# Cysteine and Selenocysteine Deprotection Chemistry in Peptide Synthesis

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**Abstract:** One of the most challenging areas in the field of peptide science is the chemistry of cysteine and the need for multiple protecting groups that can be removed orthogonally to facilitate regioselective disulfide bond formation. This article reviews the methods and reagents used to remove the various protecting groups of cysteine derivatives with subsequent disulfide bond formation used in peptide synthesis. Also reviewed is the related amino acid selenocysteine, which requires benzyl-type protecting groups during synthesis. As these benzyl groups are difficult to remove using older methods, we highlight the use of electrophilic, aromatic disulfides for the removal of selenocysteine (and cysteine) protecting groups with concomitant disulfide and diselenide bond formation.

**Key Words:** Cysteine, selenocysteine, protecting groups, deprotection, peptides.

## INTRODUCTION

As the field of peptide synthesis advances, new and innovative practical research initiatives are required in order to satisfy the architectural demands of increasingly complex systems. Peptide templates and peptidomimetic drug design have become progressively more popular targets for the peptide chemist, posing significant synthetic challenge. As such, one must be equipped and well-versed in a wide spectrum of assembly methodologies in order to gain synthetic entry into challenging systems.

Central to the successful synthesis of many challenging peptidyl systems is the judicious choice of protection protocol for the potentially reactive amino acid side-chain functionalities during chain elongation, especially when post-synthetic manipulation of the completed peptide is desired. For no amino acid residue is the variety of potential protecting groups more abundant than in the case of the sulfhydryl side-chain of cysteine, mostly in response to recent advances in directed disulfide formation in cysteine-containing peptides. The necessity for multiple layers of protecting group orthogonality in the design and sequential construction of disulfide bridges within a peptide template demands a wide variety in protection protocol, ultimately allowing a greater measure of control over this oxidative process. While directed disulfide formation in peptide systems accounts for the majority of the uses of orthogonal cysteine protection protocol, other peptidyl post-synthetic manipulations requiring cysteine sulfhydryl deactivation include applications in Native Chemical Ligation (NCL), selective S-alkylation, and cysteine-caged protein systems. Although used to a much lesser extent in solid-phase peptide synthesis (SPPS), selenocysteine (Sec, U) has been finding increasing application in synthetic peptide and protein construction due to its unique chemistry and oxidative properties. In stark contrast to its chalcogen analog cysteine, existing protecting group protocol for selenocysteine is fairly sparse, due primarily to the limited application this amino acid has historically held in peptide synthesis. With protecting group architecture populated almost exclusively with substituted benzyl motifs, selenocysteine deprotection protocol has traditionally had to rely on harsh reaction conditions. Of late, however, gentler new deprotection methodologies have emerged for these traditionally steadfast systems. Further, with the advent of new protection protocol for Sec, the facile post-synthetic manipulation of these selenium-containing systems is within grasp.

Although efforts have been made in this review to offer a complete snapshot of existing cysteine and selenocysteine deprotection methodologies, it is by no means a comprehensive listing. While several other book volumes [1-4] and journal reviews [5-8] in this arena are inventory-focused, the present effort attempts to categorize existing protocol already in use, emphasizing their utility in practical applications and post-synthetic manipulation beyond simply that of constructing the peptide sequence itself. Emphasis will also be placed upon cysteine protection typically utilized in Fmoc SPPS as this methodology has, for the most part, become the industry standard by which most present peptide synthesis is generated. Although efforts are made to categorize protecting group architecture, certain groups defy classification based on specific criteria or are removable by overlapping sets of conditions. As presented in Table **1**, although the overall taxonomy of placement may not apply to some of these systems, they are nevertheless included as a "best-fit" in order to highlight their use in practical orthogonal application with other protecting groups.

## ACID-LABILE, BENZYL-TEMPLATED CYSTEINE PROTECTION

In Fmoc SPPS, the primary vector of orthogonality to the base-mediated conditions of peptide elongation is variation in acidity. Conditions of low pH classically accomplish the dual purpose of cleaving the peptide from the solid support and removing standard sidechain protecting groups. In the case of acid-labile cysteine protection, it is possible to take advantage of gradations in acid stability of protecting groups within the same structural family in order to offer additional opportunities for orthogonal layering in areas such as disulfide topography design. Indeed, a significant number of protecting components described in this review, both acid-labile and acid-stable, have as their underlying structural basis a benzyl architecture. With acid-stability of these benzyl-derived functionalities dependant upon *degree* of benzylation and number of *o,p*-electron-donating substituents decorating the aromatic rings [9], a wide spectrum of acid-catalyzed protecting-group lability becomes possible, allowing full orthogonality between certain members of this family.

The triphenylmethyl (Trt) protecting group (**1**) is by far the most standard protocol in Fmoc SPPS for the production of free-sulfhydryl, cysteine-containing peptides [10] and, as the least substituted member of this triply-benzylic family, is considered the most acid stable. Typically, following peptide elongation, the Trt moiety is easily removed at the peptide cleavage step through treatment with 95% trifluoroacetic acid (TFA) accompanied by various cation scavengers, although effective removal with acidic cocktails as low as 5% TFA has been reported [11]. In addition to having the ability to act as an orthogonal blocking component to various acid-stable Cys protective functionalities, the Trt group can also be cleaved under neutral conditions using molecular iodine [12] or heavy metal salts [12,13]. As will be illustrated, Trt protection can be strategically disposed within an orthogonal cysteine pro-

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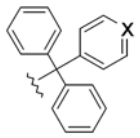
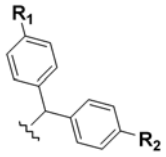
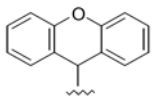
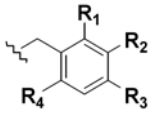
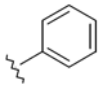
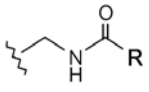
**Table 1-1.**

| | Name | Structure | Deprotection Conditions | Ref. |
|---|---|---|---|---|
| **Acid-labile Benzyl-Type** | Trt (**1**)<br>X = C-H |  | 96:2:2 TFA/$iPr_3SiH/H_2O$<br>$I_2$<br>$HgOAc_2$ | [10]<br>[12]<br>[13] |
| | Mmt (**2**)<br>X = C-$OCH_3$ | | 1:4:95 TFA/$iPr_3SiH$/DCM | [14] |
| | Pdm (**3**)<br>X = N | | $HgOAc_2$ or $I_2$ | [17] |
| | Dpm (**4**)<br>$R_1,R_2$ = H |  | Na/liq. $NH_3$ or<br>2N HBr in AcOH | [18] |
| | Ddm (**5**)<br>$R_1,R_2$=$OCH_3$ | | TFA/anisole | [19] |
| | Xan (**6**) |  | 1:0.5:98.5 TFA/$Et_3SiH$/DCM | [20] |
| | Tmob (**7**)<br>$R_1,R_3,R_4$=$OCH_3R_2$ = H | | 7:1:1:90 TFA/$Et_3SiH/H_2O$/DCM | [22] |
| **Acid-stable Benzyl-Type** | Tmb (**8**)<br>$R_1,R_3,R_4$ = $CH_3$ $R_2$ = H | | TFA (reflux) | [26] |
| | Dmb (**9**)<br>$R_2,R_3$ = $CH_3$ $R_1,R_4$ = H |  | HF, anisole | [27] |
| | Mob (**10**)<br>$R_3$ = $OCH_3$ $R_1,R_2,R_4$ = H | | 10% DMSO/TFA<br>DTNP/TFA/(PhSMe) | [37]<br>[38] |
| | Meb (**11**)<br>$R_3$ = $CH_3$ $R_1,R_2,R_4$ = H | | 5% DMSO/TFA<br>DTNP/TFA/PhSMe (partial) | [55]<br>[39] |
| | Bzl (**12**)<br>$R_1,R_2,R_3,R_4$ = H | | Na/liq. $NH_3$ | [32] |
| Ph (**13**) | |  | Pd(II)/TBDMS-H [Cys]<br>$NaIO_4$/DCM/MeOH [Sec] | [44]<br>[45] |
| Acm (**14**)<br>R = $CH_3$ | | | $Hg(OAc)_2$<br>$I_2$<br>DTNP/PhSMe/TFA | [47]<br>[48]<br>[38] |
| Tacm (**15**)<br>R = *t*Bu | |  | $I_2$<br>$AgBF_4$ | [50]<br>[51] |
| Phacm (**16**)<br>R = $CH_2Ph$ | | | Penicillin amidohydrolase | [52] |

Most of the monobenzyl-derived protecting groups are easily removable with HF. However, since this review is not primarily focused on Boc SPPS (of which HF is the primary cleavage medium), it is not mentioned here as a deprotectant if less harsh conditions are applicable.

