversion **196** → **197**, Scheme 49).<sup>137</sup> These azides can be very useful to expand the chain or ligate peptides using click chemistry. The reader is directed to the review by Spring *et al.* to find other interesting examples of photocatalysis out of the scope of this review.<sup>10</sup>

A brief comment should also be devoted to the modification of C-terminal positions with enzymes. Most of these modifications are ligation reactions, which are not covered by this review. For instance, Tubulin Tyrosine Ligase (TTL) recognizes the VDSVEGEGEEEGEE motif (Tub-Tag) in the C-terminus and attaches tyrosine derivatives, including modified tyrosines with extra functionalities.<sup>138</sup> Subtiligase ligates the C-terminal ester of a peptide and the N-terminal amine of other peptide, without requiring a recognition motif.<sup>139</sup> However, some enzymes not only attach fragments but also remove sections of “signal” motifs.

This is the case of Sortase A and Butelase 1 (Scheme 50). Sortase A is a transpeptidase from *Staphylococcus aureus*, which recognizes the LPXTG sequence on a protein, and cleaves the T-G union to form a reactive thioester between the threonine residue and a cysteine unit from the SrtA active pocket.<sup>140</sup> Then the thioester is cleaved by addition of a glycine-derived nucleophile, for instance a GGG-peptide2 chain, generating a new ligated protein (*e.g.* protein1-LPXT-GGG-peptide2) (conversion **198** → **199**, Scheme 50). It must be said that SrtA is able to recognize the LPXTG motif both in the C- and N-terminus of a protein.

Butelase 1 was isolated from the medicinal plant *Clitoria ternatea* and is an Asx peptide ligase that attaches C-terminal NHV or DHV sequences to the N-terminal domains of other proteins (conversion **200** → **201**, Scheme 50). Although specific for the C-terminal residue, it accepts most N-terminal residues.<sup>141</sup> Butelase is highly active (around 20 000 times faster than SrtA) and has been used for protein cyclization and peptide ligation.<sup>141c</sup>



Scheme 50 Some site-selective modifications catalyzed by enzymes at C-terminal positions.



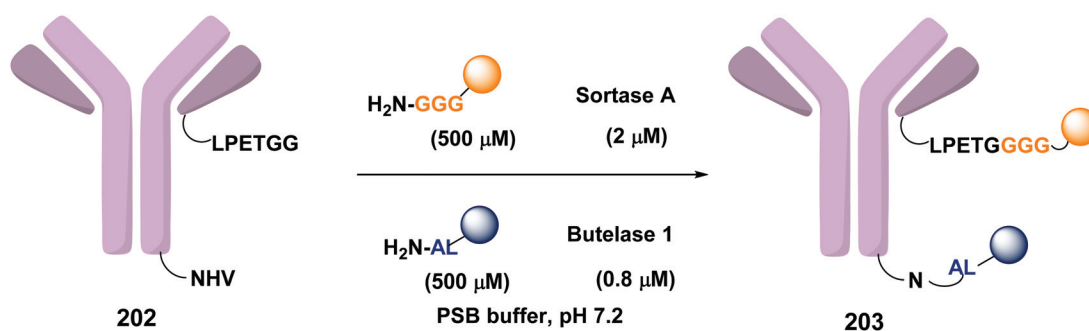
Many applications have been developed;<sup>141–144</sup> for instance, SortA-catalyzed PEGylation has been used to attach proteins or protein conjugates to the surface of cells.<sup>143</sup> Tam *et al.* have used butelase to label ubiquitin and green fluorescent protein in more than 95% yield.<sup>142</sup>

In a recent example, Ploegh *et al.* have reported the one-pot dual labeling of the immunoglobulin IgG1 with two different groups, based on the orthogonal specificity of butelase1 and sortase A (Scheme 51, conversion **202** → **203**).<sup>145</sup> The same group ligated two proteins through a linker **204**, which had one of its end prepared to react with sortase, and the other prepared to react with butelase (Scheme 52, conversion **205/206** → **207** using linker **204**). The nature of such linkers can influence the properties of the peptide/peptidomimetic backbone.<sup>145</sup> More examples will likely appear in the next future.

