Advances in Fmoc solid-phase peptide synthesis

Raymond Behrendt, a Peter White b and John Offer c*

Today, Fmoc SPPS is the method of choice for peptide synthesis. Very-high-quality Fmoc building blocks are available at low cost because of the economies of scale arising from current multiton production of therapeutic peptides by Fmoc SPPS. Many modified derivatives are commercially available as Fmoc building blocks, making synthetic access to a broad range of peptide derivatives straightforward. The number of synthetic peptides entering clinical trials has grown continuously over the last decade, and recent advances in the Fmoc SPPS technology are a response to the growing demand from medicinal chemistry and pharmacology. Improvements are being continually reported for peptide quality, synthesis time and novel synthetic targets. Topical peptide research has contributed to a continuous improvement and expansion of Fmoc SPPS applications. © 2015 The Authors. Journal of Peptide Science published by European Peptide Society and John Wiley & Sons, Ltd.

Introduction

The success of peptide drugs, notably glucagon-like peptide 1 receptor agonists, and a promising pipeline of peptide drugs has renewed interest in synthetic peptides [1,2]. Additionally, the rapidly emerging field of peptide-based biomaterials has further stimulated demand [3]. The majority of synthetic peptides are now prepared by Fmoc solid-phase peptide synthesis (SPPS) [4]. Classical t-butyloxycarbonyl (Boc) SPPS is now generally only used for specialist applications. Initially, the success of the Fmoc chemistry was due to its rapid adoption by non-chemists as biologists realised they could quickly prepare peptides suitable for antibody production using inexpensive machines and avoid the use of anhydrous hydrogen fluoride (HF) [5]. Fmoc SPPS was easy to automate because there was no need for corrosive TFA in the synthetic cycles and because deprotection released a fluorene group with strong UV absorption properties that gave a useful indicator of synthesis success [6]. For peptide chemists themselves, Fmoc chemistry provided a solution to the previously limiting conditions of the Boc method as the deprotection conditions were compatible with modified peptides, such as phosphorylated and glycosylated peptides and for peptide libraries [7]. The concern with the Boc technique had always been the lack of complete differentiation in the reaction conditions for cleavage of the Boc group and semipermanent side-chain protection. The iterative use of TFA could cleave small amounts of the side-chain protecting groups at each cycle and cause progressive loss of peptide from the polymer support. In contrast, Fmoc SPPS provided an orthogonal combination of temporary and permanent protecting groups. Fmoc belongs to a set of urethane protecting groups including the benzyl carbamate (benzyloxycarbonyl) and Boc protecting groups that suppress racemisation during activation and coupling. Carpio and Han introduced the Fmoc group for solution chemistry, but it proved unsuitable [8,9]. The initial cleavage product, dibenzofulvene, is reactive and can reattach to the liberated amine or be potentially difficult to separate from the product, in contrast to Boc where the deprotection product, butylene, is volatile. It was notable for its exceptional liability to bases, particularly secondary amines. When screened alongside several other base-labile candidates for its application to solid phase, the Fmoc group found its métier, as on the solid support, the dibenzofulvene and any associated adducts could be simply washed away [10,11]. Furthermore, the release of the Fmoc group gave a unique method to monitor deprotection [12].

There have been considerable advances in the length of peptides synthesised. Partly, this is a consequence of the improvements in purity of the Fmoc building blocks. Mostly, however, it has been due to the success of applying pseudoprolines [13,14] and backbone protection [15] to the synthesis of long peptides overcoming the difficult sequence problem [16]. Although the figure of around 50 amino acids is often given in publications as the average target
that can be routinely synthesised, in practice, this figure is meaningless as many much shorter sequences are extremely problematic and synthetic success is not guaranteed.

Whilst the combination of side-chain protecting groups used by peptide chemists for routine Fmoc-tert-butyl (tBu) chemistry has remained largely unchanged for more than 15 years (Table 1), many recognise that for some amino acids, particularly arginine, asparagine, aspartic acid, histidine and cysteine, the choice is suboptimal. However, the adoption of new and superior protecting groups by peptide chemists has been slow mainly because the standard ones are produced cheaply and ultra pure in industrial scales for good manufacturing practice peptide production. In this review, we will highlight some new developments, with the hope that by bringing their benefits to a wider audience, we will encourage their take-up by peptide chemists and help stimulate innovation in the development of basic peptide synthesis reagents. For further information on the origins of the methodology and current practice, a number of excellent reviews are available [7,17–21].

**Developments in N°-Fmoc amino acid derivatives**

**Purity of N°-Fmoc amino acids**

The industrialisation and regulation of Fmoc-protected amino acid derivatives have led to a significant improvement in the quality of the 20 standard Fmoc-protected amino acid building blocks [29]. Most Fmoc amino acids are now available in remarkably high RP-HPLC purity of >99% although a number of well-documented side reactions can occur during the introduction of the Fmoc group to the N°-amine of an amino acid. The most frequently encountered is the Lossen-type rearrangement, which leads to the formation of Fmoc-β-Ala-OH and Fmoc-β-Ala-Xaa-OH using 9-fluorenylmethoxy carbonyl N-hydroxysuccinimide [30,31], and the unwanted carboxyl activation, which generates the Fmoc-Xaa-Xaa-OH dipeptide using 9-fluorenylmethyl chlorofluoromate [32,33]. These impurities will be incorporated into the growing peptide chain. Therefore, it is important when using HPLC analysis for quality control of the Fmoc building block that these impurities do not co-elute.

To prevent this, an intermediate silylation with chlorotrimethylsilane has been proposed to protect the carboxylic acid and prevent amino acid oligomerisation during Fmoc protection [34]. The requirements for improved oxime base reagents have been developed for the clean introduction of the N°-Fmoc protecting group aiming to replace the N-hydroxysuccinimide activation [35,36].

The International Conference on Harmonisation for standards of active pharmaceutical ingredient production (Q11) requires optical purity, acetic acid content and free amine content to be specified of the amino acids [37]. Enantiomeric purity can be quantified greater than 99.9% by gas chromatography (GC)-MS [38].

The presence of acetic acid in Fmoc-amino acid derivatives is a serious problem, as it cannot be detected by RP-HPLC and causes permanent capping. Some commercial preparation of trifunctional amino acids like Fmoc-Arg[2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf)]-OH and Fmoc-Asn/Asn[triphenylmethyl (Trt)]-OH can contain significant amounts. As acetic acid has an Mr of only 60, negligible amounts lead to significant chain terminations during peptide assembly. Levels <0.02% are required for a clean SPPS.

Another consideration is the content of free amino acid in an Fmoc-amino acid derivative. This can result in incorporation of multiple copies of the target amino acid into the peptide chain. Furthermore, free amino acid can compromise long-term storage as traces of the free amine promote autocatalytic Fmoc cleavage. However, quantification of the free amine today is problematic. Suppliers provide either a GC-based method with a limit of detection of 0.2% or a semiquantitative TLC-ninhydrin assay.
Advances in side-chain protection

Aspartic acid. The most serious side reaction during Fmoc chemistry is aspartimide formation (Scheme 1) [39]. It is caused by exposure of the peptide sequence containing aspartic acid to strong base. Aspartimide formation is therefore a major problem for the synthesis of long peptides and sequences containing multiple aspartic acid residues. Aspartimide formation is particularly pernicious as it can lead to the formation of nine different by-products, some of which will co-elute with the target peptide. Attack by water yields the undesired α-/β-aspartyl peptides in a ratio of 3:1 to the α-aspartyl peptide [40–42]. Ring opening by piperidine gives a mixture of α-/α-piperidides and α-/β-piperidides. Ring opening by amino groups leads to the formation of dipeptides or cyclic peptides [43]. In most cases, α-piperidides and β-piperidides are easily separated from the target peptide by RP-HPLC; however, resolution of the epimerised α-aspartyl peptide is very difficult or impossible [44]. The extent of aspartimide formation is highly dependent on the nature of the amino acid following the aspartyl residue. Sequences that are particularly aspartimide prone are -Asp-Gly-, -Asp-Asp-, -Asp-Asn-, -Asp-Arg-, Asp-Thr- and -Asp-Cys- [45,46] with Asp-Gly being the worst case (Table 2) [47]. Ser and Thr need to be side chain protected [45]. The importance of the aspartyl side-chain protection has been demonstrated in many studies [48]. Aspartimide formation was exacerbated by the use of less bulky protecting groups: ODie > OMpe > OtBu > O-1-adamantyl [52], trityl-based [51] > OBzl, OAll [48,51] and O-phenacyl [53].

Moreover, aspartimide formation also depends on the peptide conformation [54]. The influence of conformation on aspartimide formation has been exploited by the use of pseudoproline dipeptides immediately before an aspartimide-prone Asp(OAll) residue that enabled the synthesis of N-glycopeptides containing the Asn-Xaa-Ser/Thr sequence [55,56].

Figure 1. Structures of novel aspartate protections Die (1,1-diisopropyl-ethyl) 1, Epe (3-ethyl-3-pentyl) 2 and Bno (5-butyl-5-nonyl) 3.

Recently, novel derivatives have been developed to directly address aspartimide formation [57]. These derivatives incorporate trialkylcarbinol-based esters Epe 2 and Bno 3 (Figure 1) which appear to provide excellent protection against this side reaction, as illustrated by the data presented in Table 3.

The addition of acidic modifiers has long been known to reduce the problem of aspartimide formation in Fmoc SPPS [42]. Recently, Subirós-Funosas et al. [58] have shown that the addition of 1 M ethyl cyano(hydroxyimino) acetate (Oxyma Pure) in 20% piperidine in dimethylformamide (DMF) reduces the levels of aspartimide-related impurities. In the case of the Asp(OtBu)-Gly-containing test peptide, fragment 1-6 scorpion toxin II, impurities are reduced from 44% to 15% for a 6 + 6-h treatment [58].

The only strategy, however, that currently offers complete protection from aspartimide formation is backbone protection of the aspartyl α-carboxyamide bond [47,48,59–61]. However, this comes with its own associated problems because of difficulties with acylating the secondary amine formed by introduction of the backbone protection and is generally only routinely used for the synthesis of long peptides.

Table 2. Aspartimide sensitive sequences, Asp-Xaa

| Xaa | Degree of aspartimide formation |
|-----|---------------------------------|
| Gly | ++++++                          |
| Asn(Trt) | +++                         |
| Asp(OtBu) | ++                           |
| Arg(Pbf) | ++                           |
| Ser/Thr | ++                           |
| Cys(Acm) | ++                           |
| Cys(Trt) | +                            |
| Thr(tBu) | +                            |
| Ala | +                             |

From highly sensitive (+++++) to weakly sensitive (+).

Table 3. Composition of crude products obtained from peptide resins VKDXYI after treatment with 20% piperidine in DMF at room temperature

| Asp(OR) R  | Aspartimide per cycle for X = Asn | d-Asp for X = Asn | Aspartimide per cycle for X = Arg | d-Asp for X = Arg |
|------------|---------------------------------|------------------|---------------------------------|------------------|
| tBu | 1.65 (9.1) (1.24) (25.1) | 0.49 (4.2) (0.4) (11.0) | 0.19 (2.2) (0.13) (3.1) | 0.06 (0.9) (0.06) (1.4) |
| Mpe | 0.06 (0.9) (0.06) (1.4) | 0.19 (2.2) (0.13) (3.1) | 0.49 (4.2) (0.4) (11.0) | 1.65 (9.1) (1.24) (25.1) |
| Epe | 0.19 (2.2) (0.13) (3.1) | 0.06 (0.9) (0.06) (1.4) | 0.49 (4.2) (0.4) (11.0) | 1.65 (9.1) (1.24) (25.1) |
| Bno | 0.06 (0.9) (0.06) (1.4) | 0.19 (2.2) (0.13) (3.1) | 0.49 (4.2) (0.4) (11.0) | 1.65 (9.1) (1.24) (25.1) |

aCalculation by first order decay.
bBased on 10-min treatments [57].

Scheme 1. Aspartimide formation.
of peptides containing Asp-Gly, for which a number of building blocks are commercially available: Fmoc-Asp(OtBu)-(Dmb)Gly-OH (Dmb 11), Fmoc-(Dmb)Gly-OH and Fmoc-(FmocHmb)Gly-OH (Hmb 12) [15,62] (Table 5).