Trityl = triphenylmethyl; Mmt = 4-methoxytrityl; TMTr = 4,4',4''-trimethoxyphenylmethyl; Pdm = pyridyldiphenylmethyl; Dpm = diphenylmethyl; Ddm = 4,4'-dimethoxyphenylmethyl; Xan = 9H-xanthen-9-yl; Tmob = 2,4,6-trimethoxybenzyl; Tmb = 2,4,6-trimethylbenzyl; Dmb = 3,4-dimethylbenzyl; Mob = 4-methoxybenzyl; Meb = 4-methylbenzyl; Bzl = benzyl; Ph = phenyl; Acm = acetamidomethyl; Tacm = trimethylacetamidomethyl; Phacm = phenylacetamidomethyl.

**Table 1-2.**

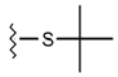
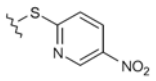
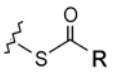
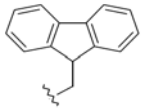
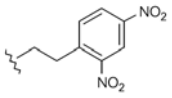
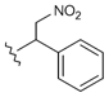
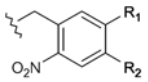
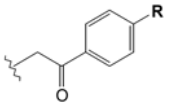
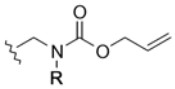
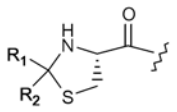
| | Name | Structure | Deprotection Conditions | Ref. |
|---|---|---|---|---|
| | *t*Bu (**17**) |  | NpsCl/AcOH<br>5% DMSO/TFA<br>DTNP/PhSMe/TFA | [54]<br>[55]<br>[39] |
| **Reductively-labile** | S*t*Bu (**18**) |  | $(n\text{-Bu})_3P$<br>20% βME/DMF/NMM<br>DTNP/PhSMe/TFA | [59]<br>[58]<br>[39] |
| | 5-Npys (**19**) |  | βME | [63 |
| | SZ (**20**)<br>R = OBn |  | HBr/AcOH | [65] |
| | Scm (**21**)<br>R = $OCH_3$ | | RSH | [66] |
| | Snm (**22**)<br>R = $N(CH_3)Ph$ | | DTT/NMM/$CHCl_3$ | [67] |
| **Base-labile** | Fm (**23**) |  | 20% piperidine/DMF | [69] |
| | Dnpe (**24**) |  | 20% piperidine/DMF | [74] |
| | Npe (**25**) |  | Mild alkaline | [75] |
| **Photo-labile** | *o*-Nb (**26**)<br>$R_1$,$R_2$ = H |  | hν (366 nm) | [79] |
| | NDMD (**27**)<br>$R_1$,$R_2$ = $OCH_3$ | | hν (340-400 nm) | [81] |
| | Phenacyl (**28**)<br>R = H |  | Mg/AcOH | [88] |
| | *p*-Hp (**29**)<br>R = OH | | hν (312 nm) | [89] |
| **Pd-labile** | Allocam (**30**)<br>R = H |  | $Bu_3SnH/PdCl_2(PPh_3)_2$/AcOH | [94] |
| | Fnam (**31**)<br>R = 2,3,5,6-tetrafluoro-4-(N'-piperidino)-phenyl | | | [95] |
| | Fsam (**32**)<br>R = 2,3,5,6-tetrafluoro-4-(phenylthio)-phenyl | | $PhSiH_3/Pd(PPh_3)_4$/AcOH | [96] |

**Table 1-2. contd….**

| | Name | Structure | Deprotection Conditions | Ref. |
|---|---|---|---|---|
| | Thz (**33**) |  | HCl, $H_2O$, MeOH [$R_1$,$R_2$ = $CH_3$] | [97] |
| | | | Methoxyamine HCl [$R_1$,$R_2$ = H] | [98] |
| | | | 3-mercaptopropiosulfonic acid [$R_1$,$R_2$ = Ninhydryl] | [99] |

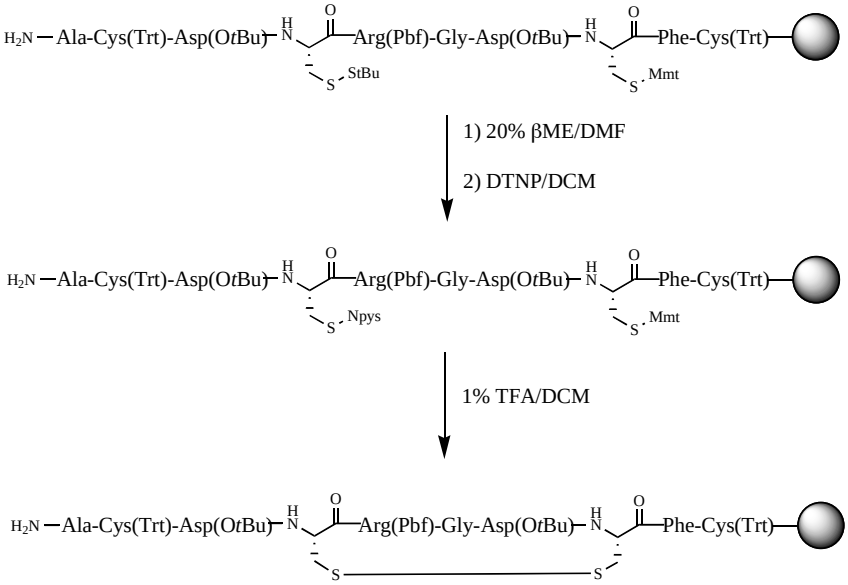
*t*Bu = *tert*-butyl; S*t*Bu = *tert*-butylmercapto; 5-Npys = 5-nitro-2-pyridinesulfenyl; SZ = carbobenzyloxysulfenyl; Scm = *S*-carbomethoxysulfenyl; Snm = (*N*'-methyl-*N*'-phenylcarbamoyl)sulfenyl; Fm = 9-fluorenylmethyl; Dnpe = 2-(2,4-dinitrophenyl)ethyl; Npe = 2-nitro-1-phenylethyl; oNb = *o*-nitrobenzyl; NDMD = 2-nitro-4,5-dimethoxybenzyl; *p*-Hp = *p*-hydroxyphenacyl; Allocam = allyloxycarbonylaminomethyl; Fnam = *N*-[2,3,5,6-tetrafluoro-4-(*N*'-piperidino)-phenyl], *N*-allyloxycarbonylaminomethyl; Fsam = *N*-[2,3,5,6-tetrafluoro-4-(phenylthio)-phenyl], *N*-allyloxycarbonylaminomethyl; Thz = thiazolidine.

tection scheme as an integral component of a stepwise deprotection cascade (*vide infra*).