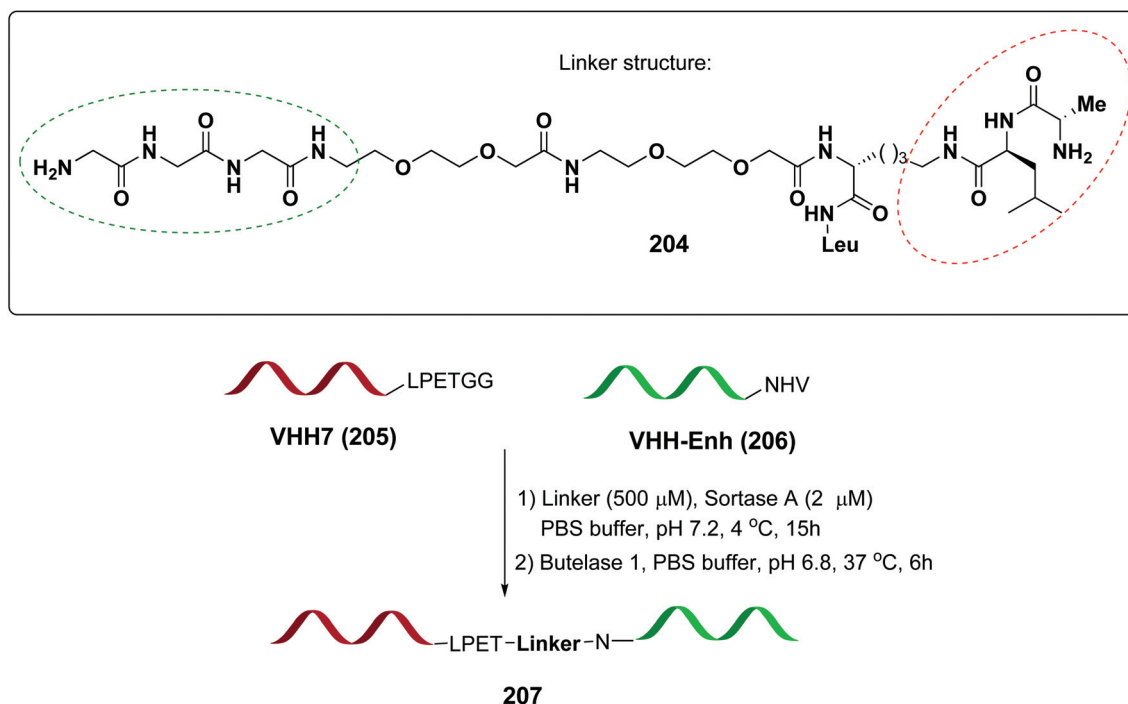
## 6. Selective conversion of N-terminal units

Unlike the site-selective transformation of backbone internal positions and C-terminal units, the selective conversion of N-terminal units has been covered by several reviews, and the reader will find a comprehensive treatment of the subject therein.<sup>1a,2,3a,e,g,146</sup> Nevertheless, in this section a selection of methods has been compiled in Table 1, and commented below.

Electrophiles such as aldehydes react preferentially with the N-terminal residue over other basic units (such as lysine), as shown in entries 1–5. This selectivity is due to the fact that the N-terminal amine presents an  $\alpha$ -electron-withdrawing carboxyl



