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Review

Selenopeptide chemistry

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Abstract: This review focuses on the chemical aspects of the 21st proteinogenic amino acid, selenocysteine in peptides and proteins. It describes the physicochemical properties of selenium/sulfur and selenocysteine/cysteine based on comprehensive structural (X-ray, NMR, CD) and biological data, and illustrates why selenocysteine is considered the most conservative substitution of cysteine. The main focus lies on the synthetic methods on selenocysteine incorporation into peptides and proteins, including an overview of the selenocysteine building block syntheses for Boc- and Fmoc-SPPS. Selenocysteine-mediated reactions such as native chemical ligation and dehydroalanine formation are addressed towards peptide conjugation. Selenopeptides have very interesting and distinct properties which lead to a diverse range of applications such as structural, functional and mechanistic probes, robust scaffolds, enzymatic reaction design, peptide conjugations and folding tools. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: selenium; selenocysteine; SPPS; selenoproteins; selenopeptides

INTRODUCTION

The growing field of research of selenoproteins combined with the ability to synthesise proteins by native chemical ligation [1] has fostered a growing interest in the chemistry of selenopeptides and selenoproteins. Synthetic access to selenopeptides by solid-phase peptide synthesis (SPPS) has become increasingly well established and is the preferred alternative to recombinant approaches. Such access is especially important for drug design and structure activity relationship (SAR) studies, where non-natural chemical modifications are incorporated in the synthetic design. Selenopeptides themselves are a very interesting class of compounds and have found a place in a wide range of applications (Table 1). To understand the chemistry of selenopeptides it is necessary to look at the physicochemical properties of selenium.

Selenium

The non-metal, selenium occurs in the earth's crust in inorganic forms as selenide (Se^{2-}), selenate (SeO_4^{2-}) and selenite (SeO_3^{-}), and its abundance is about four magnitudes lower than that of sulfur, which is also reflected in the natural abundance of these elements in biological systems. A number of radionuclides and six stable isotopes exist for selenium with the most abundant isotopes ^{78}Se (23.52%) and ^{80}Se (49.82%) being responsible for the unique mass peak distribution (Figure 1).

The element was discovered in 1818 by the Swedish chemist Jöns Jakob Berzelius when he was investigating a disease among workers at a sulfuric acid plant [41]. It was named after the Greek goddess of the moon, $\Sigma\varepsilon\lambda\eta\nu\eta$, in reference to the previously discovered and closely related element tellurium (Latin: tellus – earth). The element belongs to the group of chalcogens. Except for tellurium these elements are fundamental constituents of functional groups of amino acids and are important contributors to the chemistry and structure of peptides and proteins. Various oxidation states of selenium within proteins have been observed such as the reactive selenol, selenic acid, selenoxide, selenylsulfide and the recently discovered diselenide bond [42]. For a period, selenium was considered a poison in biology especially when field research indicated that selenium poisoning was the leading cause of alkali and blind staggers, a disease which threatened livestock upon eating selenium-accumulator plants of the genus *Astragalus* during periods of droughts in western USA and China [43,44]. Furthermore, laboratory studies led to declaring selenium a potential carcinogen [45,46]. However, the groundbreaking work of Schwarz and Foltz in 1957 changed that view significantly when they identified selenium as an essential trace element for bacteria, birds and mammals [47]. Later, Flohe *et al.* [48] demonstrated in 1973 that selenium is an integral part of the active site of the mammalian glutathione peroxidase – covalently bound in stoichiometric quantities. This triggered a continuous increase in biomedical interest and the emergence of selenium biochemistry as a field of research [49]. Many selenoproteins have been identified in all lineages of life, [50,51] the largest repertoire found in fish with 30 individual selenoproteins, followed by humans and rodents with 25

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BIOGRAPHY

Markus Muttenthaler was born in 1978 in Austria. He obtained his MSc Degree in Applied Synthetic Chemistry in 2004 from the University of Technology, Vienna. During his Masters Program he took up the opportunity to specialise in Organic Synthesis at the City College of New York for a year. He entered the field of Peptide Chemistry in 2005 under the PhD supervision of Professor Paul Alewood at the Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia. His research areas cover SPPS, venom peptide drug discovery and development, peptide folding and development of selective tools for neuroscience. Apart from his interests in science his passion lies in travelling, sports and different cultures.