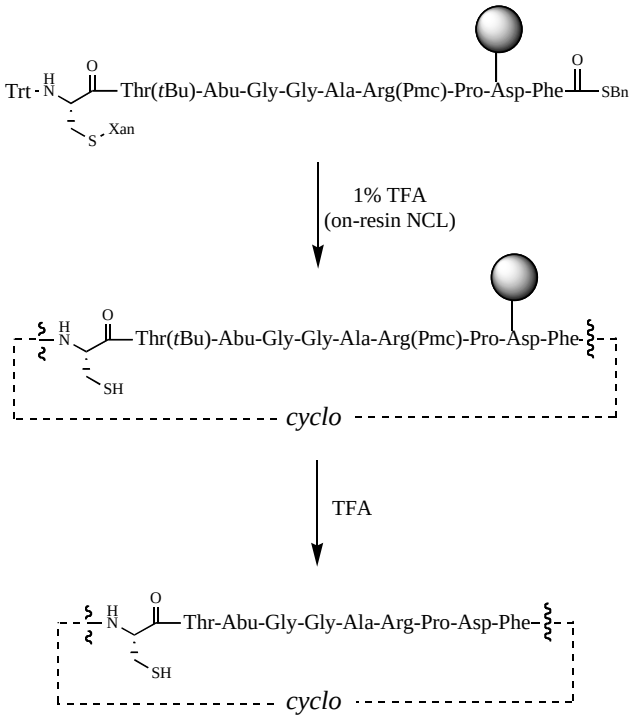
When electron-donating substituents are strategically placed at the *ortho* and/or *para* positions on the Trt aromatic rings, a higher degree of acid lability is conferred upon the protecting component. Typically in the form of methoxy-substitution, their electron-donating ability serves to further stabilize the Trt cation released during acidolytic cleavage, thus requiring only a catalytic amount of acid (and appropriate scavenger) for its complete removal [9]. A very commonly used protecting group of this type is the 4-methoxytrityl (Mmt) group **2** [14], whose singular methoxy substituent allows for deprotection conditions of 1% TFA. Although sparingly used as a sulfhydryl protectant since its introduction in 1995, it has begun to find increasing application in post-synthetic on-resin cysteine manipulation [15]. The dilute acidic conditions required for its removal allow for a degree of orthogonality to the higher acidic concentration required for cleavage of the peptide from the resin. Galande and coworkers were able to carry out an on-resin directed disulfide formation using the orthogonal deprotection of Cys(Mmt) as a key step in the synthesis (Scheme **1**). Other variations on the trityl architecture have been reportedly utilized for cysteine protection, but have not found noteworthy or strategic utility since their discovery and subsequently will not be elucidated here beyond their mention. The triply-oxygenated 4,4',4"-Trimethoxyphenylmethyl (TMTr) group and the Pyridyldiphenylmethyl (Pdm) group **3** have been reported, requiring dilute (1%) TFA and electrolytic reduction for their respective removal [16,17].

Although there are several examples of doubly-benzylic cysteine protection design in the literature, most of them have never been used to any significant extent in cysteine manipulation. The Diphenylmethyl (Dpm) group **4** and the Dimethoxyphenyl-methyl (Ddm) group **5**, requiring varying degrees of acidity for their removal, are rarely mentioned in the literature beyond the synthetic procedures required for their manufacture [18,19]. The only example of this subtype which has shown practical utility in peptide synthesis is the Xanthenyl (Xan) group **6**, developed by Barany and coworkers [20]. Constraining the two phenyl rings with a *bis*-ortho-ether linkage, the Xan motif bears a planar tricyclic substructure. Typical protocol for its removal are treatment with very dilute (~1%) TFA, surprisingly mild conditions given its doubly-benzylic architecture and, ostensibly, less stable cationic potential than its Trt-type analogs. Presumably, this enhanced acid-lability results from its constrained planarity, allowing for facile electron flow from the *ortho*-situated oxygen atom through the permanently-aligned, conjugated $\pi$-orbital system upon deprotection. Barany went on to prove the utility of this blocking protocol in many instances, particularly in the case of using an N-terminal Trt-Cys(Xan)-OH derivative as part of an intramolecular native chemical ligation [21] in the construction of a solid-supported cyclic peptide template (Scheme **2**).

Cysteine and selenocysteine protecting groups based on a mono-benzylic architecture are almost exclusively TFA-stable. The only derivative of this type which bears acid-lability is the highly oxygenated 2,4,6-trimethoxybenzyl (Tmob) group **7**, developed in 1991 by Barany as a highly acid-labile alternative to the tradition-




**Scheme 1.**



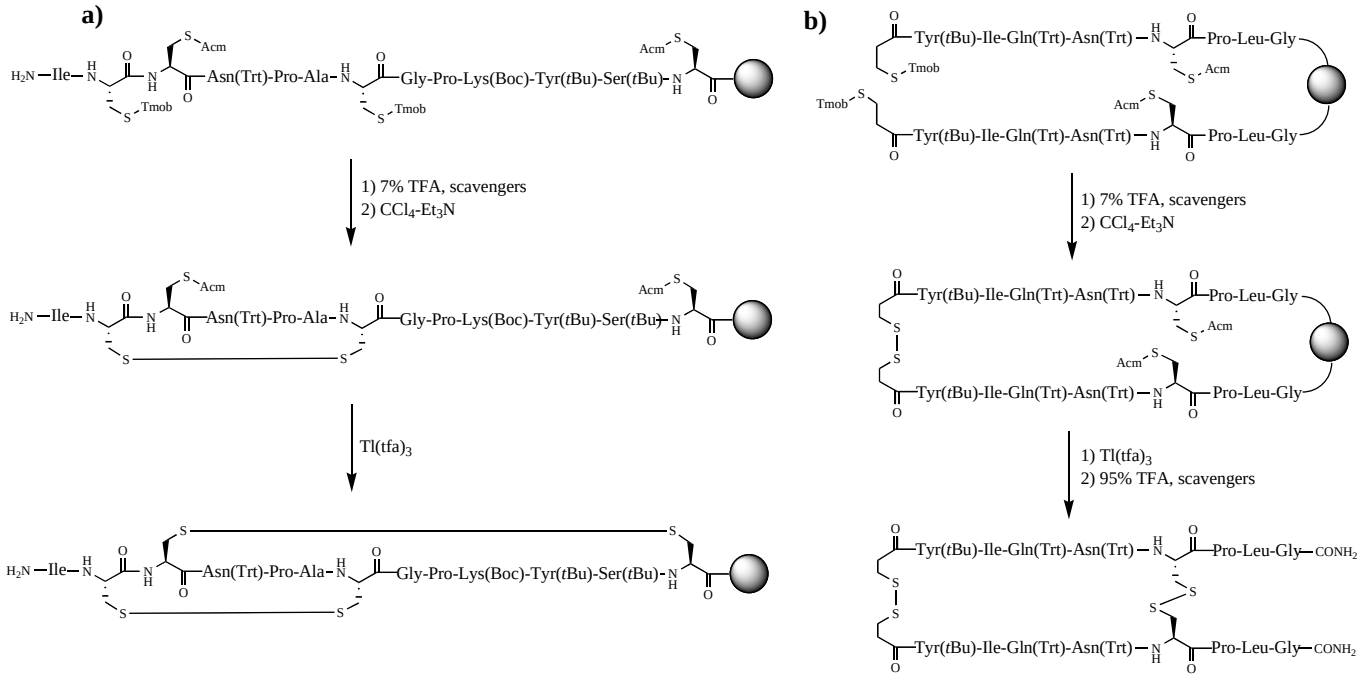

**Scheme 2.**

ally-stable benzyl family of protection design [22]. Predating the exquisitely-acid-labile, oxygenated, multi-benzylic protecting protocol, Tmob typically requires at least 7% TFA in its removal conditions. It was, however, a significant step forward in chalcogen protection, as all other benzyl-type design required much harsher removal conditions (*vide infra*). Utilization of this protecting component quickly gained favor as an effective orthogonal component to the classic acetamidomethyl group in the design of multiple disulfide-containing systems. Barany was able to make use of the Tmob/Acm orthogonality in an early syntheses of α-conotoxin SI [23] and deamino-oxytocin [24] (Scheme **3**) by way of selective removal of the Tmob components (and subsequent oxidation) of the first pair of sulfhydryls before debocking and oxidizing of the Acm-protected counterparts. In the latter case, an on-resin parallel dimer of the desired product was formed. A potential drawback to the use of Tmob protection is its enhanced propensity to form Tmob-conjugated adducts, even during scavenger-assisted deprotection. As a noteworthy solution to this problem, Garvey and coworkers were able to obtain superior results by carrying out the deprotection in aqueous acidic media, utilizing L-cysteine as the sole scavenger [25].