Arginine. 4-Methoxy-2,3,6-methylbenzenesulfonyl was initially the standard protecting group for arginine but frequently required overnight or longer deprotection with TFA/thioanisole cocktails. The 2,2,5,7,8-pentamethylchromanyl-6-sulfonyl (Pmc) protecting group, introduced in 1987, showed a dramatic reduction in deprotection time [63,64]. The unprotected but conceptually similar Pbf has subsequently become the standard protection; it was introduced in 1993 and was reported to be slightly more labile than Pmc [22]. Removal of Pbf is usually complete within 1–2 h; however, with peptides containing multiple arginine residues, extended cleavage times are still required. A new derivative, Fmoc-Arg(MIS)-OH, with improved deprotection kinetics compared with Fmoc-Arg(Pbf)-OH has been introduced. The MIS 4 group (1,2-dimethylindole-3-sulfonyl), (Figure 2) was completely cleaved from a model peptide with 1:1 TFA/DCM in 30 min compared with 4% of the peptide protected with Pbf [65]. The apparent drawback of this derivative is the release of dimethylindole-3-sulfonic acid co-precipitates in ether with the product peptide when water is used in the scavenger cocktail.

Surprisingly, dibenzo[b]sulfanyl 5 and dibenzo[b]sulfonyl 6 have never found broad application [66] despite being removed under the mildest reported conditions whilst reducing δ-lactam [67,68] and ornithine formation [69] (Figure 2).

Cysteine. Cysteine racemisation occurs with base-mediated activation methods, such as those using phosphonium or uronium reagents [70,71]. It is particularly high with microwave heating and preactivation [72]. It can be avoided in all these cases by using carbodiimide activation [73,74]. Several alternatives to the standard trityl protecting group have been reexamined in studies to overcome cysteine racemisation [75] using the model peptide H-Gly-Cys-Phe-NH2 (Table 4). Couplings were performed under basic conditions using HCTU/6-Cl-HOBt/DIPEA (4/4/8) activation. In the case of MBom 9 (Figure 3) was most effective, reducing the formation of D-Cys to 0.4%. Nevertheless, the preparation of MBom 9′, Dpm (diphenylmethane) 7, Ddm (4,4′-diphenylmethane) 8, MBom (4-methoxybenzyl-oxymethyl) 9 and THP (2-tetrahydropyranyl) 10.

Historically, histidine has been a notoriously race- misation-prone residue. Histidine is conventionally introduced using Fmoc-His(1-Trt)-OH without special precautions. However, this residue can undergo significant racemisation during coupling, particularly when base-mediated couplings are used or the reaction is slow. The racemisation-prone character of histidine is caused by the imidazole π-nitrogen promoting the enolisation of histidine active esters [81–84]. Therefore, the most effective approach to preserving the chiral integrity of histidine is to employ imidazole π-nitrogen protection. Jones and co-workers were the first to describe such protection introducing the N°-benzoxymethyl (Bom) group, which is used in Boc chemistry [85]. Later, they introduced the analogous derivative, Fmoc-His(Bum)-OH, for Fmoc SPPS based on the TFA-labile t-butoxymethyl (Bum) group [86]. Others also described N°-1-adamantylmethyl protection [87].

This topic has been revisited with the introduction of Fmoc-His(MBom)-OH [78,88]. Loss of histidine chiral integrity was compared for Fmoc-His(Trt)-OH and Fmoc-His(MBom)-OH using HCTU/6-Cl-HOBt/DIPEA (4/4/8) activation. In the case of Fmoc-His(Trt)-OH, the level of racemisation increased with the preactivation time from 1% without preactivation to 7.8% with 5 min of preactivation. In contrast Fmoc-His(MBom)-OH reduced epimerisation to 0.3% with 5 min preactivation. Microwave heating at 80°C and N°-Trt protection gave racemisation of 16.6% whereas the N°-MBom group gave 0.8%. Nevertheless, the preparation of Fmoc-His(MBom)-OH remains expensive, and as previously mentioned, the MBom group is associated with a number of undesirable side reactions.

Generally, acidic coupling conditions, such as disopropyl carbodiimide (DIC)/HOBT at ambient temperature, are sufficient to maintain histidine stereochemistry even with Fmoc-His(Trt)-OH [74]. However, histidine racemisation will always be a risk with the

---

**Table 4. Influence of cysteine side-chain protecting groups on cysteine racemisation using basic activation conditions and 1-min preactivation [75]**

| Conditions         | Racemisation [%] (D-Cys/L-Cys peptide) |
|--------------------|----------------------------------------|
|                    | Trt | Dpm | Ddm | Bl | Mob | Tmob | MBom |
| Conventional SPPS  | 8.0 | 1.2 | 0.8 | 5.3 | 1.7 | 0.6  | 0.4  |
| 50°C               | 10.9| 3.0 | 1.8 | n.d.| n.d.| n.d. | 0.8  |
| 80°C               | 26.6| 4.5 | 2.5 | n.d.| n.d.| n.d. | 1.3  |

---

![Figure 2. Structures of the novel arginine protecting groups MIS (1,2-dimethylindole-3-sulphonyl) 4, and dibenzo[b]sulfonyl 5, dibenzo[b]sulfonyl 6.](image-url)
current range of N'-protected derivatives. The search of a cost-efficient solution for this problem remains unresolved.

**Enhancing Fmoc SPPS efficiency**

The greatest problem of peptide chemistry is peptide insolubility, of either unprotected peptides in aqueous buffer [89–92] or fully protected peptides in organic solvents [89,93,94]. This obstacle has prevented the development of many areas of peptide chemistry: for example, a general method for the assembly of protected peptide fragments.

Poor solubility was recognised from the very beginning of SPPS to be the direct cause of synthetic problems [95]. Furthermore, it can complicate or prevent chemical characterisation. Although poor solubility can often be anticipated, as it is predominantly associated with hydrophobic sequences, prediction remains difficult [96,97]. Chemists have dealt with poor peptide solubility by further functionalising the peptide with hydrophilic groups such as polyarginine or polylysine to enhance solubility. Notable results have been achieved with this approach including the synthesis of membrane proteins [98,99] and the notoriously insoluble insulin A-chain [100].

The presence of N-alkylated amino acids such as sarcosine and proline at regular intervals along a peptide sequence has long been known to guarantee high peptide solubility [90,96]. This improvement in peptide solubility is directly related to maintaining an open-chain, disordered conformation by removing hydrogen bonds. The effect occurs on the solid phase where proline residues, evenly spaced throughout a sequence, assist peptide synthesis, presumably again by maintaining a fully solvated, disordered conformation [101]. Modifying the peptide backbone promises a more systematic approach to peptide solubility. However, development of this was slow because many peptide chemists reasoned that adding hydrophobic groups to the backbone would decrease solubility of the peptide, whereas the opposite effect, a dramatic increase is observed.

The possibility of using a reversible group (Table 5) for temporarily increasing peptide solubility was first suggested by Weygand and co-workers using 2,4-dimethoxybenzyl (Dmb) [102]. The optimal distancing of the backbone substitution (every six residues) was identified by Narita and co-workers [89,103]. Sheppard proposed using backbone protection as a strategy for overcoming difficult sequences [104] as the difficult sequence phenomenon could be considered as the reappearance of poor peptide solubility on resin [16]. Therefore, installing backbone protection before the onset of aggregation and thereafter at every six residues would prevent the onset of difficult sequences. Adding a substituent to the amide bond would prevent its participation in intermolecular hydrogen bonding and prevent interchain aggregation [96]. The use of Dmb introduced a new problem: the problematic acylation of hindered secondary amines [105]. Nevertheless, DmbGly dipeptide building blocks are commercially available and have found widespread use against difficult sequences and aspartimide formation [15]. The Sheppard group overcame the obstacle of steric hindrance with the introduction of Hmb [12]. Acylation could occur at the accessible 2-hydroxy position, and subsequent intramolecular transfer assists subsequent acylation of the secondary amine [106]. Hmb protection entered widespread use; however, it had a major drawback: the ease of acylation of Hmb-protected amines differs between residues, and the reaction requires DCM as solvent with lengthy (overnight) coupling times. However, an unintended consequence of using Hmb, but one that gave it a great advantage over alternative methods of backbone protection, was its stability to TFA treatment when the 2-hydroxyl group was acetylated [107]. This was found useful for the purification on HPLC of β-amyloid, as the deprotected peptide retaining backbone protection was much easier to handle [108,109]. A wide variety of other backbone protections have been proposed including dicyclopentylmethyl [110] and other structures [111].

The problem of Hmb is the lengthy time required to effect complete O,N-acyl transfer. This can be accelerated by introducing electron-withdrawing groups to the benzene ring. In one approach (Scheme 2, Hmsb [14]), a sulfone group was positioned para to the 2-hydroxyl position, which generates an active ester when acylated, that is favourably positioned for intramolecular acyl transfer [114,115,118]. Mild reduction to the sulfide makes the group labile to acidolysis, an approach also employed in the Mmsb group [16] [117]. Alewood and co-workers achieved similar results with a photolabile group, Hnb [15]. Acylation generates an activated nitrophenol ester in situ and accelerates acyl transfer. Hmsb, Mmsb and Hnb backbone protection could be retained on the side-chain-deprotected peptides for their solubilising properties [116].

An important advance was the demonstration of on-resin reduction of Hmb onto the peptide resin [112]. The reductive amination was quantitative with a single equivalent of the salicylaldehyde precursor. This was also successfully applied to Hnb and Hmsb, simplifying the introduction of the backbone protection and enabling automation.

One limitation of the benzyl-type protection is the formation of long-lived benzyl cations during deprotection that can react with the peptide, a particular concern with tryptophan. Additionally, on some long peptides and near-basic residues, prolonged acidolysis is required for removal.

The most popular approach to date is unrelated to classical benzyl-type amide substitution but instead exploits dimethyloxazolidine derivatives of serine or threonine, referred to as pseudoproline dipeptides [13]. The related cysteine equivalent, the thiazolidine had been used by the Kemp group as a protecting group for ligation [119] and had been identified as being particularly resilient to epimerisation [120]. Mutter used a different rationale from that used for Hmb and hypothesised that by ‘kinking’ the peptide, they broke structure and made the peptides more soluble. The key to their popularity is that no special conditions are required for installation of the pseudoproline dipeptide, two residues are incorporated in a single step and TFA deprotection is uncomplicated [14,113]. Unsurprisingly, many of the applications of pseudoprolines mirror those of Dmb-derived backbone protection, such as improved cyclisation, aspartimide suppression and epimerisation-free segment coupling. However, they are limited to sequences containing serine and threonine at convenient positions. Impressively long peptides have been synthesised using pseudoprolines, notably fas [13], ubiquitin [62] and D2 domain of vascular endothelial growth factor receptor 1 [121].

Another approach for overcoming chain association is the O-acyl isopeptide method (Scheme 3) [122–124]. The desired peptide is first synthesised as a depsipeptide derived from a serine or threonine residue. Such depsipeptide analogues of aggregation-prone peptides are more soluble and consequently more easily purified. Once purified, the depsipeptide is converted to the native form by adjusting the pH to 7.4, when spontaneous O-acyl to N-acyl migration occurs (Scheme 3) [125,126].