**Scheme 51** Selective labeling of IgG1 with butelase and sortase by Ploegh *et al.*<sup>145</sup>



**Scheme 52** Selective protein coupling using an adaptable linker by Ploegh *et al.*<sup>145</sup>





**Table 1** Summary of site-selective modifications at N-terminal positions

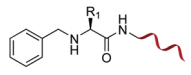
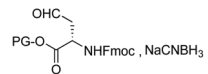
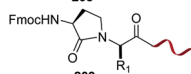
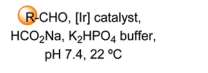
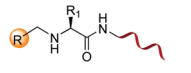
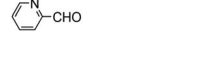
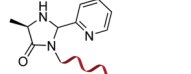
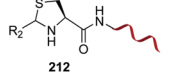
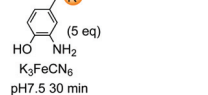
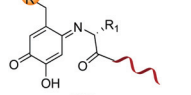
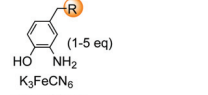
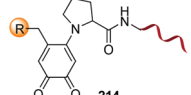
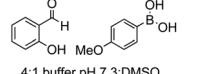
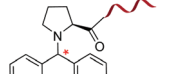
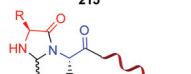
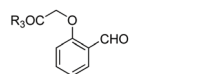
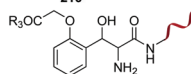
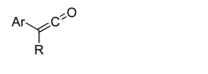
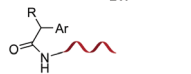
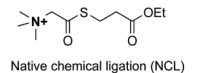
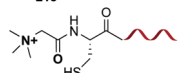
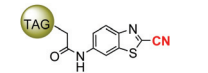
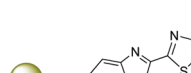
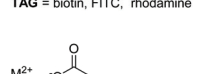
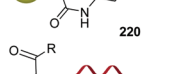
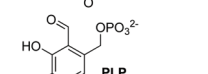
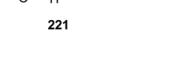
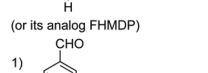
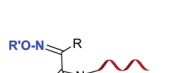
Entry	N-terminal residue	Reaction conditions	Product/s (%)	Ref.
1	Any	PhCHO NaCNBH <sub>3</sub>		148
2	Any	 deprotection (-PG) & cyclization		149
3	Any	 R-CHO, [Ir] catalyst, HCO <sub>2</sub> Na, K <sub>2</sub> HPO <sub>4</sub> buffer, pH 7.4, 22 °C		150
4	Any			151
5	Cys	R <sub>2</sub> -CHO		152
6	Any except Pro, Hyp, Pip	 HO-NH <sub>2</sub> (5 eq) K <sub>3</sub> FeCN <sub>6</sub> pH 7.5 30 min		3i and 153
7	Pro, Hyp	 HO-NH <sub>2</sub> (1-5 eq) K <sub>3</sub> FeCN <sub>6</sub> pH 7.5 30 min		3i and 26a
8	Pro, others	 4:1 buffer pH 7.3:DMSO		154
9	Any	R'-CH=CH-B(OH) <sub>2</sub> pH 7.4, rt Na ascorbate (50 mM)		25a
10	Gly			155
11	Any			24b
12	Cys	 Native chemical ligation (NCL)		158
13	Cys	 TAG = biotin, FITC, rhodamine		26b
14	Any			160
15	Any	 PLP (or its analog FHMDP)		161–163
16	Any	1)  2) R'-ONH <sub>2</sub>		25b and 164



Table 1 (Contd.)

Entry	N-terminal residue	Reaction conditions	Product/s (%)	Ref.
17	Ser, Thr	1) NaIO <sub>4</sub> 2) R-NH-NH <sub>2</sub> NaOAc	223	165b
18	Any	 pH 8.0	224	167
19	Val, other acyclic unit	Pd(OAc) <sub>2</sub> (10 mol%) Mo(CO) <sub>6</sub> (0.33 eq.) BQ (2.0 eq.), AgOAc (1.5 eq.), HFIP, 110 °C	225	168 and 169
20	Any =	 Subtiligase	226	170
21	G-N-E-A-S-Y-P-L	 NMT	227	171–173

group, which lowers its pK value.<sup>147</sup> Therefore, by controlling the pH (at slightly acidic values) it is possible to differentiate the amino group of the N-terminal residue (pK<sub>a</sub> ca. 8) and those of lysine, ornithine (pK<sub>a</sub> ca. 10) and arginine (ca. 12). Thus, Chou *et al.* selectively labeled the N-terminus of several proteins using a reductive amination, by treatment with aldehydes and sodium cyanoborohydride at pH ca. 6.1 (entry 1).<sup>148</sup> Under this slightly acidic pH, the Nε-amino group in lysine was protonated and was unable to form the imine intermediate. Using reductive amination is preferable to N-alkylation with alkyl halides or similar electrophiles, since it avoids side reactions such as overalkylation.