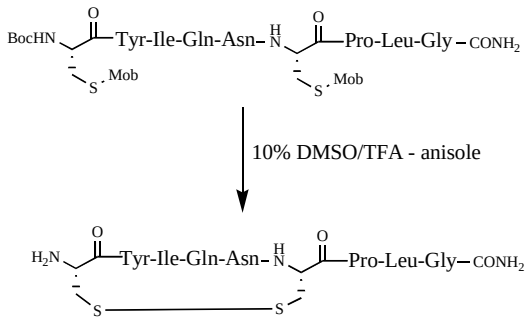
## ACID-STABLE, BENZYL-TEMPLATED CYSTEINE/SELENOCYSTEINE PROTECTION

As previously mentioned, the remainder of the benzyl-type scaffold of protection are TFA-stable. Although there invariably exist some obsolete and unused examples of this general architecture {i.e: the 2,4,6-Trimethylbenzyl (Tmb) group **8** [26] and 3,4-dimethylbenzyl (Dmb) group **9** [27]}, the 4-Methoxybenzyl (Mob) **10**, 4-Methylbenzyl (Meb) **11**, and Benzyl (Bzl) **12** structural variants have historically found great utility in Cysteine protection [28-30]. The Bzl group **12**, introduced by du Vigneaud and coworkers, is rarely utilized in Fmoc SPPS (although occasionally still used in Boc processes), as its classic removal conditions, HF [31] or metallic sodium in liquid ammonia [32], typically prove too harsh and have been known to damage the final peptide products [33].

The choices of Meb or Mob cysteine protection are much more common due to the varying electron-donating abilities of the *para* phenyl substituents, allowing for deblocking under somewhat less forceful conditions. It is, however, somewhat of a rare occurrence for Meb and Mob functionalities to be utilized in Fmoc SPPS, as their removal typically requires the use of heavy metals [34], HF [35], or the strongly-oxidizing conditions of diphenylsulfoxide-tetramethylsilane [36]. Illustrative of the continual quest for gentler deprotection conditions for these benzyl derivatives, Fujii carried out a simultaneous deprotection and oxidation of a Mob-protected Oxytocin precursor (Scheme **4**) to demonstrate the utility of the relatively gentle deprotection cocktail of DMSO in trifluoroacetic acid which he had developed [37]. Recently, Hondal and coworkers




**Scheme 3.**

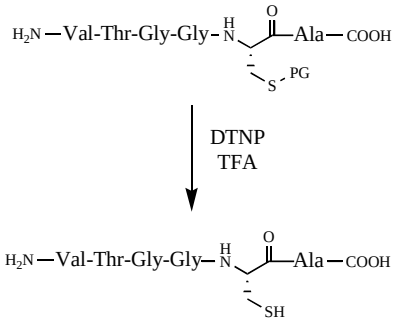



**Scheme 4.**

benzyl Sec protection which is well-established for cysteine (ie: *t*Bu, Acm, S*t*Bu; *vide infra*) appears to be nonexistent, as well as any hypotheses which would preclude their use in SPPS).

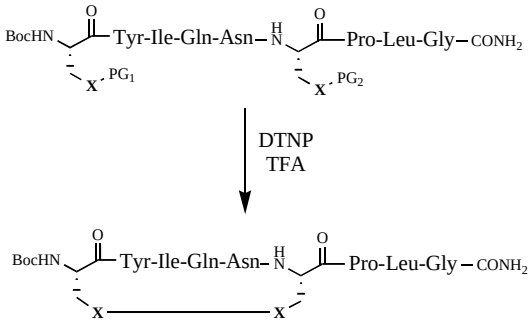
Deblocking conditions for the three commonly used benzyl functionalities (ie: Mob, Meb, Bzl) are practically identical for both Cys and Sec conjugates, with a few notable exceptions. The methodology of Hondal is even more efficient at Sec(Mob) removal, requiring no added thioanisole and only catalytic amounts of DTNP in the deprotection mixture [38]. Taking advantage of this orthogonality in thioanisole requirement, the researchers directed a stepwise selenylsulfide bond formation on a β-turn mimetic substrate, separating the deprotection/activation step of one partner from that

**a)**




| PG | % Yield | Equivalents DTNP | Thioanisole |
|---|---|---|---|
| Acm | 100 | 15 | Yes |
| *t*Bu | 100 | 15 | No |
| S*t*Bu | 90 | 15 | Yes |
| Mob | 100 | 2 | Yes |
| Meb | 10 | 15 | Yes |
| Bzl | no reaction | 15 | No |

**b)**



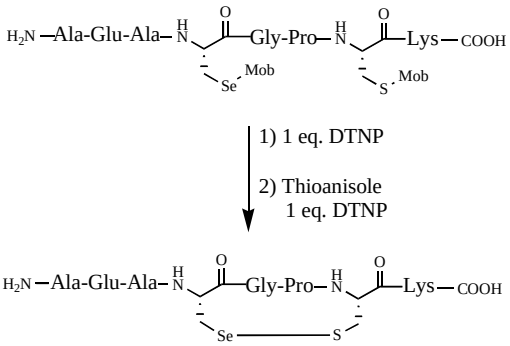

| X | $PG_1$ | Y | $PG_2$ | Equivalents DTNP | Thioanisole |
|---|---|---|---|---|---|
| **S** (Cys) | Acm | **S** (Cys) | Acm | 15 | Yes |
| **S** (Cys) | *t*Bu | **S** (Cys) | *t*Bu | 15 | Yes |
| **S** (Cys) | Mob | **S** (Cys) | Mob | 6 | Yes |
| **Se** (Sec) | Mob | **S** (Cys) | Mob | 1 | Yes |
| **Se** (Sec) | Mob | **Se** (Sec) | Mob | 1 | Yes |
| **Se** (Sec) | Mob | **Se** (Sec) | Mob | 3 | No |

**Scheme 5.**

introduced a comparatively more gentle methodology for Mob deprotection which doesn't require the forcing removal conditions previously required. The researchers found that treatment of a Cys(Mob)-containing peptide with 2 equivalents of 2,2′-dithiobis(5-nitropyridine) (DTNP) in 2% thioanisole/TFA solvent resulted in complete Mob deprotection within one hour [38]. This methodology was further utilized to simultaneously deprotect and assemble both native and non-native oxytocin from Mob-protected precursor peptides [39] (Scheme **5**). It has been found that, although this protocol is very efficient for Mob deprotection, it is only partially effective for Meb-protected variants.

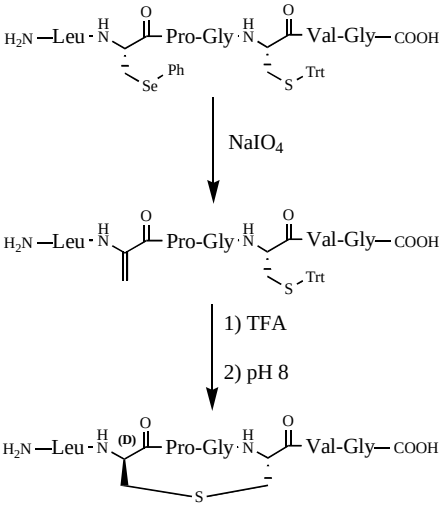
The aforementioned major benzyl-type functionalities (Mob, Meb, Bzl) also happen to be the only major Selenium-protecting components which have been utilized for selenocysteine incorporation in SPPS [40-42]. The striking paucity of selenocysteine protecting protocol as compared with that of cysteine has been commented on by Moroder [43], in which it is postulated that Sec blocking groups of the trityl variety promote loss of α-stereointegrity due to enhanced deselenation effects by the trityl cation during deprotection. Interestingly, literature mention of non-

of the other through the measured addition of thioanisole to the reaction mixture (Scheme **6**).