BIOGRAPHY

Paul Alewood completed his undergraduate chemistry degree at the University of New South Wales in Sydney (Australia), and his PhD in Organic Chemistry at the University of Calgary (Canada). He is currently Professor and Head of the Division of Chemical and Structural Biology at the Institute for Molecular Bioscience, University of Queensland, Australia. He was formerly Associate Professor in the Centre for Drug Design and Development (University of Queensland), Assistant Professor of Peptide Chemistry at Bond University, and Lecturer at the Victorian College of Pharmacy, Melbourne, Australia. His research focus is on bioactive peptides and proteins and he is the author of over 200 publications in high-quality journals. He is also an inventor of 10 patents and has maintained a strong involvement in the fledgling Australian biotechnology industry. His research group comprises approximately 20 research students, assistants and post-doctoral Fellows in the broad fields of peptide, protein and medicinal chemistry. Research projects include the development of the chemical synthesis of proteins, the design and synthesis of drugs from venoms, peptidomimetics and proteomics. Current research targets involve structure-activity studies of a wide range of toxins, development of mediators of neuropathic pain, inflammatory proteins, helical mimetics, milk proteomics and ion channel inhibitors.

and 24, respectively [52]. Selenium has been established as a biologically essential element for cellular redox balance, immune responses, cancer prevention and inflammation protection [53–55]. Some of these selenoproteins are already well characterised, such as glutathione peroxidase and thioredoxin reductase, though the precise function of many is still unknown leading to a growing interest in the synthesis and study of selenopeptides and selenoproteins.

Selenocysteine (Sec, U)–The 21st Proteinogenic Amino Acid

Selenium is predominantly present in biological systems in the form of the naturally occurring amino acid selenocysteine, which Cone *et al.* showed for the first time in 1976, on the analysis of the selenoprotein component of clostridial glycine reductase [56]. It can be found in proteins of all three lines of descent: eukaryota, archaea and eubacteria [57]. Selenomethionine is another important form in which selenium has been observed in proteins [58,59]. Selenocysteine is often found in enzymatic active sites, where its known function is either acting as a nucleophile, a metal ligand or a redox element [60,61]. The importance of selenoproteins became very apparent when the selenocysteine-tRNA gene (necessary for the incorporation of Sec into proteins) knock-out experiment in mice resulted in early embryonic death [62]. Bioincorporation of selenocysteine is genetically controlled and occurs by a specific

Table 1 Applications involving selenocysteine

Structural, functional and mechanistic probe	^{77}Se-NMR spectroscopy [2–5], X-ray crystallography [6–9], SAR studies [10–23], specific radiolabelling [24], PET (positron emission tomography) studies [25,26]
Robust drug scaffold design	Improvement of bioavailability of disulfide bond rich peptides in reducing environment [14,27]
Directed peptide and protein folding	Induction of selective folding and examination of trapped intermediates [8,20,28,29]
Enzyme function and kinetics	Change of specificity or function of enzymes by placing selenocysteine into the active site [13,21,30–37]
Peptide conjugation	Introduction of dehydroalanine as a site-specific precursor for nucleophilic addition (e.g. for the preparation of lanthionines, glycopeptides or lipopeptides) [38–40]

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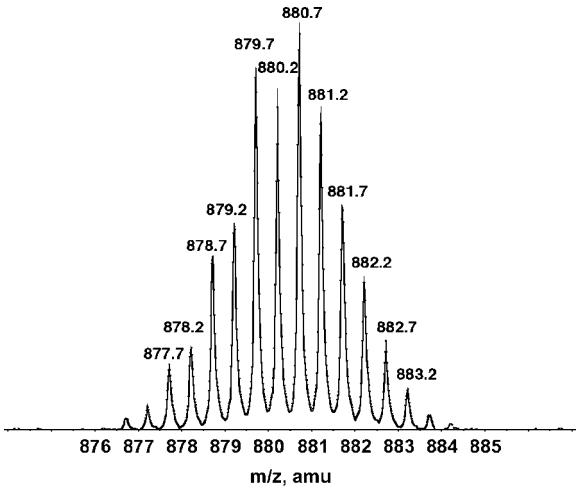

Figure 1 Characteristic isotopic selenium abundance seen in an MS spectrum of a peptide with one diselenide bond.

mechanism [60,63,64], and selenocysteine can therefore be referred as the 21st proteinogenic amino acid [65].

The comparable physicochemical properties of selenium and sulfur (Table 2) indicate that similar effects can be expected from an exchange of these two elements. However, the question to what extent the chemical, electrochemical and pharmacological properties of biomacromolecules will vary through such an interchange and how this could be of use to the scientific community has captured the interest of many research groups and will be a main focus in this review.