It has been predicted that the future of synthetic protein chemistry would come from a combination of native chemical ligation
### Table 5. Backbone amide protection in use in Fmoc SPPS

| Introduction | Acylation | Removal | Safety catch | Reference |
|--------------|-----------|---------|--------------|-----------|
| Dmb 11       | Automated SPPS | FmocDmbGly building block | Standard coupling to DmbGly; all others are very sterically hindered | TFA | No | Weygand et al., 1966 [102]; Blaakmeer et al., 1991 [105]; Cardona et al., 2008 [15] |
| Hmb 12       | Automated SPPS | FmocHmbXaa building block | Standard coupling to HmbGly; for all others, FmocAA symmetric anhydride in DCM onto HmbAA unless both are beta-branched | TFA | Yes, acetylated Hmb is TFA resistant [107] | Johnson et al., 1993 [106]; Ede et al., 1996 [112] |
| Pseudoproline 13 | Automated SPPS | Dipeptide building block; limited to XaaSer or XaaThr | Standard coupling | TFA | No | Wöhr et al., 1995 [14,113] |
| Hmsb 14      | Building block | Coupling with standard conditions to HmsbAla and HmsbLeu demonstrated | NH₄I/TFA; TmsBr/EDT/TFA/ thioanisole | Yes sulfone/sulfide safety catch | Offer et al., 1997 [114]; Abdel-Aal et al., 2014 [115] |
| Hnb 15       | On-resin reduction | Intramolecular acyl transfer assists subsequent acylation; good tolerance for a range of residues | hν | Yes, UV irradiation orthogonal to acidolysis | Miranda et al., 2000 [116] |
| Mmsb 16      | Fmoc building block | Repeat couplings required to quantitatively acylate | NH₄I/TFA | Yes, sulfone/sulfide safety catch | Paradis-Bas et al., 2014 [117] |

### Scheme 2. Principle of the safety-catch backbone protecting groups using Hmsb: (i) 1.1 eq. of the corresponding salicylaldehyde in DMF; (ii) NaBH₄, DMF; (iii) continuing SPPS; (iv) standard TFA cleavage; (v) NH₄I/DMS reduction; (vi) standard TFA cleavage.
and backbone protection [127]. Recent work supports this. The first application of backbone protection in ligation was reported for the synthesis of bovine pancreatic trypsin inhibitor with the unprotected peptide thioester fragment possessing an Hmb [128]. Liu and co-workers reported the synthesis of the M2 ion channel using a version of Hmb showing greatly improved solubility in aqueous buffer [129]. Noteworthy in this context is the Ag⁺-promoted thioester ligation of Tim-3 using the related O-acyl isopeptide approach to improve segment solubility [130]. In contrast to pseudoprolines, the acyl isopeptide analogues can be retained after side-chain deprotection. Any general method of backbone protection must be easy to install, easy to acylate and simple to remove; it is also desirable that it can be retained after the rest of the peptide has been deprotected. The most important consideration for it to be practically useful and widely used is, however, cost.

Post-translational modifications

Understanding the role of protein post-translational modifications (PTMs) in cell signalling, gene expression, and protein processing and translocation is of enormous interest [131–134]. Ready access to peptides containing post-translational modified amino acid residues, for use as probes, for use as inhibitors or for raising antibodies against protein PTMs, has been crucial for advances in these areas.

Fmoc SPPS is generally the method of choice for the synthesis of such modified peptides because many of the most important PTMs, such as glycosylation and phosphorylation, are not stable to HF cleavage conditions. The milder chemistry of the Fmoc method allows almost all PTMs to be introduced during chain elongation using the appropriate preformed protected amino acid building blocks. The most notable exceptions are ubiquitylation and farnesylation, which will be discussed in the following.

Table 6 contains the most frequently utilised building blocks for the synthesis of peptides bearing the most commonly encountered PTMs.

Phosphorylation

Reversible phosphorylation of proteins is involved in signal transduction and on/off control of enzymes [160]. In eukaryotes, phosphorylation occurs on serine, threonine, tyrosine and histidine [161] residues, whereas in prokaryotes, it is also observed on lysine and arginine [162].

The synthesis of peptides containing phosphorylated serine, threonine and tyrosine is well established and has been reviewed [163]. There are two approaches: postsynthetic global phosphorylation and introduction of a preformed phosphoamino acid building block. Global phosphorylation (Scheme 4) involves selective phosphitylation of the appropriate hydroxylamino acid on the solid phase, with a protected phosphoramidite, followed by oxidation of the resultant P(III) triester to the P(V) triester. The favoured approach utilises dibenzyl-N,N-diisopropylphosphoramidite with oxidation using anhydrous t-butyl hydroperoxide [164].

This method has largely been superseded by the building block approach, owing to its greater convenience [165]. For introduction of phosphotyrosine, free phosphate, phosphodiester and triester, phosphoamidate-based building blocks are available (Table 6). Fmoc-Tyr(PO(OBzl)OH)-OH [140] is seldom used owing to issues with pyrophosphate formation between adjacent Tyr(PO(OBzl)OH) residues [166,167]. Fmoc-Tyr PO(Brz)OH)-OH [137] is the most frequently employed reagent; however, the acidic hydroxyl group causes problems during coupling reactions [135], as piperidine counterion to the phosphate consumes activated amino acid derivative, necessitating an additional equivalent of amino acid to be employed for every monobenzyl phosphate introduced.

In contrast, Fmoc-Tyr(PO(2Bz)2)OH [138,168,169] couples smoothly but is converted into Fmoc-Tyr PO(Bz)OH)-OH by piperidine and so suffers from the same issues as the monoproTECTED derivative during subsequent chain extension [170].

Fmoc-Tyr[PO(NMe2)2]-OH [141,171] presents no problems during peptide assembly; however, regeneration of phosphotyrosine from the phosphodiamicidate requires overnight treatment with TFA containing 10% water [141].

With phosphoserine and phosphothreonine, the situation is further complicated by the propensity of their phosphotriesters to undergo β-elimination under basic conditions [172]. For these amino acids, it is necessary to use a partially protected phosphodiester for their introduction. The favoured derivatives are monobenzyl esters, Fmoc-Ser PO(Bz)OH)-OH [173,174] and Fmoc-Thr PO(2Bz)OH)-OH [137,174]. The acidic phosphate is thought to become deprotonated during Fmoc deprotection, thereby inhibiting deprotonation of the amino acid α-proton and subsequent β-elimination. In practice, these derivatives do not offer complete protection from elimination, particularly in the case of phosphoserine and during microwave-accelerated synthesis [173,175].

Problems can occur with these benzyl-protected derivatives during the TFA cleavage reaction as a result of alklylation of sensitive residues by the released benzyl carbocation.

Nonhydrolysable analogues of all three O-phosphoamino acids have been developed (Table 7), to aid in the preparation of phosphopeptide tools for use as phosphatase inhibitors and as antigens for raising antibodies. However, at present, only analogues of phosphotyrosine are commercially available: Fmoc-Pmp-OH [144], Fmoc-Fpmp-Oh [145,176] and Fmoc-Ppa(benzyl)-OH [143].

The former isostere appears to be a poor substitute for phosphotyrosine, as its use often leads to a significant reduction in biological activity, which is interpreted as being caused by the lack.
### Table 6. Commercially available building blocking blocks for introduction of principle PTMs

| PTM                         | Introduction                      | Comments                                                                 | Reference          |
|-----------------------------|-----------------------------------|--------------------------------------------------------------------------|--------------------|
| Phosphorylation Ser/Thr     | Fmoc-Ser(PO(OBzl)OH)-OH **17**    | Best coupled using imminium-based reagents [135]                        | Wakamiya et al.,   |
|                             |                                   |                                                                         | 1994 [136]         |
|                             | Fmoc-Thr(PO(OBzl)OH)-OH **18**    | Best coupled using imminium-based reagents [135]                        | White and Beythien,|
|                             |                                   |                                                                         | 1996 [137]         |
|                             | Fmoc-Tyr(PO(OBzl)2)-OH **20**     | Compatible with all coupling methods; monodemethylated by piperidine; requires TMSBr/TFA for side-chain deprotection | Kitas et al.,      |
|                             |                                   |                                                                         | 1989 [138]         |
|                             | Fmoc-Tyr(PO(OBzl)2)-OH **21**     | Best coupled using imminium-based reagents [154]                        | White and Beythien,|
|                             |                                   |                                                                         | 1996 [137]         |
|                             | Fmoc-Tyr(PO(OBzl)2)-OH **21**     | Compatible with all coupling methods; monodebenzylated by piperidine   | Perich and Reynolds,|
|                             |                                   |                                                                         | 1991 [139]         |
| PTM | Introduction | Comments | Reference |
|-----|--------------|----------|-----------|
| Fmoc-Tyr(PO₃H₂)-OH | Best coupled using imminium-based reagents; issues with pyrophosphate formation [143] | Ottinger et al., 1993 [140] |
| Fmoc-Tyr(PO(NMe₂)₂)-OH | Compatible with all coupling methods; deprotected with TFA/water (9:1) | Chao et al., 1995 [141] |
| Fmoc-Tyr(PO(OMDPE)₂)-OH | Compatible with all coupling methods; MDPSE groups removed with TFA | Chao et al., 1994 [142] |
| Fmoc-Ppa(Bzl)-OH | Best coupled using imminium-based reagents | Chauhan et al., 2007 [143] |
| Fmoc-Pmp-OH | Best introduced with HATU/DIPEA coupling | Marseigne et al., 1988 [144] |
| Fmoc-F₂Pmp-OH | Best introduced with HATU/DIPEA coupling | Gordeev et al., 1994 [145] |
| PTM         | Introduction | Comments                                      | Reference       |
|-------------|--------------|-----------------------------------------------|-----------------|
| Sulfation Tyr | Fmoc-Tyr(SO$_3$P)-OH 28 | Neopentyl ester is stable to TFA; cleaved with sodium azide/DMSO or aq. ammonium acetate | Simpson and Widlanski, 2006 [146, 147] |
|             | Fmoc-Tyr(SO$_3$DCV)-OH 29 | DCV ester stable to TFA; DCV cleaved by Zn/ AcOH reduction | Ali and Taylor, 2009 [148,149] |
| Methylation Arg | Fmoc-Arg(Me,Pbf)-OH 30 | For introduction of monomethyl arginine | White, 2006 [150] |
|             | Fmoc-ADMA(Pbf)-OH 31 | For introduction of asymmetric dimethylarginine | White et al., 2006 [150] |
|             | Fmoc-SDMA(Boc$_2$)-ONa 32 | For introduction of symmetric dimethylarginine | White et al., 2006 [150] |
| PTM                        | Introduction                              | Comments                                                                 | Reference                  |
|----------------------------|-------------------------------------------|--------------------------------------------------------------------------|----------------------------|
| Methylation Lys            | Fmoc-Lys(3Me,Boc)-OH 33                   | For introduction of monomethyl lysine                                    |                            |
|                            |                                           |                                                                          |                            |
|                            | Fmoc-Lys(3Me)-OH 34                      | For introduction of dimethyl lysine, basic side chain can promote Fmoc loss and double insertions during synthesis [151] |                            |
|                            |                                           |                                                                          |                            |
|                            | Fmoc-Lys(3MeCl)-OH 35                    | For introduction of trimethyl lysine                                     |                            |
| Citrullation               | Fmoc-citrulline-OH 36                     | For introduction of citrullation                                         |                            |
| Glycosylation Asn          | Fmoc-Asn(3β-D-GlcNAc(Ac)3)-OH 37          | Building block for introduction of monosaccharide fragment of N-linked glycoproteins | Meldal and Bock, 1990 [152]|
|                            |                                           |                                                                          |                            |
|                            | Fmoc-Asn(3β-D-GlcNAc(Ac)3-(1-4))-β-D-GlcNAc(Ac)2)-OH 38 | Building block for introduction of chitobiose fragment of N-linked glycoproteins | Meinjohanns et al., 1998 [153]|
| Glycosylation Ser (R = H)/Thr (R = Me) | Fmoc-Ser/Thr(3α-D-GlnNAc(Ac)3)-OH 39 | Building block for introduction of Tn antigen oligosaccharide fragment | Paulsen and Adermann, 1989 [154]|

(Continued)
of the H-bond acceptor phenyl oxygen and incomplete ionisation of the phosphonic acid at neutral pH (Pmp, pKa 7.22, vs pTyr, pKa 6.22) [187] F2Pmp 51 in contrast has a pKa of 5.71 and is therefore fully ionised at neutral pH, and the methylene fluorine atoms can undergo H-bonding. Peptides substituted with F2Pmp exhibit higher binding affinities to SH2 domains than Pmp analogues [176]. Enhancements of 1000-fold in affinities of F2Pmp-containing peptide compared with those containing Pmp are reported [188,189].

Unfortunately, for peptides containing phosphorylated basic amino acids, the building block approach is not appropriate as N-phosphates are not stable to TFA [161,190]. For phosphohistidine, nonhydrolysable analogues have been developed to overcome this problem, a phosphofurylalanine 53 analogue [184] and phosphotriazolyl alanine analogues 54 [185,186]. The Fmoc-protected derivative of 55 shows particular promise because peptides containing this moiety have been able to elicit antiphosphohistidine antibodies [194,195]. Recently, the synthesis of pLys-containing peptides has been reported using a strategy involving postassembly N-phosphorylation followed by saponification from a base-labile resin (Scheme 5) [196].