**Scheme 6.**

The Phenyl group (Ph, **13**), has garnered application as a chalcogen protectant for both Sec and Cys, although it has never been utilized for peptide synthesis in the case of the latter [44]. For sele-




**Scheme 7.**

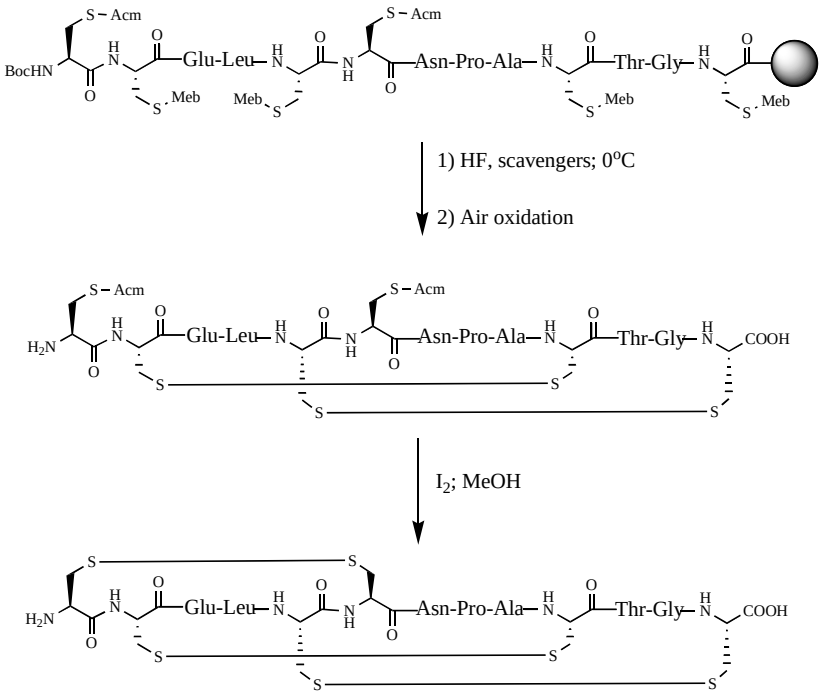
nocysteine, the Phenyl group offers a blocking protocol for a specific purpose: that of acting as a synthetic precursor to a dehydroalanine (Dha) residue. Nα-Fmoc-protected Se-phenyl selenocysteine has been introduced by van der Donk as a SPPS-compatible masked Dha residue in his synthesis of lantibiotic ring fragments [45] (Scheme **7**). In other utilization, Shirahama and coworkers used Sec(Ph) protection to illustrate different Dha-forming reaction conditions favoring syn and anti elimination pathways respectively [46].

## NON-BENZYL CYSTEINE PROTECTION

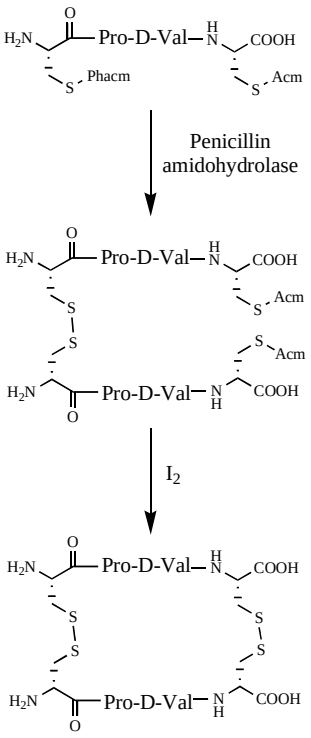
One of the most enduring functionalities in cysteine protection is the acetamidomethyl (Acm) group **14** [47]. Traditionally utilized exclusively for Boc SPPS, its amenity toward removal under a multitude of conditions have allowed it to become commonplace in Fmoc-based syntheses as well. Traditional methods for its removal, however, employ relatively harsh and potentially toxic conditions such as heavy metal ions [24, 25] and iodine [10], although the gentler cocktail of DTNP/thioanisole/TFA has been shown to be effective in some cases [38]. As a testament to its robust nature, in multiple disulfide formation processes, an Acm-protected cysteine pair is typically deblocked (and subsequently modified) as the *final* manipulation in a multi-step sequence. In their synthesis of the double disulfide-containing α-Conotoxin SI [24], Munson and coworkers used $Tl(tfa)_3$ to simultaneously deblock and oxidize a resin-bound Cys(Acm) pair as the final step (Scheme **3**). In another example, Takeda carried out an $I_2$-mediated deprotection/oxidation of a Cys(Acm) pair as the ultimate post-synthetic modification in the synthesis of the triple disulfide-containing fragment of Enterotoxin [48] (Scheme **8**). Acm has also been used as the N-terminal cysteine protectant for the central peptide fragment in native chemical ligation efforts, in particular Kent's stepwise synthesis of Crambin [49].

There do exist variations on the Acm architecture which have found some limited utility in peptide synthesis. The trimethylacetamidomethyl (Tacm) group **15** [50,51] and phenylacetamidomethyl (Phacm) group **16** [52] each bear the sturdy protective features of their Acm counterpart and, as such, typically require similar forcing conditions to effect their removal. Unique in its relationship to the Acm group, however, the deprotection of the Phacm functionality can also be effected enzymatically using penicillin amidohydrolase (PAH). When carried out in the presence of a pairing of Cys(Phacm) residues, the deprotection results in concomitant conversion to the disulfide linkage. Illustrating the utility of this methodology, and of the marked orthogonality between Acm and Phacm, Royo and coworkers established a stepwise interchain pairing of two disulfides in tetramer Ac-Cys(Phacm)-Pro-D-Val-Cys(Acm)-$NH_2$ [52] (Scheme **9**).

The *tert*-Butyl (*t*Bu) group **17** represents another robust thiol protecting functionality, typically requiring forcing removal conditions similar to those for Acm abstraction [53]. In stark contrast to its facile removal as an oxygen protectant, *tert*-butyl blocking in the case of a thiol is very resistant to acidolysis. Typical classical conditions for *t*Bu removal are HF and Hg(II), as well as with strong electrophiles such as NpsCl [54], although gentler methods have recently been discovered and have begun to gain synthetic favor.




**Scheme 8.**



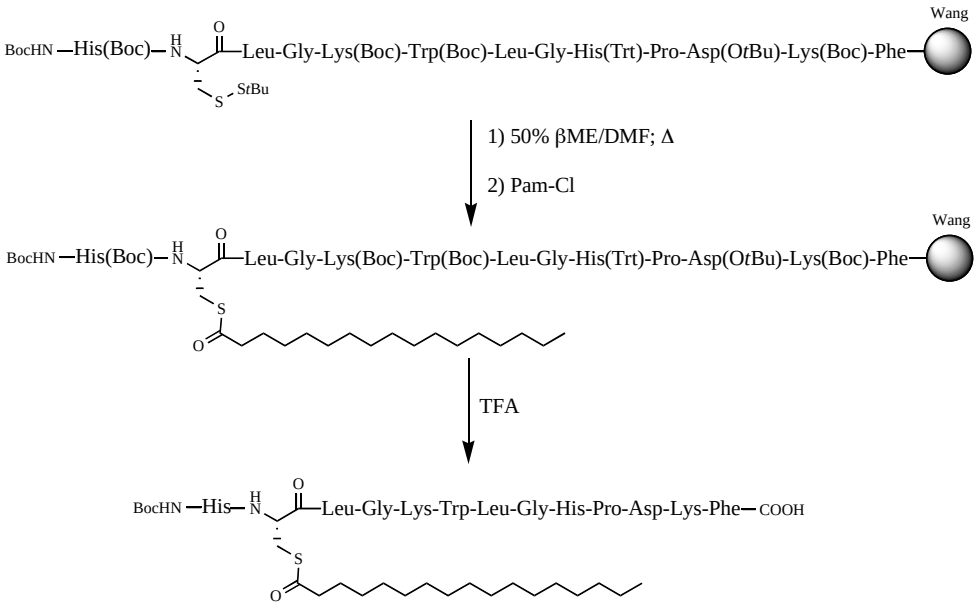

**Scheme 9.**

The DTNP method of Hondal [39] as well as DMSO in aqueous acid [37] are comparatively mild protocols, yet are completely effective at removing this protecting group. In a very elegant use of nearly-identical conditions to achieve orthogonality between two protecting components, Indrevoll's synthesis of an α-conotoxin [55] used 5% DMSO at room temperature for 30 minutes to selectively deprotect and oxidize a Cys(*t*Bu) pair in the presence of a Cys(Meb) pair (Scheme **10**).