INCORPORATION OF SELENOCYSTEINE INTO PEPTIDES AND PROTEINS

Selenocysteine has been used as a mechanistic probe for SARs since the beginning of selenium chemistry and there is more than one method to incorporate selenocysteine into peptides and proteins. This review focuses purely on chemical approaches. Incorporation of Sec through the biosynthetic cell machinery, such as expressed protein ligation, transfection of eukaryotic cells and recombinant selenoprotein production techniques, has been extensively reviewed elsewhere [60,63,64,67,68].

Peptide Synthesis

Chemical incorporation of Sec residues into peptides is largely achieved by SPSS, and in combination with native chemical ligation it is regarded as a very effective tool in rational peptide/protein design. Selenocysteines are usually assembled in the form of the Fmoc-Sec(Mob)-OH derivative for the Fmoc/tBu strategy, or as the Boc-Sec(MeBzl)-OH derivative for the Boc/Bzl strategy. These building blocks behave like their Cys analogues with high coupling efficiency and smooth chain assembly. There are many examples in the literature where this method has been successfully applied (Table 3).

The early syntheses of Sec-peptides were performed in solution and the benzyl (Bzl) group was used exclusively for the selenol protection, which was introduced predominantly as the Z-Sec(Bzl)-OH intermediate [22,69–74,82]. Its lability in alkaline media and the deprotection step with sodium in liquid ammonia provoked significant β-elimination of the phenylmethaneselenolate (BzlSe–) resulting in low yields. It is now well known that $BzlSe^-$ is a much better leaving group than the corresponding thiolate [83]. An even better leaving group is the benzeneselenolate $PhSe^-$ which is used as a precursor for the introduction of dehydroalanine through mild oxidative elimination (see Section 8.5.2) [38,40]. Following the development of Cys protecting groups that were compatible with Fmoc/tBu and Boc/Bzl chemistry, the two protecting groups 4-methoxybenzyl (Mob) and 4-methylbenzyl (MeBzl) in the form of Fmoc-Sec(Mob)-OH [11,84] and Boc-Sec(MeBzl)-OH [75] were introduced. Mob protection was used intensively by various groups [11,17,20,23,39,40,78–80,84,85], but was shown to be prone to racemisation during activation and coupling steps. Another drawback is the high tendency

Table 2 Physicochemical properties of the chalcogen elements [27,66]

Properties		X = oxygen	X = sulfur	X = selenium	X = tellurium
Electronegativity		3.44	2.58	2.55	2.1
Covalent radius	(Å)	0.73	1.02	1.17	1.35
Bond length C^{β}–X^{γ}	(Å)	1.43	1.82	1.95–1.99	2.4
Bond length X^{γ}–X^{δ}	(Å)	—	2.03	2.33	—
Distance C^{α}	(Å)	—	5.7	5.1	—
Van der Waals radii	(Å)	1.52	1.8	1.9	2.06
Torsion angle χ^3–C^{β}–C^{γ}–X^{δ}–C^{ε}–	(°)	180	179.7	174.4	180
Torsion angle χ^3–C^{β}–X^{γ}–X^{δ}–C^{ε}–	(°)	—	98.3	93.9	—

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Table 3 Summary of synthesised peptides and proteins with incorporated selenocysteine

Name	Ref.	Year	Chemistry	#Residues	#S–S bonds	#Sec	Purpose	Yield
Selenoglutathione	69,70	1964	Solution	3	0	1	SAR	NA
Selenooxytocin	71	1964	Solution	9	1	2	SAR	NA
1-deamino-[C6U]-oxytocin	22,70,72	1965	Solution	9	1	1	SAR	NA
1-deamino-[C1,6U]-oxytocin	73	1966	Solution	9	1	2	SAR	NA
[C1U]–oxytocin	22,70	1967	Solution	9	1	1	SAR	NA
[C3,14U][8w]-somatostatin	74	1980	Solution	14	1	2	SAR	9%
Selenosubtilisin	31,37	1989	Solution	379	0	1	SAR	50%
Metalloselenoein	75	1991	Boc-SPPS	25	0	7	SAR	2%
Selenoglutathione	76	1993	Solution	3	0	1	SAR	9%
[C7,23U]-rANP (7–28)	11	1993	Fmoc-SPPS	28	1	1	SAR	NA
SA[a] of glutaredoxin (10–17)	77,78	1996	Fmoc-SPPS	8	1	1,2	Redox	NA
[Sec 3,11][Nle7]-endothelin-1	20	1998	Fmoc-SPPS	21	2	2	Folding	3%
SA[a] of apamin	17,29	1999	Fmoc-SPPS	16	2	2	Folding	3–4%
SA[a] of interleukin-8 (4–72)	10	1999	Boc-SPPS	69	2	1	SAR	NA
[C754U]-RNA (745–761)	79	2001	Fmoc-SPPS	17	1	1	Ligation	NA
[C110U]-RNase A	23	2001	Fmoc-SPPS	124	4	1	Ligation	NA
[C38U]-BPTI	80	2001	Fmoc-SPPS	58	3	1	Ligation	NA
SA[a] of α-conotoxin ImI	14	2006	Boc-SPPS	12	2	2,4	SAR	30%
SA[a] of glutaredoxin 3	13	2006	Boc-SPPS	83	1	1,2	SAR	NA
Selenoglutathione	12	2007	Fmoc-SPPS	3	1	1	Redox	33%
Se15P	18	2007	Ligation	15	0	1	SAR	25%
Selenooxytocin	81	2007	Fmoc-SPPS	9	1	1,2	Folding	NA
PTVTGCUG (vicinal)	19	2007	Fmoc-SPPS	8	1	1	SAR	NA