Recently, a method for pyrophosphorylation of pSer has been reported [197], which enables the investigation of pyrophosphorylation [198].

Sulfation

It is believed that up to 1% of all protein tyrosine residues in eukaryotes are sulfated; however, the biological role of tyrosine sulfation is

| PTM                          | Introduction                                      | Comments                                      | Reference        |
|-------------------------------|---------------------------------------------------|-----------------------------------------------|------------------|
| Fmoc-Ser/Thr(β-Asp)           | Building block for introduction of Tfn antigen     | Building block for Tfn antigen                | Irazoqui et al., 1999 [155] |
| β-Galulosyl(Ac)2-α-GlnNAc     | Antigen oligosaccharide fragment                  |                                               |                  |
|                               |                                                   |                                               |                  |
| Fmoc-Ser/Thr(sialylOMe(Ac)4-α-| Building block for introduction of STn antigen     | Building block for STn antigen               | Liebe and Kunz, 1997 [156] |
|     β-Gal(Ac)3-α-GlnNAc(Ac)2-| Antigen oligosaccharide fragment                  |                                               |                  |
|     OH                         |                                                   |                                               |                  |
| Fmoc-Ser/Thr(β-Asp)           | Building block for introduction of Tfn antigen     | Building block for Tfn antigen                | Komba et al., 1999 [157] |
| β-Galulosyl(Ac)2-α-GlnNAc     | Antigen oligosaccharide fragment                  |                                               |                  |
|                               |                                                   |                                               |                  |
| Fmoc-Ser/Thr(β-Asp)           | Building block for introduction of β-GlcNAc       | Building block for β-GlcNAc modification      | Arsequell et al., 1994 [159] |
| GlcNAc(Ac)3-OH                | Building block for β-GlcNAc modification; building blocks tend to racemise |                                               |                  |

Scheme 4. Global phosphorylation strategy (phosphitylation–oxidation method): (i) (BzlO)2-PN(i-Pr)2/tetrazole; (ii) tBuOOH; (iii) standard global TFA cleavage.
poorly understood [199,200]. Sulfation is thought to be involved in the modulation of the extracellular protein–protein interactions of secreted and transmembrane proteins [201,202]. It is also an essential requirement for maintaining the biological activity of a number of peptide hormones such as gastrin II, cholecystokinin and caerulein [203,204].

One of the principal hurdles to studying tyrosine sulfation is the difficulty in obtaining site-specifically sulfated peptides for use as biological probes or antigens for raising antibodies. This is because tyrosine sulfate esters are rapidly degraded in acid and fragment during mass spectrometry, making their synthesis and characterisation highly problematic. The basic principles for the synthesis of sulfated peptides have been elaborated by methods in solution and then transferred to SPPS. For an exhaustive review, see [205].

Recent advances were achieved by protecting the sulfate, which stabilises it during the TFA cleavage, enabling standard reaction conditions to be used without significant loss of the sulfate. The use of four protecting groups has been examined in detail: azidomethyl [206], trichloroethyl [149,207], dichlorovinyl (DCV) [148] and neopentyl (nP) [146]. Of these, nP protection appears to offer particular promise as the group is stable to piperidine and TFA but can be removed postcleavage with either sodium azide or ammonium acetate [147]. Fmoc-sulfotyrosine building block s are available, bearing DCV and nP protecting groups: Fmoc-Tyr(SO$_3$DCV)-OH and Fmoc-Tyr(SO$_3$nP)-OH.

Farnesylation

S-Farnesylation of protein C-terminal cysteinyl residues is thought to be involved in modulating protein–membrane and protein–protein interactions [208,209]. Stepwise synthesis of farnesylated peptide probes is challenging as the unsaturated farnesyl group is subject to addition reactions during TFA cleavage. Therefore, the S-farnesyl group is generally introduced by treating cysteinyl peptides that are either protected with base-labile groups or fully deprotected, with farnesyl bromide in solution [210] or on resin [211]. For an exhaustive review, see [212].

In the context of Fmoc SPPS, solution and solid-phase approaches to farnesylation are nicely exemplified by the following reported syntheses of yeast mating pheromone a-factor. The synthesis of this peptide is complicated by the fact that it contains not only a farnesyl group but also a C-terminal cysteine methyl ester.

**Table 7. Nonhydrolysable analogues of phosphoamino acid residues.**

| Structure | Name | Reference |
|-----------|------|-----------|
| PO$_3$H$_2$ | Phosphonomethylalanine | Engel, 1977 [177] |
| F$_2$PO$_3$H$_2$ | Difluorophosphonomethylalanine | Berkowitz et al., 1994 [178] |
| PO$_3$H$_2$ | β-(Phosphonomethyl) aminobutyric acid | Ruiz et al., 1994 [179] |
| F$_2$PO$_3$H$_2$ | (Difluorophosphonomethyl) aminobutyric acid | Berkowitz et al., 1996 [180] |
| PO$_3$H$_2$ | Phosphonomethyl phenylalanine | Marseigne et al., 1988 [144] |
| OH | Hydroxyphosphonomethyl phenylalanine | Burke et al., 1993 [181] |
| PO$_3$H$_2$ | Fluorophosphonomethyl phenylalanine | Burke et al., 1993 [181] |
| F$_2$PO$_3$H$_2$ | Difluorophosphonomethyl phenylalanine | Burke et al., 1993 [182] |
| Phosphonophenylalanine | Ppa | Lui et al., 2002 [183] |
| O$_3$P(OH)$_2$ | 2-Phospho-4-furylalanine | Schenkels et al., 1999 [184] |
| N$_3$N | 1-(2-Phosphonoethyl)-1H-1,2,3-triazol-4-ylalanine | Yang et al., 2011 [185] |

(Continues)
Cys residue is farnesylated. Oxidation followed by methanolysis releases the desired peptide methyl ester. Approaches to the peptidyl cysteine methyl ester involving side-chain anchoring of the C-terminal cysteine methyl ester [216] are not recommended as cysteine esters are not optically stable in the presence of piperidine [80].
Methylation

Protein methylation occurs predominantly on lysine and arginine residues of cytosolic and nuclear proteins and is involved in protein targeting and signalling and in the epigenetic control of gene expression [134,217–220]. Methylation of arginine is mediated by peptidylarginine methyltransferases (PRMTs), giving monomethyl, symmetric dimethyl and asymmetric dimethyl modifications. Lysine methyltransferases effect methylation of lysine, producing monomethyl, dimethyl and trimethyl modifications [221].

Building blocks are available for the introduction during Fmoc SPPS of all biologically relevant methylated lysine [Fmoc-Lys(Me, Boc)-OH 33, Fmoc-Lys(Me3)-OH 34 and Fmoc-Lys(Me3Cl)-OH 35] and arginine residues [Fmoc-Arg(Me,Pbf)-OH 30, Fmoc-ADMA(Pbf)-OH 31, Fmoc-SDMA(Boc2)-ONa 32] (Table 6). In the case of the methylated lysine, the only point of note is that there is anecdotal evidence to suggest the basic side chain of dimethyllysine can promote Fmoc cleavage during subsequent coupling reactions, leading to double additions [151].

For dimethylarginine, side-chain-protected [150] (31 and 32) and unprotected derivatives are available. However, the use of the latter are not recommended because of low reactivity resulting from γ-lactam formation and the potential for ornithine formation.

Citrullination

Conversion of arginine residues in proteins to citrulline is performed by enzymes known as peptidylarginine deiminases. Antibodies against citrullinated fibrin and fibrinogen are associated with rheumatoid arthritis and other autoimmune diseases, and citrullination is involved in epigenetic gene control by modification of histones [161,222]. For chemical synthesis, the introduction of citrulline into synthetic peptides is usually performed using a side-chain-protected building block. Coupling is slow, presumably as a result of competing γ-lactam formation. The Pbf side-chain-protected derivative Fmoc-citrulline(Pbf)-OH has been described and should provide a more robust approach [151].

Glycosylation

Most secreted proteins are glycosylated, conferring heterogeneity to the glycoprotein by the type of sugar occupying the glycosylation site (glycoform) and the site occupancy. Only extremely rarely are proteins naturally expressed as a single glycoform. The dissection of the roles of the oligosaccharide is an area of great interest and has demanded a source of defined, chemically homogeneous glycoproteins.

A great deal of this demand has been met by breakthroughs in the expression of single-glycoform proteins, for example, the use of the glycosidase inhibitors such as kifunensine in mammalian expression systems or the use of engineered yeast or cell lines [223]. Nevertheless, important work in dissecting the role of glycosylation continues to be performed with synthetic glycopeptides, for example, its role in stabilisation of protein folds [224].

The synthesis of glycoproteins is one of the grand challenges of organic chemistry and continues to stretch the frontiers of organic synthesis. Notable achievements have been the synthesis of ribonuclease C [225] and synthesis of part of gp120 [226]. The synthesis of glycoproteins has been reviewed [227–231].

Glycoproteins are made by native chemical ligation. These glycoproteins bear either the desired side-chain oligosaccharide or a truncated oligosaccharide that can be enzymatically extended or transglycosylated post-synthesis.

The methods used to produce the smaller glycopeptide components of the glycoproteins depend on whether the sugar is linked to the peptide chain by oxygen (O-linked via serine and threonine) or nitrogen (N-linked via asparagine). The synthesis of simple glycopeptides, with detailed practical protocols, has been described [7].

The most important class of O-linked glycosides has a 2-acetamido-2-deoxy-α-D-galactopyranosyl (GalNAc) unit attached to serine or threonine. Such O-glycosides are found in a wide range of proteins, such as mucin secreted from epithelial cells, the tumor-associated sialyl-T, sialyl-Tn-antigen and gp120 from HIV [232]. These O-linked glycopeptides are generally prepared using preformed Fmoc-protected glycoamino acid building blocks, and a number are commercially available including those for the introduction of sialyl-T and sialyl-Tn antigens (Table 5, 39, 40, 41 and 42). These are stable to TFA and piperidine and are hence compatible with Fmoc SPPS methods [233]. Sequential glycosylation has been employed to elaborate synthetic glycopeptides bearing this core O-GalNAc unit, using the appropriate glycosyl transferases and nucleotide [234,235].

Modification of serine or threonine residues with 2-acetamido-2-deoxy-β-D-glucopyranosyl (GlCNac) is functionally more akin to phosphorylation than glycosylation [236]. The addition and removal of O-AcNH-β-Glc is a dynamic process controlled by a transferase, UDPGlCNac polyolpeptide transferase, and removed by a β-N-acetylgalactosaminidase. Only a single sugar is added, and the carbohydrate is not further extended. O-AcNH-β-Glc glycosylation processes are thought to be involved in transcription, signal transduction, apoptosis and glucose homeostasis. Fmoc-Ser(Ac3AcNH-β-Glc)-OH and Fmoc-Thr(Ac3AchNH-β-Glc)-OH building blocks 43 are commercially available.

Most N-linked glycans possess a trimannose-di-N-acetylchitobiose pentasaccharide core linked via a β(1→6) linkage to Asn. Like O-linked glycopeptides, N-linked glycopeptides can be prepared by the introduction of glycosylated building blocks during SPPS (37 and 38) [152,237,238]. They can also be introduced convergently by acylation of glycosylamines with a peptide aspartyl side chain either in solution by Lansbury aspartylation [239] or convergent assembly on solid phase [48,55,61,240].

Peptide thioesters by Fmoc SPPS

In the Boc method, peptide thioesters can be prepared directly [241,242], whereas for the Fmoc method, the use of piperidine at each cycle is not compatible with a thioester at the C-terminus [243,244]. Several indirect methods have therefore been proposed. But none so far match the Boc method for simplicity and yield. The use of HF in the last step of Boc SPPS precludes the preparation of peptide thioesters bearing acid-sensitive PTMs and has largely driven the development of an Fmoc method. The approaches used to prepare peptide thioesters with Fmoc protocols can be classified into two types: those that use a safety-catch approach or those that exploit an intramolecular O,S or N,S acyl transfer step to form a bond stable to Fmoc synthesis into a thioester. Currently, the safety catch approach is more prevalent.