Kodadek has reported an interesting reductive amination and cyclization, which generates a cycloalanine unit (CyAla) at the N-terminal position (entry 2). This modification is an efficient alternative to N-terminal methylation in order to obtain more protease-stable and cell-permeable peptides.<sup>149</sup>

In a variation of this method, Francis used an iridium complex to catalyse a reductive amination where sodium formate was used as the hydride source (entry 3).<sup>150</sup>

In another example, the group reported the use of 2-pyridine carboxyaldehydes (entry 4) to generate an imine that underwent intramolecular addition of the adjacent amide nitrogen, affording an aminal.<sup>151</sup> In this case, the pyridine ring acted as a base that favored the cyclization reaction. Neri showed that when cysteine occupies the N-terminal position, a thiazolidine is formed (entry 5).<sup>152</sup>

A recent labeling of N-terminal residues uses the oxidation of an aminophenol by ferricyanide, which gives an *o*-aminoquinone intermediate (entries 6 and 7).<sup>3i,153</sup> This reactive Michael acceptor undergoes addition of the N-terminal amino group. The reaction is fast (about 0.5 h) and tolerates many N-terminal units, with proline being the most reactive residue. The products are different for reaction with primary (entry 6) and secondary amines (entry 7).<sup>26a</sup> Ferricyanide offers advantages with respect to other oxidants (such as sodium periodate) since it avoids side-reactions (*e.g.* oxidation of glycans) and offers superior selectivity and

yields.<sup>3i,26a</sup> Francis has used this strategy to label many proteins, as commented later.

Boronic acids are also useful to modify the N-terminal position, as shown in the example by Raj *et al.* where a terminal secondary amine such as proline underwent a Petasis bioconjugation to give a site-selective labeled peptide (entry 8).<sup>154</sup> Angiotensin, bradykinin, insuline chain A, hyperthrehalosemic neuropeptide and creatine kinase were thus successfully labeled.

In another example, Ball *et al.* formed a heterocyclic aminal on treatment of the peptide with the boronic acid in the presence of ascorbic acid (entry 9).<sup>25a</sup> The reaction needs oxygen to take place, which suggests an “umpolung” reaction where an *in situ* formed dehydroascorbate acts as an oxidizing agent.

When glycine occupies the N-terminal position, treatment with aromatic aldehydes without reductive conditions leads to C-α alkylation, giving an aminoalcohol (entry 10).<sup>155</sup> The method can differentiate N-terminal glycine even in cell lysates. Rai *et al.* used the method to prepare pure tagged proteins.

The selective acylation of the N-terminal position has also received much attention. Since Reid showed that the growth hormone could be selectively acetylated at the N-terminus by using pH control and a small amount of acetic anhydride,<sup>156</sup> other authors have used different acylating agents (*N*-hydroxysuccinimide esters, *N*-hydroxyphthalimide, selenobenzaldehyde esters).<sup>157</sup> Che *et al.* used an isolated alkyne-functionalized ketene (entry 11) which was generated from the acid in two steps: by forming a mixed anhydride with oxalyl chloride, and then by reaction with a base.<sup>24b</sup> Che also showed this selective labeling for lysozyme, insulin, RnaseA, BcArg and other peptides.<sup>24b</sup>

An interesting application of N-terminal acylation is to generate derivatives with better solubility and less prone to aggregation. Thus, Tolbert *et al.* attached betaine, which possesses a quaternary ammonium unit, to the N-terminal residue of two peptides, using native chemical ligation (entry 12).<sup>158</sup>



When cysteine occupies the N-terminal position, selective conjugation to a variety of tags can be achieved, as shown with the reaction with a cyanobenzothiazole (entry 13).<sup>26b</sup> N-Terminal cysteine can also be used for native chemical ligation, but ligation methods are beyond the scope of this review.<sup>28</sup>

The N-terminal position can also be modified by transamination promoted by metal ions such as Cu(II) and Ni(II). Inspired by previous work by other authors,<sup>159</sup> Dixon and Moret<sup>160</sup> used glyoxal to generate an imine that coordinated the metal (entry 14). The acidity of such imine favoured proton removal from C- $\alpha$  and isomerization to a second imine which was hydrolyzed to generate a terminal  $\alpha$ -ketoacid.