[a] *SA, Selenium analogues;* NA, not available.

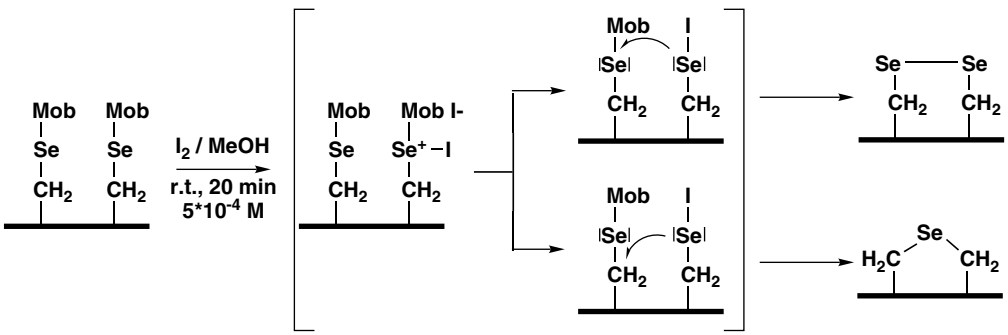
to deselenate via β-elimination during iterative piperidine Fmoc-deprotection steps resulting in dehydroalanine, and consequently, piperidyl adducts [77,85,86]. The deselenation and racemisation could be largely suppressed by keeping the exposure to piperidine to a minimum and using pentafluorophenyl esters without the addition of base [85]. To avoid these side reactions completely, Boc/Bzl chemistry came back into focus, where deprotection, coupling and cleavage are carried out in acidic to neutral medium, e.g. via the *in situ* neutralisation protocol [87]. The optical purity of the Boc-Sec(MeBzl)-OH building block and its behaviour during peptide synthesis was assessed showing that the stereochemical integrity was conserved and no major side reactions have been reported so far [13,14].

Deprotection, Cleavage

The Mob group can either be removed in TFA in the presence of strong Lewis acids such as trimethyltrifluoromethane sulfonate [11,84] or with I_2 in acetic acid, which can cause complications and low yields if other intramolecular Sec or Cys residues are present, due to selenolanthionine formation (Scheme 1) [8,77,79].

Deprotection with mercuric acetate is unsuccessful as it leads to the formation of mercuric diselenide, which is stable to treatment with excess thiols for the displacement of the heavy metal (Scheme 2) [77,85].

The most applicable method to date is deprotection in TFA in presence of DMSO, which leads directly



Scheme 1 Possible mechanism for observed predominant selenolanthionine formation during oxidative deprotection of Mob group with iodine.

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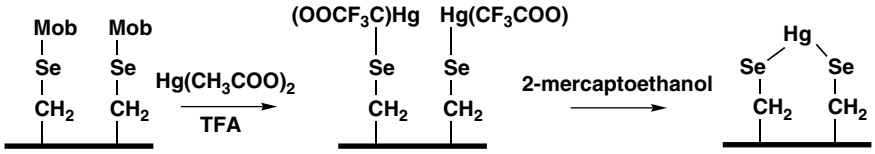
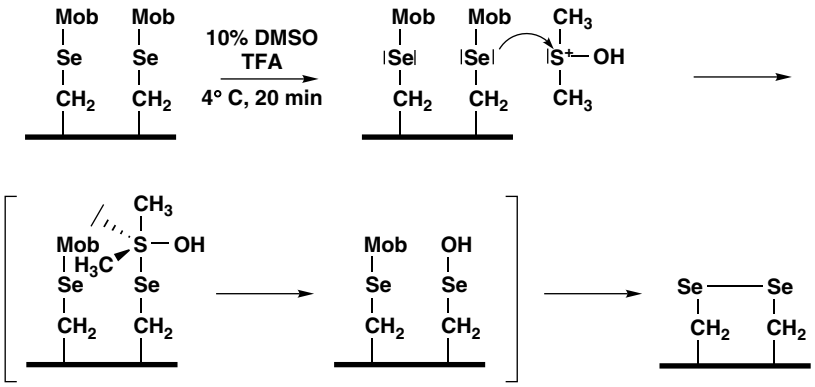


Scheme 2 Deprotection of the Mob group with mercuric acetate, which leads to the intrachain mercuric diselenolate.