The first preparation of Fmoc peptide thioesters used a safety-catch sulfamylbutyryl resin. Following chain assembly, the sulfonamide linker is activated by alkylation usually by treatment with iodoacetonitrile or TMS-diazomethane. The fully protected peptide is cleaved from the resin by sodium thiophenolate in DMF, and the resulting protected peptide thioester is treated with acid to remove
side-chain protection (Scheme 8) [244]. Numerous variations on this approach have since been developed [245].

The sulfamylbutyryl approach has been used to synthesise impressive targets, including long peptide thioesters [246], glycoproteins [225,247] and phosphoproteins [248]. However, the thiolysis step can sometimes be problematic because of poor solvation of the resin-bound peptide and difficulties with peptide recovery from DMF. It has been demonstrated that the thiolysis step is unnecessary and the methyl sulfamylbutyryl linker itself in the presence of mercaptoophenylacetic acid additive can perform ligation directly in the ligation buffer [249].

Another widely used safety-catch linker was adapted [250] from the N-acyl urea safety catch [251,252]. The ortho-di-aniline system 3,4-diaminobenzoic acid is activated after chain assembly with p-nitrophenyl chloroformate (Scheme 9). This linker approach has been successfully used to give PTM-modified protein precursors, including glycoproteins [230]. Recently, a variation of this approach has been described based on 3-amino-4-methylaminobenzoic acid [253]. This modification improves regioselectively during acylation of the linker.

Latterly, there has been much success revisiting the classical hydrazide/azide [254] as a route to peptide thioesters for their application in ligation [255–257]. Side reactions have been observed including intramolecular acylation of an adjacent lysine side chain [258]. In a notable study, biologically expressed ubiquitin bearing a C-terminal cysteine has been successfully converted to a hydrazide and subsequently converted to an azide and ligated [259]. The reaction is dependent on the ability of a C-terminal cysteine to transiently rearrange to a thioester [260]. This rearrangement has become the basis for the other major classes of approaches to the synthesis of peptide thioesters: those that use an intramolecular acyl transfer to give the peptide thioester.

The N,S-acyl transfer approach (Scheme 10) developed from the related area of ligation auxiliaries [261]. Whilst attempting to remove a ligation auxiliary, 2-mercapto-4,5-dimethoxybenzyl, from a peptide with TFA, the Aimoto group observed the appearance of a compound with a different retention time by HPLC but with the same mass, assigned as the thioester [262]. Interestingly, this migration had been previously observed [263]. Furthermore, the Aimoto group demonstrated that this could be exchanged with another thiol.

![Scheme 8](image_url)

**Scheme 8.** Principle of the sulfamyl safety-catch linker for peptide thioester Fmoc SPPS, X = butyl or phenyl. (i) Fmoc-AA (4 eq.), PyBOP, DIPEA (8 eq.); (ii) SPPS; (iii) Boc₂O; (iv) TMS-diazomethane; (v) R-SH, NaSPh; (vi) standard TFA cleavage; (vii) 6 M guanidine, phosphate buffer pH 7.8, 1% thiophenol.

![Scheme 9](image_url)

**Scheme 9.** Use of diaminobenzoic acid linker (Dbz) as safety-catch linker for peptide thioester Fmoc SPPS. (i) SPPS; (ii) 4-nitrochloroformate; (iii) DIPEA; (iv) standard TFA cleavage; (v) R-SH, NaSPh; (vi) 6 M guanidine, phosphate buffer pH 7.8, 1% thiophenol.
alkylated amides as a latent thioester was first demonstrated with conditions and can therefore be used directly in ligation. The application of MPAA to ligation using ε-alkylated cysteine also underwent this reaction [265], and this observation was extended to other tertiary amides bearing a C-terminal δ- or ε-thio alkyl or aryl group [266]. Many of these linkers are converted to a thioester in a two-step reaction, rearrangement occurs irreversibly under strong acidic conditions followed by exchange to give a peptide thioester suitable for use in ligation. Later, it was observed that most of these linkers undergo acyl transfer under neutral conditions and can therefore be used directly in ligation.

Botti and co-workers pioneered the idea of an O,S-acyl on a linker derivatised from cysteine [267]. They reported the successful synthesis of a protein, NNY-Rantes (1–68), but cautioned that the method was prone to hydrolysis. This work led others to consider the possibility that the amide bond existed in equilibrium with a thioester and could be shifted further to the thioester form by the addition of thiol additives (Scheme 10). Evidence emerged that under some conditions amides with an adjacent δ-thiol or ε-thiol functionality could participate directly in native chemical ligation. The introduction of 4-mercaptophenylacetic acid (MPAA) as a water-soluble thiol additive to ligation reactions had a major impact [268], enabling many of the amide systems that showed NS-acyl transfer in acid to be used directly in ligation. The application of MPAA to ligation using N-alkylated amides as a latent thioester was first demonstrated with the bis(sulfanylamido) (SEA) linker [269]. Transfer and ligation are performed in a single step, and they have reported the synthesis of many impressive targets with this approach (Scheme 11). The SEA linker has been used for the synthesis of 76mer SUMO, and this has in turn been conjugated [270]. The Otaka group first reported the N,S-acyl shift properties of sulfanylenylanilide in acid [271]. Later, they were able to demonstrate the use of this linker directly in ligation using MPAA [272]. They have since reported the synthesis of a glycoprotein in four pieces with their approach [273]. Both linkers, SEA and sulfanylenylanilide, are difficult to acylate.

These NS rearrangements used N-substituted amides; however, cysteine itself has been demonstrated to be in equilibrium between an amide and a thioester, and this has been exploited for the synthesis of cyclic peptides [274]. Substitution at the CA of cysteine to give α-methylcysteine further promoted NS-acyl transfer by slightly twisting the amide bond and increasing its reactivity [274] so that it could participate in intermolecular ligation reactions (Scheme 12) [128]. One advantage of using α-methylcysteine is that it is stable to many postsynthetic manipulations including hydrazine treatment that makes it compatible with the use of Hmb backbone protection and other protecting group strategies. Its lack of N-substitution made it compatible with standard linkers as it was less susceptible to diketopiperazine formation [128].

However, these N,S-acyl transfer methods are currently limited by slow-ligation kinetics in comparison with safety-catch or Boc methods and require careful control of pH and reaction conditions to achieve good results. This will undoubtedly change as this is a fast-moving area of study [275]. It is remarkable, however, that proteins are now being routinely synthesised using an amide bond as the active group. This approach has already generated interest for the preparation of dynamic peptide libraries [276]. Nevertheless, the search for a straightforward, low-cost Fmoc alternative for the preparation of thioester peptides continues because of the wealth of potential applications.

The use of heating in Fmoc SPPS

Since the inception of solid-phase synthesis, heat has been used to speed up peptide assembly [277]. A programmable heating block was incorporated into one of the first Fmoc continuous-flow peptide synthesers, LKB Biolynx, concomitant with introduction of Fmoc SPPS in the 1980s. The use of heating has been recently reviewed [18,278].

![Scheme 10](image-url)

**Scheme 10.** O,S-acyl [267] and N,S-acyl [265] shift for peptide thioester preparation. (i) R-SH, NaSPh.

![Scheme 11](image-url)

**Scheme 11.** Use of the SEA-linker for peptide thioester Fmoc SPPS. (i) SPPS; (ii) standard TFA cleavage; (iii) iodine oxidation; (iv) N,S-acyl shift; 0.2 M tris(2-carboxyethyl)phosphine/MPAA; (v) R-SH, NaSPh; (vi) 6 M guanidine, phosphate buffer pH 7.8, 1% thiophenol.
Advances in Fmoc Solid-Phase Peptide Synthesis

Scheme 12. α-Methylcysteine as a thioester surrogate.

Heating can be applied conventionally or by microwave or infrared irradiation. There are, however, associated problems exacerbated by heating: loss of peptide loading during peptide assembly [279,280], cysteine and histidine racemisation [72,281–284], aspartimide formation [72,281,285] and elevated levels of β-elimination in serine building blocks like pSer [175] and Ser-glycan moieties [278].

There is a trend to return to simple coupling reagents in conjunction with heating. A carbodiimide/hydroxybenzotriazole-based coupling protocol at 86°C accelerated the synthesis of β-amyloid (1–42) [282,283]. The group of Jensen also found that DIC/Oxyma performed very well [284,286]. This topic has been comprehensively reviewed [18,278].

Applying heat to Fmoc SPPS should reduce synthesis time and potentially suppress chain aggregation. The use of microwave heating in combination with backbone protection has been applied to the synthesis of aggregation-prone sequences like the islet amyloid polypeptide [287,288] and the influenza virus haemagglutinin [115]. A comparison of the use of peptide backbone protection and microwave heating in the synthesis of difficult sequences showed that backbone protection was more effective in preventing aggregation than heating [115].

The major concern when using heating during Fmoc deprotection is aspartimide formation. This has been partially addressed by the substitution of piperidine with pipеразине and the incorporation of acidic modifiers into the deprotection mixture [74,285]. However, despite these measures, aspartimide formation is still evident [57,285].

Conclusion

Fmoc SPPS is very widely used and effective; however, it is still far from meeting its potential. It is generally considered that, as SPPS is a stepwise process with errors compounded throughout the synthesis, it cannot ever compete with the templated process of expression. Nevertheless, as we have outlined in this review, the constant improvement of side-chain protection strategies and increasing purity of the building blocks have made previously unobtainable, lengthy targets accessible.

From the increasing number of long targets synthesised using commercially available backbone protection, there is a growing realisation that the major obstacle to peptide synthesis is peptide aggregation from interchain association on the growing peptide resin. Pseudoprolines are a good answer to address this problem and are used routinely to prevent association. They are limited by the dipeptide building blocks available, but as more researchers realise the benefits of backbone protection, there is a need for a more general backbone protection that can also be retained after synthesis to improve peptide handling properties.

Nonspecialists were responsible for the success of the Fmoc method and will be again for the future of Fmoc. The strongest upshot of Fmoc chemistry has been for the study of new materials. Chemical synthesis has the advantage of complete atom-by-atom control over the whole peptide, and multiple nonnatural or post-translationally modified residues can be added. With the advances in backbone protection, heating and improved reliability of automation, we are on the threshold of machines suitable for the nonspecialist researcher to routinely reach synthetic peptides of lengths comparable with those for chemical nucleotide synthesis of above 100 residues.

References

1. Fosgerau K, Hoffmann T. Peptide therapeutics: current status and future directions. Drug Discov. Today 2015; 20: 122.
2. Kaspar AA, Reichert JM. Future directions for peptide therapeutics development. Drug Discov. Today 2013; 18: 807.
3. Boyle AL, Woolfson DN. De novo designed peptides for biological applications. Chem. Soc. Rev. 2011; 40: 4295.
4. Sheppard R. The fluorenylmethoxycarbonyl group in solid phase synthesis. J. Pept. Sci. 2003; 9: 545.
5. Walter G. Production and use of antibodies against synthetic peptides. J. Immun. Meth. 1986; 88: 149.
6. Dryland A, Sheppard RC. Peptide synthesis. Part B. A system for solid-phase synthesis under low pressure flow conditions. J. Chem. Soc., Perkin Trans. 1 1986: 125.
7. Chan WC, White PD. Fmoc Solid Phase Peptide Synthesis, Oxford University Press: Oxford, UK, 2000.
8. Carpino LA, Han GY. 9-Fluorenylmethoxycarbonyl amino-protecting group. J. Org. Chem. 1972; 37: 3404.
9. Bodanszky M, Deshmone SS, Martínez J. Side reactions in peptide synthesis. 11. Possible removal of the 9-fluorenylmethoxycarbonyl group by the amino components during coupling. J. Org. Chem. 1979; 44: 1622.
10. Atherton E, Logan CJ, Sheppard RC. Peptide synthesis. Part 2. Procedures for solid-phase synthesis using N α-fluorenylmethoxycarbonylamino-acids on polypeptide supports. Synthesis of substance P and of acyl carrier protein 65–74 decapetide. J. Chem. Soc., Perkin Trans. 1 1981; 538.
11. Chang CD, Meldal M, Sheppard RC. Solid phase peptide synthesis using mild base cleavage of Nα-fluorenylmethoxycarbonylamino acids, exemplified by a synthesis of dihydroinosotatin. Int. J. Pept. Protein Res. 1978; 11: 246.
12. Cameron L, Meldal M, Sheppard RC. Feedback control in organic synthesis. A system for solid phase peptide synthesis with true automation. J. Chem. Soc., Chem. Commun. 1987; 4: 270.
13. White P, Keyte JW, Bailey K, Bloomberg G. Expediting the Fmoc solid phase synthesis of long peptides through the application of dimethyloxalozidine dipeptides. J. Pept. Sci. 2004; 10: 18.
14. Wühr T, Mutter M. Pseudo-prolines in peptide synthesis: direct insertion of serine and threonine derived oxazolidines in dipeptides. Tetrahedron Lett. 1995; 36: 3847.
15. Cardona V, Eberle I, Barthelemy S, Beythien J, Doerner B, Schneeberger P, Keyte J, White PD. Application of DMB-dipeptides in the Fmoc SPPS of difficult and aspartimide-prone sequences. Int. J. Pept. Res. Ther. 2008; 14: 285.
16. Kent SBH, in Peptides, structure and function, Proceedings of the 9th American Peptide Symposium (Eds.: Deber CM, Hubry VJ, Kopple KD), Pierce Chemical Company, Rockford, 1985, pp. 407.
17. Zompra AA, Galanis AS, Wrbizky O, Albericio F. Manufacturing peptides as active pharmaceutical ingredients. Future Med. Chem. 2009; 1: 361.
18. Sabatino G, Papini AM. Advances in automatic, manual and microwave-assisted solid-phase peptide synthesis. Curr. Opin. Drug. Discovery Dev. 2008; 11: 762.
19. Pires DAT, Bemquerer MP, do Nascimento CJ. Some mechanistic aspects on Fmoc solid phase peptide synthesis. Int. J. Pept. Res. Ther. 2014; 20: 53.
20. Fields GB. Molecular Biomethods Handbook, Humana: Totowa, NJ, 1998; 527.
21. Isidro-Lloret A, Alvarez M, Albericio F. Amino acid-protecting groups. Chem. Rev. 2009; 109: 2455.
Mergler M, Dick F. The aspartimide problem in Fmoc-based SPPS. Part I. J. Pept. Sci. 2003; 9: 36.