The reaction was subsequently optimized, and Francis *et al.* reported a variation where pyridoxal-5-phosphate (PLP, vitamin B6) promoted the reaction (entry 15).<sup>161</sup> Francis found that the reaction was completed in 2 h at 65 °C and 24 h at 25 °C for most amino acids,<sup>161</sup> although some residues pose problems due to their low reactivity (Pro) or because side-reactions take place (Lys, His, Trp). Other alternative to PLP is its analog FHMDP, where the phosphate has been removed, and the pyridine is forming an *N*-methyl hydrochloride salt. FHMDP is much more efficient than PLP when bulky amino acids (Val, Leu, Ile) occupy the N-terminal position.<sup>162</sup> The ketoproteins thus obtained are stable and can be readily manipulated. Francis and others have used this reaction to label different proteins, as commented later.<sup>25d,161–163</sup> Francis has also reported other alternatives to PLP, such as Rapoport salt (*N*-methylpyridinium-4-carboxaldehyde-benzene sulfonate salt), which is particularly efficient for glutamate-rich sections (entry 16).<sup>25b,164</sup> The method was applied to transamination of bacterially expressed proteins, such as cellulase mutants EES-, AEE- and AKT-EGPh in high yields.<sup>164</sup>

A particular case is when serine or threonine occupy the N-terminal position (entry 17). Fields and Dixon,<sup>165a</sup> and later Geoghegan and Stroh,<sup>165b</sup> reported that treatment with sodium periodate produced the oxidative cleavage of the hydroxymethyl group and the formation of a glyoxalate that can be trapped by amines, hydrazides, and other nucleophiles. The methodology was applied to the synthesis of modified peptides.<sup>165b</sup> A review by El Mahdi and Melnyk comments the glyoxyl transformations to obtain bioconjugates.<sup>166</sup>

Other procedures transform the N-terminal amino group into other functions. For instance, van Hest *et al.* converted the terminal amino moiety into an azido group, by treatment with 1-sulfonyl azides (entry 18).<sup>167</sup>

Other selective modifications of the N-terminus use palladium-catalyzed C(sp<sup>3</sup>)-H activation.<sup>168,169</sup> Carretero *et al.* converted acyclic residues into cyclic ones using this methodology (entry 19).<sup>168</sup>

Finally, enzymes can also be used to functionalize the N-terminus. Entries 20 and 21 illustrate sequence-specific conversions catalysed by the enzymes subtiligase and NMT.

Subtiligase conjugates C-terminal activated esters, such as thioesters, with the N-terminus of peptides and proteins (entry 20).<sup>170</sup> Its promiscuity makes it ideal to conjugate a wide range

of peptides and proteins. *N*-Myristoyltransferase (NMT) attaches a fatty acid to the N-terminal position of a protein expressing the sequence GNEASYPL, which facilitates its anchoring to the cell membrane.<sup>171</sup> NMT can accept many different fatty acids, incorporating carbonyls, halogens, azides or alkynes for further protein functionalization.

It should be said that *N*-functionalization can deeply affect the stability, solubility, aggregation and other properties of the peptide. For instance, NMT was used to convert an elastin-related polypeptide into a myristoyl derivative, which then assembled in liposomes. The nanostructures were temperature-responsive and were used to encapsulate drugs such as doxorubicin and paclitaxel.<sup>172</sup> The myristoylation has also been used to generate valuable fatty-acid-modified elastin-like polypeptides (FAMES) which assembled into different nanostructures depending on the temperature.<sup>173</sup>

Finally, Fig. 3 presents some selected examples of protein and peptide functionalization using methodologies described in Table 1. More examples are given in the commented reviews. For instance, Francis *et al.* reported the modification of the N-terminus of native RNase (228) with 2-pyridine carboxaldehyde (PCA) derivatives (entry 5).<sup>151</sup>

The same group used the oxidation of aminophenols with ferricyanide (entries 6 and 7 in the Table 1) to label many different proteins, such as wild type lysozyme MS2, Lysozyme, RNase A, myoglobin, chymotrypsinogen and wild type Green Fluorescent Protein (GFP).<sup>3i,26a</sup> The N-terminal residues in these proteins were Ala (0% and 38% modification in wtMS2 and Lysozyme, respectively), Lys (30% for RNase), Gly (34% for myoglobin), Cys (42% for chymotrypsinogen) and Met (36% for wt GFP). Interestingly, when a proline was introduced into the N-terminal position of Green Fluorescent protein (compound 229) the levels of modification increased notably.<sup>3i,26a</sup> The oxidation was also employed to pegylate the enzyme creatine kinase by using PEG-derivatised aminophenols (50–60% modification).<sup>174</sup>