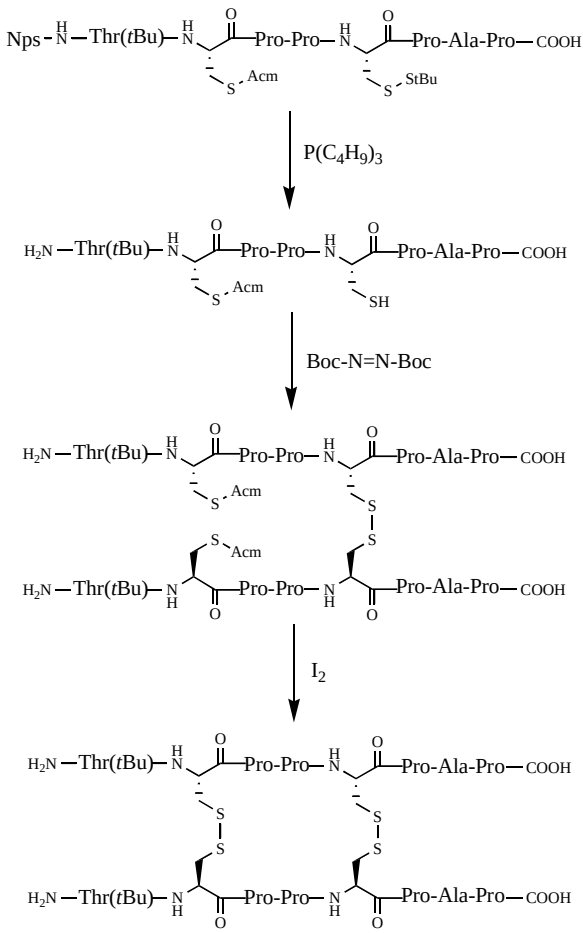
Up to the present point, acidic conditions have been the primary vehicle with which to initiate cysteine protecting group removal, with orthogonality between the methods dictated by the degree of acidity and the accompanying reagents added to the deprotection cocktail. What follows here is a departure from deprotection protocol derived from an acidic environment, rather focusing on cysteine protective species which are reductively-labile. The most recognizable member of this family is the *tert*-Butylmercapto (S*t*Bu) group **18**, taking the form of an asymmetric disulfide at the cysteine sulfhydryl [56]. The most common removal method for this protecting protocol is the use of 20% β-mercaptoethanol (βME) in DMF [15]. Since difficulties in achieving complete S*t*Bu deprotection using this methodology have been noted [57], optimization via the addition of a tertiary amine base to the deprotection cocktail has been reported [58]. S*t*Bu protection has also found utility in on-resin cysteine alkylation. In their synthesis of palmitoyl-thioester T-cell epitopes of Myelin Proteolipic Protein, Denis and coworkers were able to use this protection scheme to assist in cysteine acylation in a solid-supported model peptide by first reducing off its S*t*Bu protection followed by immediate treatment with palmitoyl chloride [57] (Scheme **11**). Phosphine reduction, typically carried out using (*n*-Bu)$_3$P, found early application in the selective removal of StBu protection in the case of solution peptide syntheses, showcasing this group's exquisite orthogonality to the majority of commonly-used cysteine protection schemes. This enhanced orthogonality was put to use by Wünsch [59] in the synthesis of the bis-cystinyl fragment 225-232/225'-232' of Human IgGl hinge region (Scheme **12**). Through the use of Cys(S*t*Bu)/Cys(Acm) orthogonal pairs, the researchers selectively deblocked the Cys(StBu) pair using (*n*-Bu)$_3$P, followed by disulfide formation using the novel sulfenyl hydrazide method [60].

The introduction of electron-withdrawing components into the protective disulfide framework creates a condition in which the sulfhydryl becomes activated toward oxidative attack (Fig. **1**). There are numerous instances of this type of blocking functionality in the literature, however it would be disingenuous to term them "protective", as their primary utility is to introduce an umpolung in reactivity at the cysteine sulfur. This reversal of reactivity can be


**Scheme 10.**




**Scheme 11.**




**Scheme 12.**

quite useful in the directed formation of disulfide architecture into a peptide from comparatively non-reactive precursors.

The most recognizable activated disulfide Cys functionalization is the Npys group **19**, nitro-substituted at the 3- or 5-position. It is derived from the reaction of the cysteine sulfhydryl with the corresponding sulfenyl halide [61] or, more frequently, the symmetrical

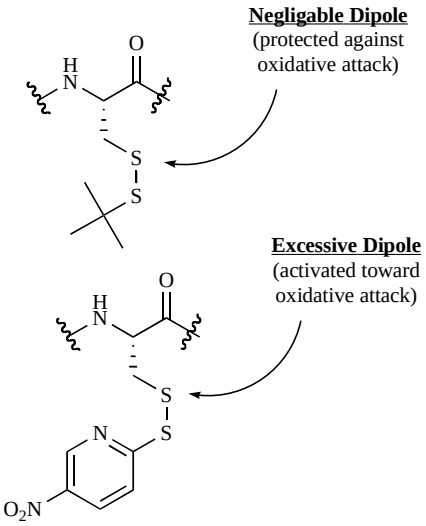
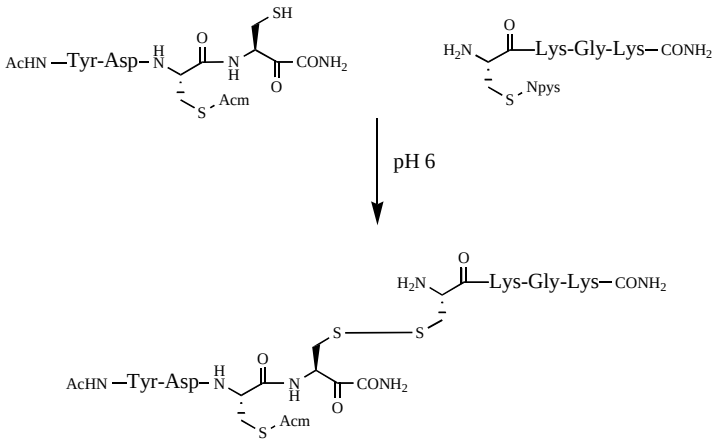


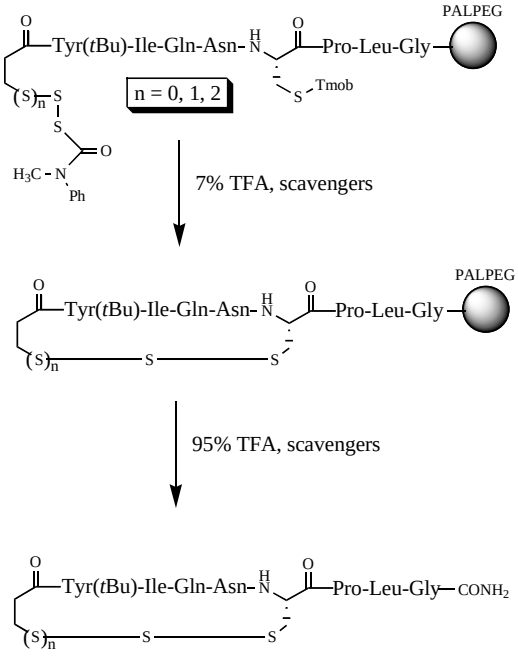

**Fig. (1).**

disulfide, 2,2'-dithiobis-(5-nitro-pyridine) (DTNP) [15,62]. Since the activated cysteine then presumably undergoes further modification (usually via oxidative attack of another cysteine), removal of the blocking moiety to regenerate the native sulfhydryl is usually not an issue. However, in the event that this is a desired outcome, complete Npys removal has been accomplished using βME as a reductant [63]. Notable examples of the utility of this blocking functionality include the activation/cyclization of a variety of disulfide-containing peptides in an on-resin, stepwise approach by Galande [15] (Scheme **1**), as well as the solution synthesis of several ω-conotoxin disulfide fragments by Simmonds and coworkers [64] (Scheme **13**).