Scheme 3 Sec(Mob) deprotection with DMSO/TFA.

Table 4 Percent deprotection/oxidation of Cys/Sec in oxytocin XYIQNXPLG*, with X = C/U

Protection group	(%)	Equiv. DTNP	Thioanisole
Cys(Acm) and Cys(Acm)	60	15	Yes
Cys(StBu) and Cys(StBu)	60	15	Yes
Cys(Mob) and Cys(Mob)	>98	6	Yes
Sec(Mob) and Cys(Mob)	>98	1	Yes
Sec(Mob) and Sec(Mob)	>98	1	Yes
Sec(Mob) and Sec(Mob)	>98	1	No

to diselenide or selenylsulfide formation (Scheme 3) [8,11,77,79,85].

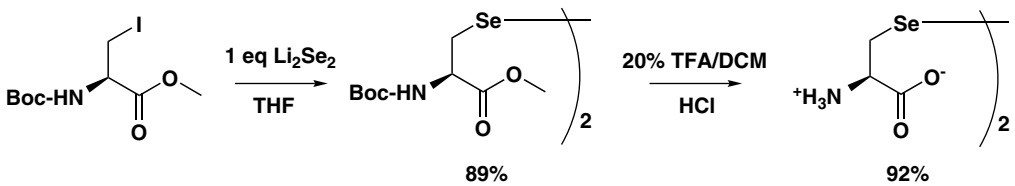
Deprotection of Sec(Mob) can also be achieved under milder conditions via the addition of sub-stoichiometric amounts of 2,2′-dithiobis(5-nitropyridine) (DTNP) in TFA, where the Mob group is converted to the 5-nitropyridyl selenylsulfide which can subsequently be cleaved by thiolysis [88]. This method can be used for desired selenylsulfide bond formation [81] and was also shown to produce vicinal selenylsulfide bonds (Table 4) [19].

In the Boc/Bzl strategy Sec(MeBzl) is deprotected by standard hydrogen fluoride methods at 0 °C within 1 h and with no observed difficulties [10,13,14,27,75]. A very interesting feature is that upon deprotection, the Sec residues are found to be already fully oxidised to the corresponding diselenide or mixed selenylsulfide bonds even in strong acid environments [13,27] (Metanis N, Dawson PE. *personal communication*). This is probably due to the high reactivity and low pK_A of the selenol.

Synthesis of Selenocysteine Building Blocks

The development of novel, robust and scalable synthetic routes to Sec building blocks was fostered by the requirement for readily accessible selenopeptides to probe biological mechanisms, instigate drug design programs and undertake SAR studies (Table 5).

Selenocystine. Selenocystine was initially prepared by Fredga in 1936 [93], but it was Soda and co-workers who made the synthesis of selenocystine feasible by reacting excess (to prevent monoselenide formation) *in situ* generated disodiumdiselenide with β-chloro-alanine [89,90,94]. Stocking *et al.* improved this method by reacting dilithiumdiselenide with the Boc-protected β-iodo-alanine-methylester yielding the Boc-protected selenocystine methylester, which upon deprotection could be converted into optically pure selenocystine (Scheme 4) [4].



Scheme 4 Synthesis of selenocystine [4].

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Table 5 Overview of Sec-building block syntheses found in the literature

Selenocysteine building blocks	Reference	Scheme	Reaction steps	Time (days)	Scale (g)	Overall yield %	Year
$(Sec)_2$	89,90	9	2	2	3	62	1985
$(Sec)_2$	4	4	2	2	0.5	82	1997
$(Sec)_2$	91	5	2	3	0.1	51	2004
Fmoc-Sec(Bzl)-OH	92	6	4	4–5	—	59	2002
Fmoc-Sec(Mob)-OH	84	8	3	3–4		42	1993
Fmoc-Sec(Mob)-OH	79	6	4	4–5	5	61	2001
Fmoc-Sec(Ph)-OH	40	7	3	2–3	3	42	2000
Fmoc-Sec(Ph)-OH	79	6	4	4–5	5	37	2001
Boc-Sec(Ph)-OH	40	7	2	2	1	47	2001
Boc-Sec(MeBzl)-OH	75	9	3	3–4	NA	NA	1991
Boc-Sec(MeBzl)-OH	14	9	3	3–4	3	32	2006
Boc-Sec(MeBzl)-OH	13	10	6	4–5	10	45	2006
Boc-Sec(MeBzl)-OH	27	9	3	3–4	20	53	2008

A different approach was introduced later by Siebum *et al.*, who took up the challenge of economically introducing the isotopes ^{13}C, ^{15}N, ^{17}O, ^{18}O and ^{77}Se to produce site-directed isotopomers for NMR studies. This method utilised the efficient Mitsunobu reaction to incorporate the expensive ^{77}Se into the building block (Scheme 5) [91].