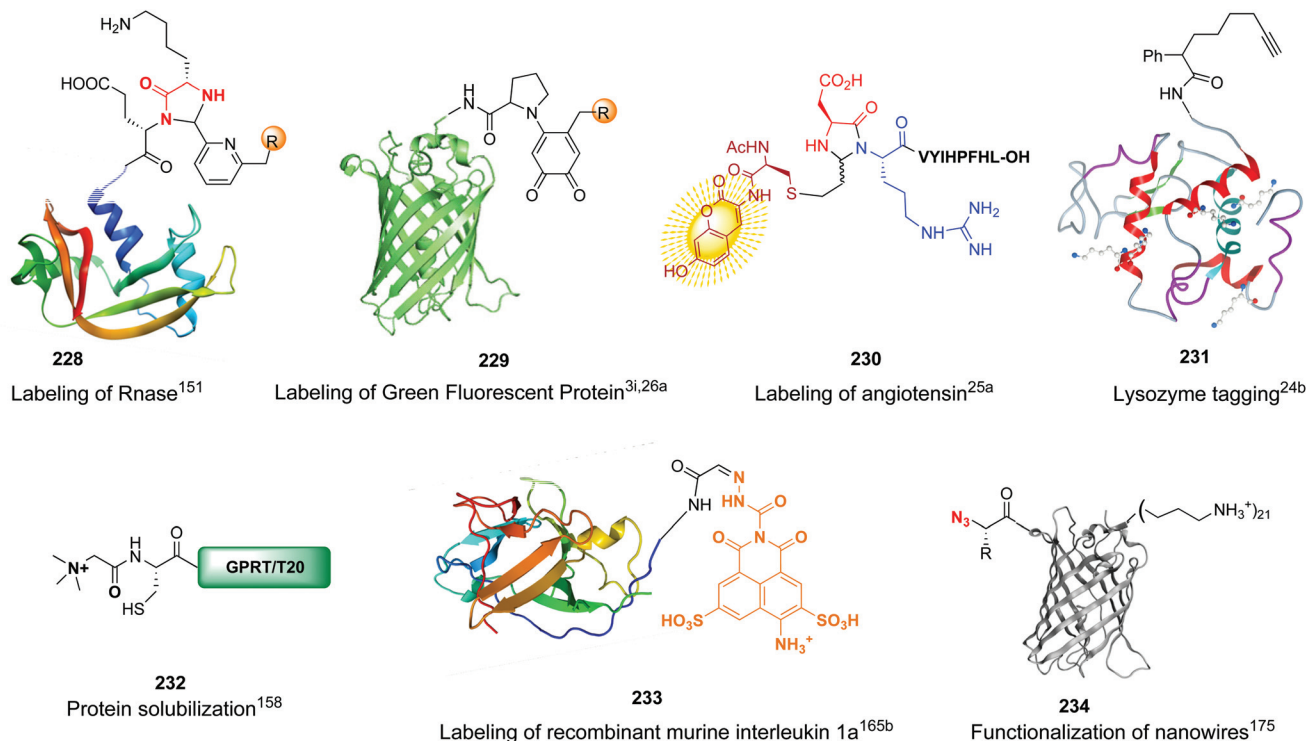
Ball *et al.* used the treatment with a boronate in the presence of ascorbic acid (entry 9)<sup>25a</sup> to label the blood-pressure regulating peptide angiotensin with a fluorophore (compound 230).