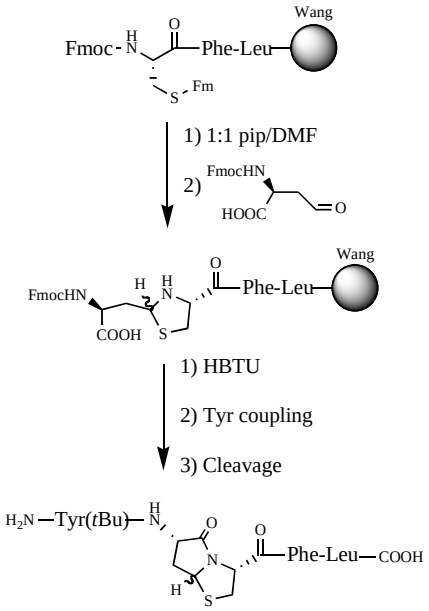
Another category of activated disulfide cysteine blocking agent of any significant import is of the sulfenyl thiocarbonate architecture. The carbobenzyloxysulfenyl (SZ, **20**) and carbomethoxysulfenyl (Scm, **21**) groups, introduced by Berndt [65] and Hiskey [66] respectively, saw relatively little utility as cysteine protectants until Scroll and Barany introduced the (*N*'-methyl-*N*'-phenylcarbamoyl)sulfenyl (Snm, **22**) group as a functionally more stable thiocarbamyl analog of this type [67]. In an elegant application of




**Scheme 13.**




**Scheme 14.**




**Scheme 15.**

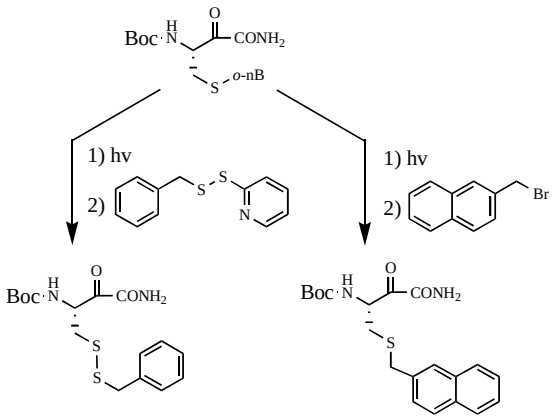
these and analogous activated cysteine derivatives, Barany carried out the syntheses of native and de-amino analogs of oxytocin, inducing the creation of both disulfide as well as *tri/tetra-sulfide* variants of these common templates [68] (Scheme **14**).

Seemingly incongruous with Fmoc SPPS are a series of cysteine protecting groups whose orthogonal lability vector is base-promoted. Piperidine-mediated α-Fmoc deprotection typically precludes the use of these types of blocking protocol. Indeed, the vast majority of the uses of this subset of cysteine protection are in the synthesis of peptides via Boc protocol. The most commonly-encountered constituent of this category is the 9-fluorenylmethyl (Fm) group **23** [69,70], structurally similar to the Fmoc group itself. Stable to TFA and HF, its use as a blocking group in post-synthetic manipulation following Boc synthesis is well-established, particularly in the arena of disulfide bond formation [71,72]. It has, however, proven useful in Fmoc syntheses as well, provided its cysteine is placed at the N-terminus of the peptide. Hruby was able to make use of Fm protection protocol in his Fmoc synthesis of various bicyclic β-turn dipeptides [73] (Scheme **15**).

There are additional examples of base-labile cysteine protecting groups, although their application in the literature is extremely limited. Further, in the occurrences in which they are employed in SPPS, syntheses are primarily Boc-oriented, and aren't significantly cited outside their original introductory publication. The 2-(2,4-dinitrophenyl)ethyl (Dnpe) group **24** and the 2-nitro-1-phenylethyl (Npe) group **25**, introduced by Royo *et al.* [74] and Jung *et al.* [75] respectively, are instances wherein these protecting protocol haven't gained significant literature popularity beyond that of the authors.

## ATYPICAL CYSTEINE PROTECTION

At the relative end of the spectrum of utility are several series of cysteine protecting protocol which, due to their limited usage in SPPS (or peptide synthesis in general), are relatively unknown or unrecognized. However, they do bear mentioning here, as their potential contribution toward cysteine manipulation remains largely unexplored. Of greatest significance are the additional vectors of orthogonality which have yet to be taken advantage of to any considerable extent in the literature beyond that of their initial introduction.



**Scheme 16.**

Photolabile blocking has been highly utilized for amino and carboxylate protection [76,77], however it has seen little use as a cysteine S-protectant outside of its extended use in protein systems which bear "caged-cysteine" topography [78]. The *o*-nitrobenzyl (*o*-Nb) group **26** [79] and structural variant 2-nitro-4,5-dimethoxybenzyl (NDMD) group **27** [80,81], although originally designed as protectants for other functional groups, have recently found a niche as a cysteine protecting group. The research group of Amos Smith has begun testing this vector of light-induced lability in their in-situ alkylation of discrete cysteine residues following photolysis of the *o*-Nb protection [82] (Scheme **16**).

hydryl have been utilized for cysteine protection. The phenacyl architecture has been gaining popularity in peptide synthesis as a photolabile alternative to the classical *o*-Nb protection [85]. Similarly to *o*-Nb protection, phenacyl blocking found its peptide synthesis origin in amino and carboxylate blocking [84,86], and its use in cysteine sulfhydryl protection is a relatively recent occurrence, primarily of use in caged, whole-protein systems [87], and only tentatively in model peptide systems [88] in its unsubstituted Phenacyl **28** form. The *p*-hydroxyphenacyl (*p*-Hp) group **29** is the only member of this family which is light-cleavable and whose architecture has specifically begun to be applied to cysteine protection in substantial peptide systems. Specht *et al.* have explored and optimized conditions for installing p-hydroxyphenacyl bromide onto cysteine, and carrying out its removal via photolysis [89]. These optimized results have potential to be utilized toward applications for this protecting group in actual peptide systems.

Another orthogonal vector which has remained largely unexplored in cysteine protection is palladium-mediated deblocking of the sulfhydryl functionality. Making use of the lability of the allyl motif via π complexation with palladium, this functionality has proven itself an effective and popular orthogonal element in the protection of amino [90] and carboxylate [91] functionalities in SPPS. To an even greater extent than the case with photolabile protection, examples of this type of protection are almost nonexistent when applied to thiol functionalization. Traditionally, applications of conventional Pd-removable allylic blocking protocol are not well suited for sulfur protection [92]. S-allyl ether systems are surprisingly stable and are not cleaved by palladium to the π-allyl species to any significant extent. Further, allyloxycarbonyl (alloc)

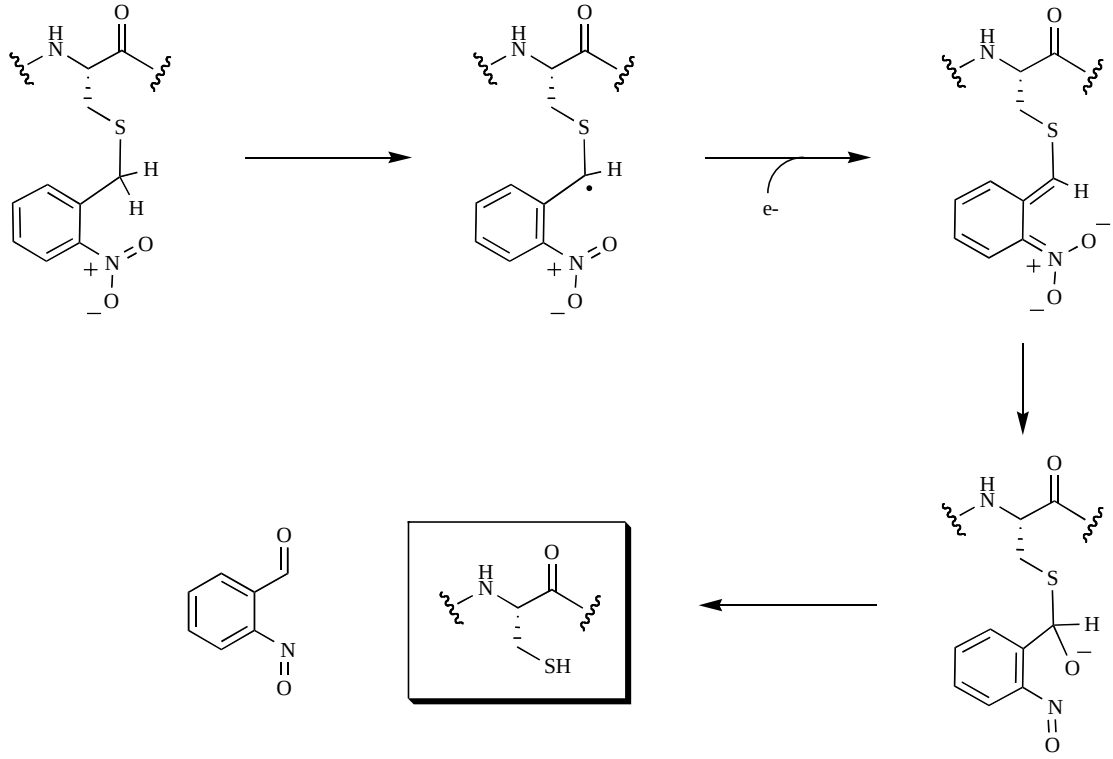
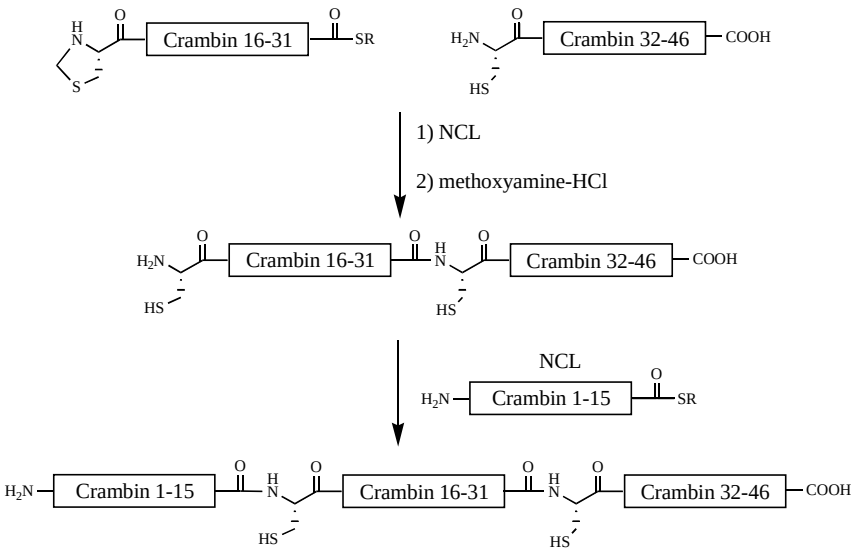