Karlstöm A, Undén A. Design of protecting groups for the β-carboxyl group of aspartic acid that minimize base-catalyzed aspartimide formation. Int. J. Pept. Protein Res. 1996; 48: 305.

Bodanszky M, Martínez J. Side reactions in peptide synthesis. 8. On the phenylacetyl group in the protection of the β-carboxy-carbonyl function of aspartic residues. J. Org. Chem. 1978; 43: 3071.

Dölling R, Beyermann M, Haenel J, Kernchen F, Krause E, Franke P, Brudel M, Bierner M. Piperidine-mediated side product formation for Asp(OBu) containing peptides. J. Chem. Soc., Chem. Commun. 1994; 7: 853.

Ullmann V, Raedisch M, Boos I, Freund J, Poehner C, Schwarzer S, Unverzagt C. Convergent solid-phase synthesis of N-glycopeptides facilitated by pseudoprolines at consensus-sequence Ser/Thr residues. Angew. Chem. Int. Ed. 2012; 51: 11566.

Wang P, Aussedat B, Vohra Y, Danishefsky JS. An advance in the chemical synthesis of homogeneous N-linked glycopeptides by convergent aspartylation. Angew. Chem. Int. Ed. 2011; 50: 11571.

Behrendt R, Huber S, Martí R, White P. New N-carboxyl protecting groups preventing aspartimide formation in Fmoc SPPS. Int. J. Pept. Sci. 2015; 21: 98.

Subirós-Funosas R, El-Faham A, Albericio F. Use of Oxyma as pH modulatory agent to be used in the prevention of base-driven side reactions and its effect on 2-chlorotrityl chloride resin. Pept. Sci. 2012; 98: 89.

Packman LC. N-2-Hydroxy-4-methoxybenzyl(Hmb) backbone protection strategy prevents double aspartimide formation in a ‘difficult’ peptide sequence. Tetrahedron Lett. 1995; 36: 7523.

Röder B, Henkeln P, Wyl WB, Weilhöfer M, Mügge C, Pätz M, Schubert U, Carpino LA, Henkeln P. On the use of N-dicyclopropylmethyl arginine for backbone amide protection. J. Pept. Sci. 2010; 16: 65.

Conroy T, Jolliffe KA, Payne RJ. Synthesis of N-linked glycopeptides via solid-phase aspartylation. Org. Biomol. Chem. 2010; 8: 3723.

El Oualid F, Merker R, Ekkebus R, Hameed DS, Smit JJ, de Jong A, Hilkmann H, Sixma TK, Ovaa H. Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin. Angew. Chem. Int. Ed. 2010; 49: 10149.

Ramage R, Green J. NG-2,5,7,8-pentamethylchroman-6-sulfonyl-L-arginine: a new acid labile derivative for peptide synthesis. J. Pept. Sci. 2012; 18: 2287.

Green J, Ogunjobi O, Ramage R, Stewart A, McCurdy S, Noble R. Application of the NG-(2,5,7,8-pentamethylchroman-6-sulphonyl) derivative of FMOC-arginine to peptide synthesis. Tetrahedron Lett. 1988; 29: 4314.

Isidro A, Latassa D, Giraud M, Alvarez M, Albericio F, 1,2-Dimethylindole-3-sulfonyl (MIS) as protecting group for the side chain of arginine. Org. Biomol. Chem. 2009; 7: 2565.

Noda M, Kiffe M. New mild acid-labile protecting groups for the guanidino function of N-4-fluorophenylalanine. J. Pept. Sci. 2001; 7: 823.

Riffel DB, Noble RB. Solid-phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. J. Pept. Sci. 1990; 15: 316.

Rink H, Sieber P, Raschdorf F. Conversion of N-G urethane protected arginine to ornithine in peptide solid phase synthesis. Tetrahedron Lett. 1984; 25: 621.
Advances in Fmoc Solid-Phase Peptide Synthesis

70 Musiol HJ, Siedler F, Quarzaro D, Moroder L. Redox-active bis-cysteiny1 peptides. I. Synthesis of cyclic cysteiny1 peptides by conventional methods in solution and on solid supports. *Biopolymers* 1994; 34: 1553.

71 Kaiser T, Nicholson G, Kohlbau H, Voelter W. Racemization studies of Fmoc-Cys (Trt)-OH during stepwise Fmoc-solid phase peptide synthesis. *Tetrahedron Lett.* 1996; 37: 1187.

72 Palasek SA, Cox ZJ, Collins JM. Limiting racemization and aspartimide formation in microwave-enhanced Fmoc solid phase peptide synthesis. *J. Pept. Sci.* 2007; 13: 143.

73 Han Y, Albericio F, Barany G. Occurrence and minimization of cysteine racemization during stepwise solid-phase peptide synthesis. *J. Org. Chem.* 1992b; 57: 62.

74 Collins JM, Porter KA, Singh SK, Vanier GS. High-efficiency solid phase peptide synthesis (HE-SPPS). *Org. Lett.* 2014; 16: 940.

75 Hibino H, Miki Y, Nishiuchi Y. Evaluation of acido-labile S-protecting groups to prevent Cys racemization in Fmoc solid-phase peptide synthesis. *J. Pept. Sci.* 2014; 20: 30.

76 Mitchell MA, Runge TA, Mathews WR, Ichipuranli AK, Ham NK, Dobrowolski PJ, Eckenrode FM. Problems associated with use of the benzoxymethyl protecting group for histidines. Formaldehyde adducts formed during cleavage by hydrogen fluoride. 1. *J. Pept. Protein Res.* 1990; 36: 350.

77 Kumiko YK, Ishizu T, Isaka S, Tamura M, Rumi OT, Nishiuchi Y, Kimura T, Nishio H, Bódi J, Kimura T, Tsuji FI, Sakakibara S. Fmoc-Cys (Trt)-OH during stepwise Fmoc-solid phase peptide synthesis. *Chem. Eur. J.* 2014; 20: 1187.

78 Atherton E, Hardy P, Harris DE, Matthews BH, in *Peptides 1990: Chemistry*. 2016; : 9047.

79 Narita M, Ishikawa K, Nakano H, Isokawa S. Tertiary peptide bond containing-oligo (Leu) s. *Int. J. Pept. Protein Res.* 1984; 24: 14.

80 Okada Y, Wang J, Yamamoto T, Sato T, Sun X, Mutter M. Pseudo-prolines as a solubilizing, structure-disrupting protection technique in difficult sequences. *J. Org. Chem.* 1999; 64: 665.

81 Clippingdale A, Macris M, Wade J, Barrow C. Synthesis and secondary structural studies of penta (acetyl-Hmb) Aβ (1–40). *J. Pept. Res.* 1999; 53: 118.

82 Nishiuchi Y, Inui T, Nishio H, Bódi J, Kimura T, Tsuji FI, Sakakibara S. Chemical synthesis of the precursor molecule of the Aequorea green fluorescent protein, subsequent folding, and development of fluorescence. *Proc. Natl. Acad. Sci.* 1988; 95: 13549.

83 Sakakibara S. Chemical synthesis of proteins in solution. *Pept. Sci.* 1999; 51: 279.

84 Hancock WS, Prescott DJ, Vagelos PR, Marshall GR. Solvation of the polymer matrix. Source of truncated and failure sequences in solid phase synthesis. *J. Org. Chem.* 1973; 38: 774.

85 Hibino H, Miki Y, Nishiuchi Y. Synthesis of defined oligomers of alanine and of lysyl-glutamyl-π histidine side chains in peptide synthesis: the use of the tert-butoxymethyl group. *J. Chem. Soc., Perkin Trans. 1* 1994; 9: 350.

86 Colombo R, Colombo F, Jones JH. π-π-protecting group of Nπ histidine side chains in peptide synthesis: the use of the Nπ protection: the tert-butoxymethyl group. *J. Chem. Soc., Perkin Trans. 1* 1980; 24: 67.

87 Blaakmeer J, Tijsse-Klasen T, Tesser GI. Enhancement of solubility by temporary dimethoxybenzyl-substitution of peptide bonds. Towards the synthesis of defined compounds of alanine and of l-lysyl-glutamyl-glycine. *Int. J. Pept. Protein Res.* 1991; 37: 556.

88 Johnson T, Quibell M, Sheppard R. Some ‘difficult sequences’ made easy. *Int. J. Pept. Protein Res.* 1994; 43: 431.

89 Atherton E, Hardy P, Harris DE, Matthews BH. Problems associated with use of the benzoxymethyl protecting group for histidines. Formaldehyde adducts formed during cleavage by hydrogen fluoride. 1. *J. Pept. Protein Res.* 1990; 36: 350.

90 Atherton E, Hardy P, Harris DE, Matthews BH. Problems associated with use of the benzoxymethyl protecting group for histidines. Formaldehyde adducts formed during cleavage by hydrogen fluoride. 1. *J. Pept. Protein Res.* 1990; 36: 350.

91 Nishiuchi Y, Inui T, Nishio H, Bódi J, Kimura T, Tsuji FI, Sakakibara S. Fmoc-Cys (Trt)-OH during stepwise Fmoc-solid phase peptide synthesis. *Chem. Eur. J.* 2014; 20: 1187.

92 Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* 2006; 75: 333.

93 Nishiuchi Y, Inui T, Nishio H, Bódi J, Kimura T, Tsuji FI, Sakakibara S. Chemical synthesis of the precursor molecule of the Aequorea green fluorescent protein, subsequent folding, and development of fluorescence. *Proc. Natl. Acad. Sci.* 1998; 95: 13549.
24

Kemp D, Carey RL. Boc-L-Dmt-OH as a fully N,S-blocked cysteine derivative for peptide synthesis by prior thiol capture. Facile conversion of N-terminal Boc-L-Dmt-peptides to H-Cys (Smc)-peptides. J. Org. Chem. 1989; 54: 3640.

Barber M, Jones JH. An alternative synthesis of 7-(thiazolidine-4-carboxylic acid)-A2M. J. Pept. Protein Res. 1977; 9: 269.

Goncalves V, Gautier B, Huguenot F, Legroux P, Garbay C, Vidal M, Inguibert N. Total chemical synthesis of the 2D domain of human VEGF receptor 1. J. Pept. Sci. 2009; 15: 417.