Che *et al.* labeled lysozyme (pdb: 2LYZ) in the N-terminal unit, in the presence of five lysines (compound 231) using ketenes (entry 11 in Table 1).<sup>24b</sup> This selective labeling was also used with insulin, RnaseA, BcArg and other peptides.<sup>24b</sup>

Tolbert *et al.* attached betaine to the N-terminal residue of bacterial xanthine-guanine phosphoribosyltransferase CG-GPRT (compound 232, entry 12 in Table 1), and antiHIV peptide CG-T20.<sup>158</sup> The two peptides have a high propensity to aggregation, but once modified with betaine, their solubility was greatly increased without loss of activity. Interestingly, the modified CG-T20 is an inhibitor of the aggregation of native CG-T20.

Geoghegan and Stroh used the oxidation of serine with sodium periodate (entry 17) to label recombinant murine interleukin 1a (pdb: 2KKI) with a Lucifer yellow dye (compound 233).<sup>165b</sup> Other applications of this reaction include PEGylation, protein conjugates, protein dendrimers, and lig-





**Fig. 3** Application of site-selective modifications at N-terminal positions to protein functionalization. The 3D structure of GFP is authored by Francis,<sup>3i,26a</sup> of Lysozyme by Che<sup>24b</sup> (reproduced with ACS permission), and that of nanowires by Meldal<sup>175</sup> (WILEY-VCH permission). Those of Rnase and interleukin 1a were created by Vossman<sup>176a</sup> and Boghog,<sup>176b</sup> respectively (public domain).

ation of protein fragments.<sup>3f,57</sup> Finally, Meldal used the method reported by van Hest (entry 18) to create nanowires (compound 234). Thus, the newly created azide was transformed using click chemistry.<sup>175</sup> The fast progress in this area will likely provide more exciting examples in the next years.

## 7. Conclusions

The site-selective modification of peptides has achieved many advances in the last years, and although most of them are reported for side-chain manipulation, promising methodologies have also appeared to modify the backbone and thus modulate the conformation, folding ability, physico-chemical and biological properties of the peptide. This review has addressed the advances in the modification of C $\alpha$ - and N-positions, from the use of “customizable units” to the residue-directed introduction of substituents. The site-selective modification of the peptide bond, *i.e.* the formation of thioamides or heterocycles, which alters backbone rigidity and ability to form hydrogen bonds or recognition by enzymes, was also described. In addition, not only the modifications in internal backbone positions, but also in the N- and C-termini were discussed.

The use of “customizable amino acid units”, which undergo cleavage of a certain C–X bond (X=H, C) and its replacement by a variety of other functions (generating new C–C,

C–N, C–O, C–P, C–S bonds, *etc.*) allow the efficient modification of the C $\alpha$ . Thus, glycine has been used by different groups to introduce an alkyl, allyl or aryl chain, in some cases with excellent stereoselectivity.

Photocatalytic methods have recently shown their promise to obtain a variety of such units with high stereoselectivity.

Glutamic acid has allowed the one-pot transformation of peptides with glutamic units into  $\alpha,\gamma$  hybrids. The scission of serine and threonine, followed by the addition of new chains, has generated interesting  $\alpha$ -alkyl and  $\alpha$ -arylglycines. It has also afforded  $\beta$ -substituted dehydroamino acids with excellent stereoselectivity. Since these amino acids can be reduced with chiral catalysts, this is an interesting way to study the role of normal L-units, and compare them with D-units and rigid dehydroamino acid residues.

Serine, threonine and alloserine have been used to create cyclic sulfamidates that were transformed into  $\alpha,\alpha$ -disubstituted amino acids. Then the rigid sulfamidate ring could be opened with nucleophiles.

The introduction of L- or D-hydroxyproline units (Hyp) which underwent scission of the C $_4$ –C $_5$  bond under very mild conditions, generated acyclic amino acids with a variety of  $\alpha$ -chains which retained the stereochemistry of the starting unit. Moreover, an *N,O*-acetal was generated, which could be manipulated independently from the  $\alpha$ -chain. The addition of nucleophiles under mild conditions afforded a variety of N-substituents, even bulky ones. Since peptide coupling of





*N*-alkylated residues is challenging and plagued by side reactions and low reaction rate, this route could offer a promising solution to introduce bulky *N*-alkyl residues at any desired position. A “doubly customizable” Hyp unit, that can be decarboxylated and cleaved at the C<sub>4</sub>–C<sub>5</sub> bond sequentially, with introduction of new chains/function at these positions, has recently been reported. Proline has also been developed as customizable unit, but in this case, the C<sub>5</sub>–N<sub>1</sub> bond is cleaved by oxidation with iron catalysts. Although these customizable units have been studied mainly in small peptides, the application of these methodologies to larger molecules should be possible, and is an open line for the future.