**Fig. (2).**

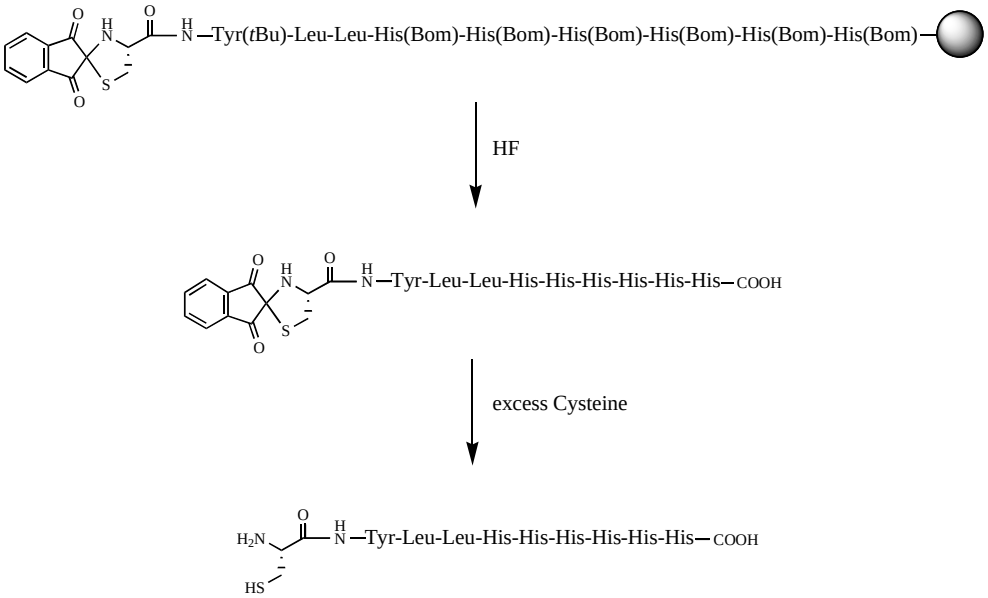
In spite of its popularity as a photolabile cysteine protectant, the *o*-Nb group does have some significant drawbacks. The cleavage process, brought about by a photo-induced Norrish Type II [83] radical cascade process (Fig. **2**), produces an aromatic aldehyde, which has been shown in some cases to conjugate the freshly-liberated thiol [84]. However, other light-sensitive groups whose deprotection by-products do not interfere with the deprotected sulf-

functionalization would not be suited for Fmoc-SPPS cysteine protection, due to the fragile nature of the thiocarbonate linkage and its accompanying lability to potential nucleophiles such as piperidine.

Indeed, only one research group has grappled with the task of applying this chemistry to the deprotection of thiol-allyl complexes. The Allyloxycarbonylaminomethyl (allocam) group **30**, developed




**Scheme 17.**




**Scheme 18.**

by Guibe [93,94], is an allylic S-protective derivative whose structure is based on the aminomethyl handle reminiscent of the Acm architecture [47]. Treatment of the allocam-cysteinyl adduct with a Pd(II) complex catalyst accompanied by tributyltin hydride/AcOH affords the deprotected sulfhydryl, partially as its $Bu_3Sn$ salt. It is noteworthy that this protection protocol has never been attempted on peptidyl systems, either in solution or on the solid support, perhaps due in part to its partial lability to TFA. The researchers attempted to address this problem in subsequent publications in which they introduced the *N*-[2,3,5,6-tetrafluoro-4-(*N*'-piperidino)-phenyl], *N*-allyloxycarbonylaminomethyl (Fnam) group **31** [95] and the *N*-[2,3,5,6-tetrafluoro-4-(phenylthio)-phenyl], *N*-allyloxycarbonylaminomethyl (Fsam) group **32** [96], allocam frameworks bearing stabilizing substitution at the aminomethyl nitrogen. Although these electron-withdrawing components significantly increased the acid and base stability of the protecting group, further application toward peptide synthesis hasn't been forthcoming.

A novel cysteine protecting moiety of somewhat limited utility is the Thiazolidine (Thz) group **33** [97], involving the condensation of the Cys sulfhydryl and the α-nitrogen onto an oxygenated carbon to afford an S,N-acetal framework. This blocking group is obviously constrained toward protection of only N-terminal cysteine residues, which might be assumed to limit its usefulness in SPPS. To the contrary, it has found application in native chemical ligation syntheses of proteins in which more than two fragments are used. Kent was able to utilize a native Thz protection in this manner, removed through treatment with methoxyamine-HCl, in his one-pot, native chemical ligation of Crambin (Scheme **17**) [98]. In another, perhaps more novel example, Pool and coworkers have devised a Thz derivative based on a ninhydrin framework [99]. As an N-terminal cysteine protectant, Nin-Thz is stable to both TFA and HF, with reductive removal being effected by treatment of the peptide with excess thiol to regenerate the native cysteine residue. Constructed to protect N-terminal cysteine-containing peptides from their incompatibility with concurrent His(Bom) deprotection, the researchers were able to first carry out His(Bom) deprotection on a test-peptide, while saving the Nin-Thz removal for the final step (Scheme **18**).

A blocking protocol of last resort for cysteine-containing peptides has been protection by the solid support itself. Although this approach has been used before to serve the dual purpose of solid support and temporal carboxylate protection [100], its use as a discrete sidechain protectant has seen limited application. Typically utilized as an alternative tether for C-terminal cysteine containing peptides, sidechain S-affixation to the resin can overcome certain challenges inherent in these systems such as Cys racemization and Dha formation via piperidine-mediated desulfurization. Barany carried out an in-depth investigation of the benefits of this approach on various peptide systems in which the C-terminal cysteine residues were anchored to the solid support through the sulfhydryl sidearm and a xanthenyl (XAL) linker [101]. It was found that the levels of racemization and Dha formation were dramatically suppressed when compared to the same peptide systems anchored to the resin through traditional carboxylate linkages.

## CONCLUSION

Sidechain protection is an essential component for successful peptide synthesis on the solid phase. As has been shown in this review, the wide variety of cysteine protecting groups in common use eclipses that of any other amino acid residue. The enhanced reactivity of the cysteine sulfhydryl as well as the importance and elegance of cysteine post-synthetic manipulation in peptide synthesis necessitates a diversity of orthogonal protection. Vectors of orthogonality include acidic and basic milieu and gradations thereof, reductive and oxidative protocol, as well as more exotic means such as enzymatic, photolytic and π-allyl-mediated. To be certain, there are significant avenues of orthogonality in cysteine protection yet to be explored or revisited in discrete application toward peptide synthesis. To this end, there exists a wide range of unexplored territory ripe with research potential for the peptide chemist.

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