Mutter M, Chandravarkar A, Boyat C, Lopez J, Santos SD, Mandle B, Mimma R, Murat K, Patiny L, Sauclidean L, Tuchserger G. Switch peptides in statu nascendi: induction of conformational transitions relevant to degenerative diseases. Angew. Chem. Int. Ed. 2004; 43: 4172.

Carpino LA, Krause E, Sferdean CD, Schümann M, Fabian H, Bienert M, Beyermer M. Synthesis of “difficult” peptide sequences: application of a depsipeptide technique to the Jung-Redemann 10- and 26-mers and the amyloid peptide Aβ(1–42). Tetrahedron Lett. 2004; 45: 7519.

Sohma Y, Sasai M, Hayashi Y, Kimura T, Kiso Y. Novel and efficient synthesis of dipeptide sequence-containing peptides through O–N intramolecular acyl migration reaction of O-acyl isopeptides. Chem. Commun. 2004; 1: 124.

Sohma Y, Kiso Y. Synthesis of O-acyl isopeptides. The Chemical Record 2013; 13: 218.

Coi N. The depsipeptide method for solid-phase synthesis of difficult peptides. J. Pept. Sci. 2010; 16: 223.

Miranda LP, Alewoud PF. Challenges for protein chemical synthesis in the 21st century: bridging genomics and proteomics. Biopolymers 2000; 55: 217.

Burlina F, Papageorgiou G, Morris C, White PD, Offer J. In situ thioester incorporation of Fmoc-Tyr (PO3Bzl, H)-OH, Fmoc-Ser (PO3Bzl, H)-OH into peptides and proteins via Fmoc chemistry. J. Pept. Sci. 2012; 18: 639.

Prabakaran S, Lippens G, Steen H, Gunawardena J. Post-translational modification: nature’s escape from genetic impairment and the basis for dynamic information encoding. Wiley Interdiscip. Rev. Syst. Biol. Med. 2012; 4: 565.

Walsh CT, Garneau-Tsodikova S, Gatto GJ. Protein posttranslational modifications: the chemistry of proteome diversifications. Angew. Chem. Int. Ed. 2005; 44: 7342.

Lotrope AP, Torres MP, Fuchs SM. Deciphering post-translational modification codes. FEMS Lett. 2013; 587: 1247.

Kouzarides T. Chromatin modifications and their function. Nat Rev Cancer 2007; 7: 286.

Kitas E, Perich J, Wade J, Johns R, Pennington MW. Efficient synthesis of protected L-phenylphophomalanine (Ppa) derivatives suitable for solid phase peptide synthesis. Tetrahedron Lett. 2007; 48: 4051.

Marseigne I, Roques BP. Synthesis of new amino acids mimicking sulfated and phosphorylated tyrosine residues. J. Org. Chem. 1988; 53: 3621.

Gordeev MF, Patel DV, Barker LD, Gordon EM. N-α-Fmoc-4-phosphono (difluoromethyl)-l-phenylalanine: a new O-phosphorysine isosteric building block suitable for direct incorporation into peptides. Tetrahedron Lett. 1994; 35: 7585.

Simpson LS, Widlanski TS. A comprehensive approach to the synthesis of sulfate esters. J. Am. Chem. Soc. 2006; 128: 1605.

Simpson LS, Zhu JZ, Widlanski ST, Stone MJ. Regulation of chemokine recognition by site-specific tyrosine sulfation of receptor peptides. Chem. Biol. 2009; 16: 153.

Ali AM, Taylor SD. Efficient solid-phase synthesis of sulfotyrosine peptides using a sulfate protecting-group strategy. Angew. Chem. Int. Ed. 2009; 48: 2024.

Ali AM, Hill B, Taylor SD. Trichloroethyl group as a protecting group for sulfonates and its application to the synthesis of a disulfonate analog of the tyrosine sulfated PSGL-143–30 peptide. J. Org. Chem. 2009; 74: 3583.

White P. In Novabiochem innovations, 2006.

Rothbart SB, Krajevski K, Strahl BD, Fuchs SM. Peptide microarrays to interrogate the ‘histone code’. Methods Enzymol. 2012; 512: 107.

Meldal M, Bock K. Pentafluorophenyl esters for temporary carboxyl group protection in solid-phase synthesis of N-linked glycopeptides. Tetrahedron Lett. 1990; 31: 6987.

Meihojanns E, Meldal M, Paulsen H, Dwek RA, Bock K. Novel sequential solid-phase synthesis of N-linked glycopolptides from natural sources. J. Chem. Soc., Perkin Trans. 1 1998; 549.

Paulsen H, Ademann K. Synthesis of O-Glycopeptid-Sequenzen des N-Terminals von Anteileen-2. Liebigs Ann. Chem. 1989; 1989: 751.

Fukumatsu F, Vides MA, Noreas GA. Structural requirements of carbohydrates to bind Agaricus bisporus lectin. Glycobiology 1999; 9: 59.

Liebe B, Kunz H. Solid-phase synthesis of a tumor-associated sialyl-tα antigen glycopeptide with a partial sequence of the ‘tandem repeat’ of the MUC-1 mucin. Angew. Chem. Int. Ed. 1997; 36: 618.

Komba S, Meldal M, Werdelin O, Jensen T, Bock K. Convenient synthesis of Thr and Ser carrying the tumor associated sialyl (2→3)-a-T antigen as building blocks for solid-phase glycopeptide synthesis. J. Chem. Soc., Perkin Trans. 1 1999; 415.

Zhang Y, Muthana SM, Farnsworth D, Ludek O, Adams K, Barchi JJ, Jr, Gildersleeve JC. Enhanced epimerization of glycosylated amino acids during solid-phase peptide synthesis. J. Am. Chem. Soc. 2012; 134: 6316.

Hargreil G, Krippner L, Dwek RA, Wong SYC. Building blocks for solid-phase glycopeptide synthesis: 2-acetamido-2-deoxy-β-D-glycosides of FmocSerOH and FmocThrOH. J. Chem. Soc., Chem. Commun. 1994; 2383.

Cohen P. The regulation of protein function by multisite phosphorylation—a 25 year update. Trends Biochem. Sci. 2000; 25: 596.

Besant PG, Attwood PV. Histidine phosphorylation in histones and in other mammalian proteins. Methods Enzymol. 2010; 471: 403.

Ciesla J, Frączyk T, Rode W. Phosphorylation of basic amino acid residues in proteins: important but easily missed. Acta Biochim. Pol. 2011; 58: 137.

Perich JW, in Houben-Weyl Methods of Organic Synthesis, Synthesis of Peptides and Peptidomimetics, Vol. E22b (Eds.: Goodman M, Felix A, Moroder L, Toniolò C, Georg Thieme, Stuttgart, 2002, pp. 375.

Andreas D, Kitchin J, Seale P. Solid-phase synthesis of a range of O-phosphorylated peptides by post-assembly phosphorylation and oxidation. Int. J. Pept. Protein Res. 1991; 38: 469.

Pettilo DE, Mowrey DR, Allwein SP, Bakale RP. A general preparation of protected phosphoamino acids. Org. Lett. 2012; 14: 1206.

Ottinger EA, Xu Q, Barany G. Intramolecular pyrophosphate formation during N'-α-9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis of peptides containing adjacent phosphotyrosine residues. Pept. Res. 2005; 19: 223.

García-Echeverría C. Potential pyrophosphate formation upon use of N'-α-Fmoc-Tyr (PO3H2)-OH in solid-phase peptide synthesis. Lett. Pept. Sci. 2002; 9: 233.

Kitas EA, Wade JD, Johns R, Perich JW, Treger GW. Preparation and use of N'-fluorenylmethoxycarbonyl-α-dibenzylphosphono-o-tyrosine in
continuous flow solid phase peptide synthesis. J. Chem. Soc., Chem. Commun. 1991; 5: 338.

169 Kitas EA, Norr R, Trzeciak A, Bannwarth W. Alternative strategies for the Fmoc solid-phase synthesis of O$_2$-phospho-L-tyrosine-containing peptides. Helv. Chim. Acta 1991; 74: 1314.

170 Pascal R, in Peptides 2000, Proceedings of the 26th European Peptide Symposium (Eds: Martinez J, Fehrentz A), Editions EDK, Paris, 2001, pp. 263.

171 Ishida A, Shigeri Y, Taniguchi T, Kameshita I. Protein phosphatases that regulate multifunctional Ca$^{2+}$/calmodulin-dependent protein kinases: from biochemistry to pharmacology. Pharm. Ther. 2003; 100: 291.

172 Lacome J, Andriamampaisoa F, Pavia A. Solid-phase synthesis of peptides containing phosphoserine using phosphate tet-buty1 protecting group. Int. J. Pept. Proteins Res. 2009; 73: 275.

173 Harris PW, Williams GM, Shepherd P, Brimble MA. The synthesis of phosphopeptides using microwave-assisted solid phase peptide synthesis. Int. J. Pept. Res. Ther. 2008; 14: 387.

174 Vorherr T, Bannwarth W. Phospho-serine and phospho-threonine building blocks for the synthesis of phosphorylated peptides by the Fmoc solid phase strategy. Bioorg. Med. Chem. Lett. 1995; 5: 2661.

175 Attard TJ, O’Brien-Simpson NM, Reynolds EC. Identification and suppression of $\beta$-elimination byproducts arising from the use of Fmoc-Ser (PO$_2$But$_2$O), HI- OH in peptide synthesis. Int. J. Pept. Res. Ther. 2009; 15: 69.

176 Burke TRJ, Smyth MS, Otaka A, Nomizu M, Roller PP, Wolf G, Case R, Shoelso NE. Nonhydrolyzable phosphotyrosyl mimics for the preparation of phosphatase-resistant SH2 domain inhibitors. Biochemistry 1999; 38: 6490.

177 Engel R. Phosphonates as analogues of natural phosphates. Chem. Rev. 1977; 77: 349.

178 Berkowitz DB, Shen Q, Maeng J-H. Synthesis of the (\alpha,\omega-difluorophosphate) phosphonate analogue of phosphoserine. Tetrahedron Lett. 1994; 35: 6445.

179 Ruiz M, Ojea V, Shapiro G, Weber H-P, Pombo-Villar E. Asymmetric synthesis of a protected phosphonate isostere of phosphothreonine, L-phosphothreonine and L-phosphohistidine. J. Org. Chem. 1996; 61: 4666.

180 Burke TR, Jr, Smyth MS, Nomizu M, Otaka A, Roller PP. Preparation of fluoro-and hydroxy-4-(phosphonomethyl)-DL-phenylalanine suitably protected for solid-phase synthesis of peptides containing hydroxymethyl stable analogs of O-phosphophoryl. J. Org. Chem. 1993; 58: 1336.

181 Burke TR, Smyth MS, Otaka A, Roller PP. Synthesis of 4-phosphophenylalanine (\alpha-phospho-L-phenylalanine and N-Boc and N-Fmoc derivatives) suitably protected for solid-phase synthesis of nonhydrolyzable phosphotyrosyl peptide analogues. Tetrahedron Lett. 1994; 35: 4551.

182 Berkowitz DB, Eggen M, Shen Q, Shoemaker RK. Ready access to fluorinated phosphonate mimics of secondary phosphates. Synthesis of the (\alpha,\omega-difluoro)phosphate analogues of L-Phosphoserine, L-phosphothreonine, and L-phosphohistidine. J. Org. Chem. 1996; 61: 4666.

183 Liu W-Q, Olszowy C, Bischoff L, Garbay C. Enantioselective synthesis of fluoro-and hydroxy-4-(phosphonomethyl)-D,L-phenylalanine suitably protected for solid-phase synthesis of nonhydrolyzable phosphonopeptide. Tetrahedron Lett. 1999; 40: 6490.

184 Moore KL. The biology and enzymology of protein tyrosine O-sulfation. J. Biol. Chem. 2003; 278: 24243.

185 Musiol HJ, Escherich A, Moroder L, in Houben-Weyl Methods of Organic Synthesis, Synthesis of Peptides and Peptidomimetics, Vol. E22b (Eds.: Goodman M, Felix A, Moroder L, Tonioi C), Georg Thieme, Stuttgart, 2002, pp. 424.

186 Young T, Kissling LL. A strategy for the synthesis of sulfated peptides. Angew. Chem. 2002; 114: 3599.

187 Bunschoten A, Kruitjzter JA, Ippel JH, de Haas CJ, van Strijp JA, Kemmink J, Liskamp RM. A general sequence independent solid phase method for the site specific synthesis of multiple sulfated-tyrosine containing peptides. Chem. Commun. 2001; 2251.