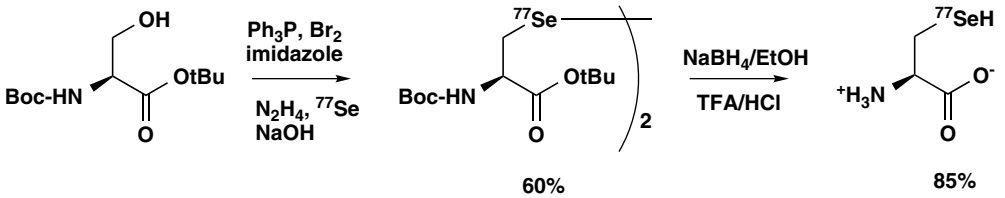
Currently, the most used selenol protection groups are the MeBzl and Mob groups, whereas Sec(Ph) protection is now predominately used as a precursor for dehydroalanine and is incorporated either as Boc-Sec(Ph)-OH or Fmoc-Sec(Ph)-OH [38,40].

Building Blocks for Fmoc/tBu Chemistry

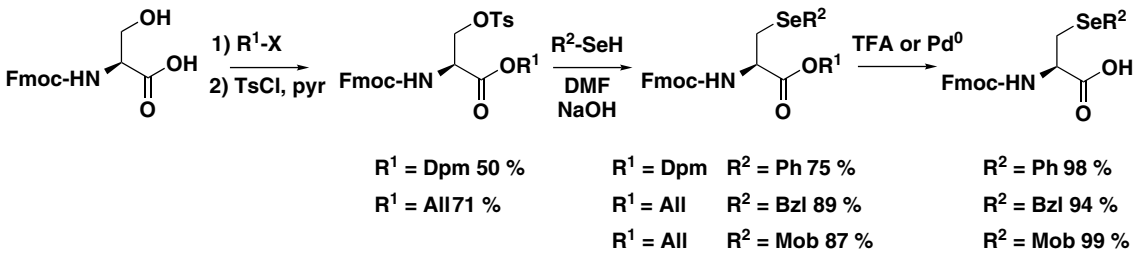
The first optically pure Sec(Bzl) derivatives were reported in the late 1960s by Walter and co-workers [70,95]. The synthetic approach involved nucleophilic displacement of the *O*-tosyl moiety of l-serine derivatives by the benzyl selenolate anion (BzlSeNa). This key reaction was developed further by the group of van der Donk into a robust and scaleable synthesis to access Fmoc-Sec(Bzl)-OH, Fmoc-Sec(Ph)-OH and Fmoc-Sec(Mob)-OH in high yields and with simple recrystallisation workup (Scheme 6) [79].

An alternative approach for the synthesis of phenylselenocysteine is the reduction of diphenyldiselenide with sodium metal followed by reaction with Boc-serine-β-lactone [96,97]. This procedure was slightly modified by Okeley *et al.*, performing the reduction with sodium trimethoxyborohydride ($NaBH(OMe)_3$) *in situ* and converting the Boc group into the Fmoc derivative (Scheme 7) [40].

Fmoc-Sec(Mob)-OH can also be obtained by reduction of selenocystine with $NaBH_4$ and *in situ* reaction with 4-methoxybenzyl chloride, followed by acylation with Fmoc succinimide (Scheme 8) [84].



Scheme 5 Synthesis route for efficient ^{77}Se incorporation into selenocysteine [91].



Scheme 6 Synthesis of Se-protected N^{α}-Fmoc-selenocysteine derivatives [79].