Dehydroamino acids are special types of “customizable units”. Due to the capto-dative character of their substituted double bonds, their reactivity is quite different from that of the other amino acids. They can undergo the addition of nucleophiles, radicals, dienes, and azacompounds. The addition of thiol derivatives, for instance, has generated a variety of labeled peptides and proteins. Dha can be readily generated from serine, threonine and cysteine. The latter is particularly useful, since it is relatively scarce in peptides and proteins, and thus biomolecules with just one Dha unit in accessible (“open”) positions are feasible in many cases. The development of write-read-erase systems is an application of Dha with important biological applications.

Dha is a scarce amino acid, and as a result, it has been used to selectively label certain positions. However, if there are several units in a peptide, it can be difficult to differentiate between them. A possible solution is to have “masked” Dha units as phenyl- or alkylselenocysteine and/or cysteines using orthogonal protecting groups. These units could be activated sequentially, by varying the reaction conditions.

Another important challenge is the introduction of *N*-substituents, since it allows modulation of the number of hydrogen bonds, and also creates steric interactions that can strongly influence the secondary structure of the peptide, its solubility and aggregation properties. In addition to the “customizable units” commented before, glycine and alanine units with removable *N*-substituents have been used to disrupt structures that were aggregation-prone or presented other undesired properties. After the peptide was completed, the substituent was cleaved and the desired challenging peptide was released in high purity. Difficult  $\beta$ -amyloid and polyalanine sequences were completed this way.

An outstanding advance in this area was the His-directed *N*-alkylation of adjacent units. Since histidine is a relatively scarce amino acid, a peptide can often be monolabeled accurately. The development of Pyr-His “tags”, which are extremely rare in natural proteins and highly reactive, also affords promise for selective protein labeling. Moreover, the recent development of Red-shift backbone photocaging systems will allow the “marking” and “unmarking” of proteins and the modulation of their functions (active-unactive).

The selective labeling of C- and N-terminal positions is quite useful. C-Terminal positions have different pK<sub>a</sub> with respect to glutamic and aspartic units, which allows differentiation of

these residues. Something similar happens with the N-terminal units and lysine, arginine and ornithine. Therefore, a variety of methods have been used to differentiate them. The selective oxidation of N-terminal units to glyoxalate followed by amination or other functionalizations is also remarkable. The oxidation of aminophenols with ferricyanide offers a mild, effective method to label large proteins at the N-terminus.

The decarboxylation of C-terminal units using photocatalytic methods has also found application in complex protein functionalization. With smaller peptides, the decarboxylation-alkylation of C-terminal “customizable units” has allowed the formation of terminal amino acid analogues, and thus  $\alpha,\beta$ - and  $\alpha,\gamma$ -hybrids.

The site-selective modification of the peptidic bond, for instance by formation of thioamides or heterocyclic rings has also been highlighted. The use of tryptophan to obtain oxazole-indole systems displaying high fluorescence is an example of the previous functionalization of the lateral chain to produce then a cyclization involving the peptide backbone. These remarkable transformations would be probably expanded in a future.

The high regio- and stereoselectivity achieved by enzymes is commented along the review. Many are quite selective, and their exquisite selectivity is at the cost of substrate limitation. But some enzymes have been reported that are promiscuous with respect to the whole substrate, recognizing a certain motif in different molecules. This is the case of enzymes such as sortase or butelase1 that recognize specific sequences in the peptide or protein and make modifications at these points. Since the discovery and engineering of new enzymes advances at a rapid pace the range of possible reactions will likely increase in the next future.

In conclusion, the site-selective backbone modification is a rapidly developing topic, with continuous promising incorporations to the synthetic toolbox. The ability to selectively modify the peptide backbone will be crucial to understand peptide conformation, folding and other physicochemical properties, and their biological action. This understanding will facilitate the design of tailor-made peptides for use in a variety of fields, such as biomedicine and materials science.

## Conflicts of interest

There are no conflicts to declare.

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