188 Peters C, Wagner M, Völkert M, Waldmann H. Bridging the gap between cell biology and organic chemistry: chemical synthesis and biological application of lipidated peptides and proteins. Naturwissenschaften 2002; 89: 381.

189 Novelli G, D'Apice MR. Protein farnesylacylation and disease. J. Inherit. Metab. Dis. 2012; 35: 917.

190 Naider FR, Becker JM. Synthesis of prenylated peptides. Biopolymers 1997; 43: 3.

191 Kragol G, Lumbiieres M, Palomo JM, Waldmann H. Protein synthesis: solid-phase synthesis of lipidated peptides. Angew. Chem. Int. Ed. 2004; 43: 5839.

192 Pelegarao S, Moroder L, in Houben-Weyl Methods of Organic Synthesis, Synthesis of Peptides and Peptidomimetics, Vol. E22b (Eds.: Goodman M, Felix A, Moroder L, Tonioi C), Georg Thieme, Stuttgart, 2002, pp. 333.

193 O'Reilly N, Charbin A, Lopez-Serra L, Uhmann F. Facile synthesis of budding yeast a-factor and its use to synchronize cells of a mating type. Yeast. 2012; 29: 283.

194 Kaderet D, Deck P, Heinemann I, Waldmann H. Acid-labile protecting groups for the synthesis of lipidated peptides. Chem. Eur. J. 2001; 7: 1184.

195 Millington CR, Quarrall R, Lowe G. Aryn hydrazides as linkers for solid phase synthesis which are cleavable under mild oxidative conditions. Tetrahedron Lett. 1998; 39: 7201.

196 Diaz-Rodriguez V, Mullen DG, Ganusova E, Becker JM, Distefano MD. Synthesis of peptides containing C-terminal methyl esters using trityl side-chain anchoring: application to the synthesis of a-factor and a-factor analogs. Org. Lett. 2012; 14: 5648.

197 Palk WK, Palk DC, Kim S. Historical review: the field of protein methylation. Trends Biochem. Sci. 2007; 32: 146.

198 Tian X, Fang J, Curren A. Peptide mimetics on histone demethylases. Acta Biochim. Biophys. Sin. 2007; 39: 81.

199 Clarke SG. Protein methylation at the surface and buried deep: thinking outside the histone box. Trends Biochem. Sci. 2013; 38: 243.

200 Bedford MT, Clarke SG. Protein arginine methylation in mammals: who, what, and why. Mol. Cell 2009; 33: 1.
Martin C, Zhang Y. The diverse functions of histone lysine methylation. Nat. Rev. Mol. Cell Biol. 2005; 6: 838.

Chirivi RG, Jennikens GJ, Raats JM. Anti-citrullinated protein antibodies as novel therapeutic drugs in rheumatoid arthritis. J. Clin. Cell Immunol. 2013; 4: 2.

Dabiel M, Crispin M, Scanlan CN, Zitzmann N, Dwek RA. Emerging Chirivi RG, Jenniskens GJ. Anti-citrullinated protein antibodies. J. Am. Chem. Soc. 2010; 132: 11110.

Hiedler P, Link A. N-Acyl-N-alkyl-sulfonamide anchors derived from Kenne's safety catch linker: powerful tools in bioorganic and medicinal chemistry. Biorg. Med. Chem. 2005; 13: 585.

Mende F, Beisswenger M, Seitz O. Automated Fmoc-based solid-phase synthesis of peptide thioesters with self-purification effect and application in the construction of immobilized SH3 domains. J. Am. Chem. Soc. 2013; 135: 13113.

Macmillan D, Bertozzi CR. Modular assembly of glycopolymers: towards the synthesis of GlyCAM-1 by using expressed protein ligation. Angew. Chem. Int. Ed. 2004; 116: 13791.

Flavell RR, Huse M, Goger M, Trester-Zedlitz M, Kuriyan J, Muir TW. Efficient semisynthesis of a tetraphosphorylated analogue of the type TGFβl receptor. Org. Lett. 2002; 4: 165.

Burflina F, Morris C, Behrendt R, White P, Offer J. Simplifying native chemical ligation with an N-acetyl-sulfonamide linker. Chem. Commun. 2012; 48: 2579.

Blanco-Canas JB, Dawson PE. An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. Angew. Chem. Int. Ed. 2008; 47: 6851.

Pascal R, Chavey D, Sola R. Carboxyl-protecting groups convertible into activating groups. Carbamates of O-aminoaldehydes are precursors of reactive N-acetyl ureas. Tetrahedron Lett. 1994; 35: 6291.

Zheng J-S, Tang S, Huang Y-C, Liu L. Development of new thioester equivalents for protein chemical synthesis. Acc. Chem. Res. 2013; 46: 2475.

Zheng J-S, Tang S, Qi Y-K, Wang Z-P, Liu L. Chemical synthesis of proteins using peptide hydrazides as thioester surrogate. Nat. Protoc. 2013; 8: 2483.

Reif A, Siebenhaar S, Tröster A, Schmalzleinsin C, Velsetty P, Gottwald K, Pöhrner C, Boos I, Schubert V. Semisynthesis of biologically active glycoforms of the human cytokine interleukin 6. Angew. Chem. Int. Ed. 2014; 53: 12125.

Siman P, Karthikeyan SV, Nikolov M, Fischle W, Brik A. Convergent chemical synthesis of histone H2B protein for the site-specific ubiquitination at Lys34. Angew. Chem. Int. Ed. 2013; 52: 8059.

Koike Y, Kajihara Y, Nakahara Y, Takeda A, Nakahara Y, Ohtani E. Solid-phase synthesis of O-glycopeptide antigens for therapeutic exploitation of glycosylation. Houben-Weyl Methods of Organic Chemistry: Synthesis, Synthesis of Peptides and Peptidomimetics (Eds.: Gabius H-J, Birk J, Fischle W, Brik A, Engler H, Clayton E). By: European Peptide Society and John Wiley & Sons, Ltd. 2013; 60.

1973: 17917. 1979; 179.

Angew. Chem. Int. Ed. 2013; 124: 2288.

Wilson RM, Stockdill JL, Wu X, Li X, Vadola PA, Park PK, Wang P, Daneshfsy SJ. A fascinating journey into history: exploration of the world of isonitriles en route to complex amides. Angew. Chem. Int. Ed. 2012; 51: 2834.

Sharon N, Lis H, in Glycosciences: Status & Perspectives (Eds.: Gabius H-J, Gabius Sj, Chapman& Hall, Weinheim, 1997; pp. 133.

Sjölin P, Elofsson M, Kihlberg J. Removal of acyl protective groups from glycopeptides: base does not epimerize peptide stereocenters, and β-elimination is slow. J. Org. Chem. 1996; 61: 560.

Takano Y, Kojima N, Nakahara Y, Hojo H, Nakahara Y. Solid-phase synthesis of core 2 O-linked glycopeptide and its enzymatic sialylation. Tetrahedron 2003; 59: 8415.

Leppänen A, Mehta P, Ouyang Y-B, Tu J, Helin J, Moore KL, van Dei I, Canfield WM, McEvoy RP, Cummings RD. A novel glycosulfopropionate binding to P-selectin and inhibits leukocyte adhesion to P-selectin. J. Biol. Chem. 1999; 274: 24838.

Galán MC, Benito-Alifonso D, Watt GM. Carbohydrate chemistry in drug discovery. Org. Biomol. Chem. 2011; 9: 3598.

Otvos L, Merch PE, Fischle W, Brik A. Convergent chemical synthesis of histone H2B protein for the site-specific ubiquitination at Lys34. Angew. Chem. Int. Ed. 2013; 52: 8059.

Siman P, Karthikeyan SV, Nikolov M, Fischle W, Brik A. Convergent chemical synthesis of histone H2B protein for the site-specific ubiquitination at Lys34. Angew. Chem. Int. Ed. 2013; 52: 8059.
Ollivier N, Dheur J, Mhidia R, Blanpain A, Melnyk O. Bis (2-sulfanylethyl) amino native peptide ligation. Org. Lett. 2010; 12: 5238.

Boll E, Drobecq H, Ollivier N, Blanpain A, Raibaut L, Desmet R, Vicogne J, Melnyk O. One-pot chemical synthesis of small ubiquitin-like modifier protein–peptide conjugates using bis (2-sulfanylethyl) amido peptide latent thioester surrogates. Nat. Protoc. 2015; 10: 269.

Tsuda S, Shigenaga A, Bando K, Otaka A. N→S acyl-transfer-mediated synthesis of peptide thioesters using anilide derivatives. Org. Lett. 2009; 11: 823.

Sato K, Shigenaga A, Tsuji K, Tsuda S, Sumikawa Y, Sakamoto K, Otaka A. N-Sulfanylethylanilide peptide as a crypto-thioester peptide. ChemBioChem 2011; 12: 7469.

Terrier VP, Adihou H, Arnould M, Delmas AF, Aucagne V. A straightforward method for automated Fmoc-based synthesis of bio-inspired peptide crypto-thioesters. Chem. Sci 2015. DOI: 10.1039/C5SC02630J.

Cowper B, Shariff L, Chen W, Gibson SM, Di W-L, Macmillan D. Expanding the scope of N→S acyl transfer in native peptide sequences. Org. Biomol. Chem. 2011; 13: 7469.

Ruff Y, Garavini V, Giuseppone N. Reversible native chemical ligation: a facile access to dynamic covalent peptides. J. Am. Chem. Soc. 2014; 136: 6333.

Yu HM, Chen ST, Wang KT. Enhanced coupling efficiency in solid-phase peptide synthesis by microwave irradiation. J. Org. Chem. 1992; 57: 4781.

Pedersen SL, Tofteng AP, Malik L, Jensen KJ. Microwave heating in solid-phase peptide synthesis. Chem. Soc. Rev. 2012; 41: 1826.

Echaller C, Al-Halifa S, Kreiter A, Enjalbal C, Sanchez P, Ronga L, Puget K, Verdié P, Amblard M, Martinez J. Heating and microwave assisted SPPS of C-terminal acid peptides on trityl resin: the truth behind the yield. Amino Acids 2013; 45: 1395.

Yang X, Lin H, Lu W, Wang D. Compatibility study of Merrifield linker in Fmoc strategy peptide synthesis. Prot. Pept. Lett. 2013; 20: 140.

Loffredo C, Assuncao NA, Gerhardt J, Miranda M. Microwave-assisted solid-phase peptide synthesis at 60 °C: alternative conditions with low enantiotomerization. J. Pept. Sci. 2009; 15: 808.

Bacsa B, Horváti K, Bősze S, Andreae F, Kappe CO. Solid-phase synthesis of difficult peptide sequences at elevated temperatures: a critical comparison of microwave and conventional heating technologies. J. Org. Chem. 2008; 73: 7532.

Bacsa B, Bősze S, Kappe CO. Direct solid-phase synthesis of the β-amyloid (1–42) peptide using controlled microwave heating. J. Org. Chem. 2010; 75: 2103.

Roodbeen R, Pedersen SL, Hosselini M, Jensen KJ. Microwave heating in the solid-phase synthesis of N-methylated peptides: when is room temperature better? Eur. J. Org. Chem. 2012; 2012: 7106.

Nissen F, Kraft TE, Ruppert T, Eisenhut M, Haberkorn U, Mier W. Hot or not—the influence of elevated temperature and microwave irradiation on the solid phase synthesis of an affibody. Tetrahedron Lett. 2010; 51: 6216.

Tofteng AP, Jensen KJ, Schäffer L, Hoeg-Jensen T. Total synthesis of desB30 insulin analogues by biomimetic folding of single-chain precursors. ChemBioChem 2008; 9: 2989.

Marek P, Waym AM, Sutton K, Zanni MT, Raleigh DP. Efficient microwave-assisted synthesis of human islet amyloid polypeptide designed to facilitate the specific incorporation of labeled amino acids. Org. Lett. 2010; 12: 4848.

Harris PWR, Kowalczyk R, Hay DL, Brimble MA. A single pseudoproline and microwave solid phase peptide synthesis facilitates an efficient synthesis of human amylin 1–37. Int. J. Pept. Res. Ther. 2013; 19: 147.