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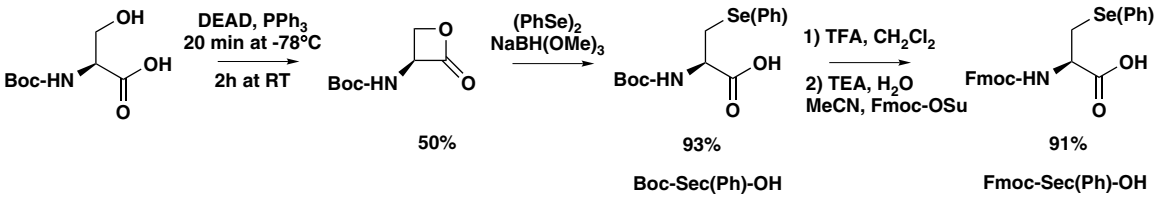
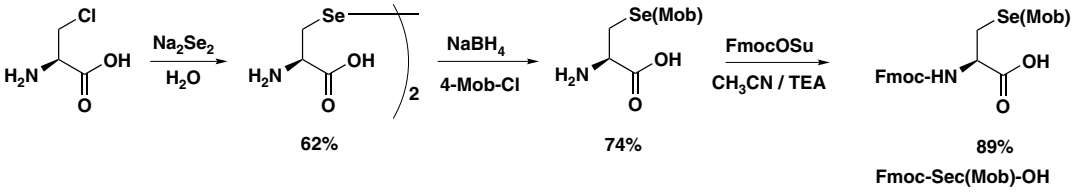


Scheme 7 Synthesis of N^{α}-Fmoc-phenylselenocysteine [96,97].

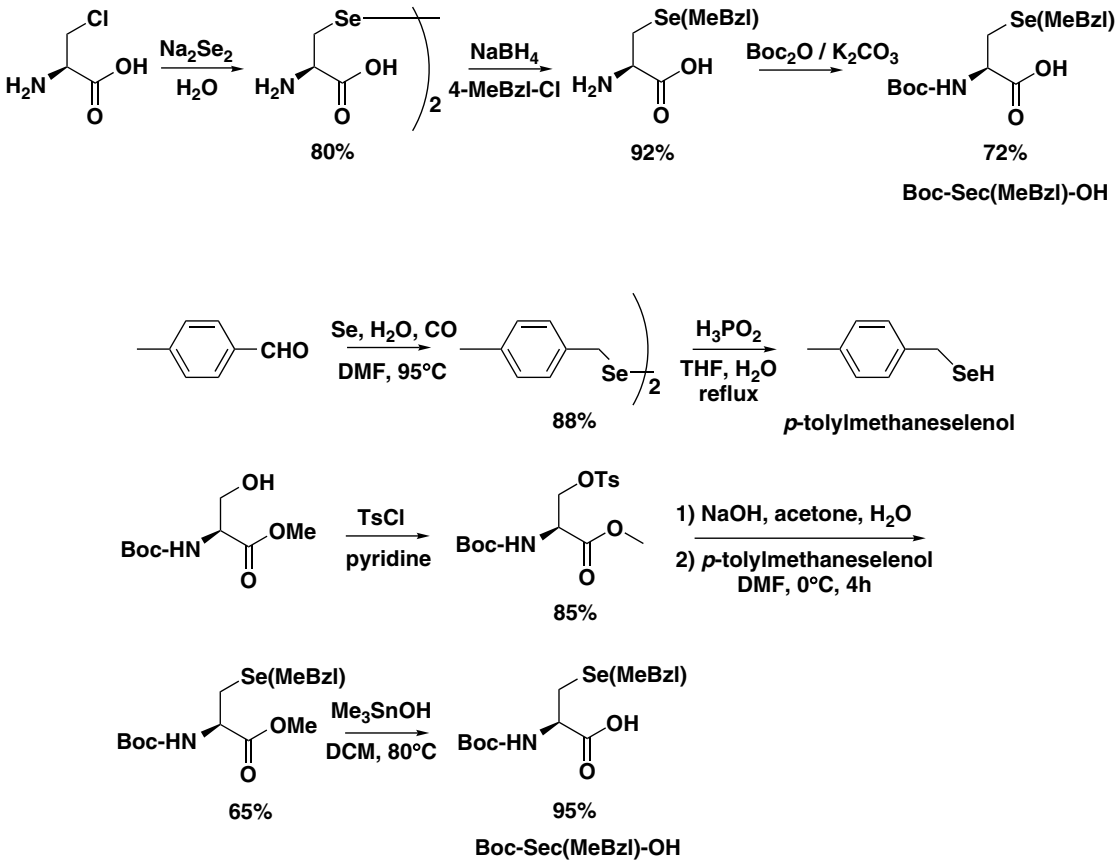


Scheme 8 Synthesis of Fmoc-Sec(Mob)-OH [84].

Building Blocks for Boc/Bzl Chemistry

Boc-Sec(MeBzl)-OH was synthesised by reaction of suitable β-halo-alanines with *in situ* generated disodium or dilithiumdiselenide [4,90,91], leading to optically pure selenocystine, which is used as a precursor for further building blocks [75,89]. This method was originally introduced by Oikawa *et al.* [75], but it was Alewood and co-workers who developed it into a fast and efficient synthesis for Boc-protected Sec building blocks, amendable to a 20 g scale-up with easy workups (Scheme 9) [14,27].

Boc-Sec(MeBzl)-OH can also be obtained by synthetic routes analogous to those followed in the synthesis of the Fmoc building blocks (Scheme 9). One robust scaleable approach includes the synthesis of the 4-methylbenzyl-diselenide, using elemental selenium under bubbling CO [98], and the mild deprotection of the methyl ester using Me_3SnOH [13,99].



Scheme 9 Synthesis of N^{α}-Boc, Se-MeBzl protected selenocysteine [13,14,27].

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PROPERTIES OF SELENOPEPTIDES

The similarities between sulfur and selenium discussed earlier (Table 2) suggest that a mutation of cysteine to selenocysteine should represent a very conservative substitution. This hypothesis has been put to test on a wide range of bioactive peptides such as oxytocin [22,71–73], somatostatin [74], α-rat atrial natriuretic peptides [11], endothelin-1 [8,20], apamin [17], interleukin-8 [10], BPTI [80], ribonuclease A [23], glutaredoxin 3 [13] and the α-conotoxins [14,27] (Table 3). In all cases, the selenium analogues folded correctly with NMR structural analysis (Figure 2) and CD spectroscopy confirming the isosteric character of selenocysteine. Importantly, full biological activity was observed in all cases. Indeed, the substitution of a Cys residue with Sec has significant advantages over a substitution with other chemical moieties such as carba [100,101], lactam [102], thioether [103], homocysteine or penicillamine [10], which can impart structural distortions that may compromise bioactivity and selectivity.

pK_A, Nucleophilicity and Reactivity

Despite the resemblance between the elements selenium and sulfur, selenocysteine and cysteine exhibit significantly distinct chemical properties. Even though Sec has similar electronegativity to Cys, it is a stronger nucleophile [23,105,106] and a better leaving group than its sulfur analogue [15]. Diselenides were shown to be more susceptible to nucleophilic attack than disulfides [84,107]. Furthermore in pK_A determination studies selenocysteine exhibited a much higher acidity than cysteine (pK_A (Sec) = 5.24–5.63, pK_A (Cys) = 8.25) [105,108,109], which suggests at physiological pH the Sec residue will be present largely in its reactive anionic form, the selenolate, while the cysteine residue would remain largely protonated. In pH-dependent titration studies it was also shown that selenocysteine reacted with iodoacetate or iodoacetamide at pH values far below the pK_A of the selenol [105]. Generally it is well established that exposed selenols are a very reactive species and readily oxidised by air [110]. ^{1}H-NMR studies of selenoenzyme selenosubtilisin revealed that the enzyme-bound selenol and seleninic acid have unusually low pK_A values when compared to typical selenium compounds and were found to be deprotonated at all accessible pHs [111]. Intriguingly, Sec residues in peptides are found to be fully oxidised directly upon deprotection via hydrogen fluoride [27] (Metanis N, Dawson PE. *personal communication*). Investigations towards pK_A determination of Sec residues incorporated in peptides and proteins though not trivial would certainly be of great value.

Considering the low pK_A and high reactivity of the selenolate at physiological pH the question arises as to which form the catalytic selenium is present within a selenoenzyme. Crystallographic data on the selenoenzyme glutathione peroxidase showed that well conserved amino acid residues tryptophan and glutamine constitute a catalytic triad in which the selenolate is both stabilised and kept activated by hydrogen bonding with the imino group of the tryptophan residue and with the amido group of the glutamine residue (Figure 3) [112,113]. In the case of selenosubtilisin the active selenolate is likewise stabilised by hydrogen bonding of a histidine and asparagine residue in close spatial proximity (see Figure 3) [114].

Most human selenoenzymes have cysteine homologues that are generally weaker catalysts. Mutations of the Sec residue to Cys in selenoenzymes confirm this, showing a 100- to 1000-fold decrease in catalytic activity [15,115–117]. Kinetic analysis of thioredoxin reduction by selenium analogues of glutaredoxin 3 suggested that it is the selenium's nucleophilicity that is the primary reason for the strong increase in activity rather than its role as the better leaving group [13]. This difference in pK_A and nucleophilicity can be used to discriminate between Sec and Cys, and is the underlaying principle of the BESThio assay. BESThio (3′-(2,4-dinitrobenzenesulfonyl)-2′,7′-dimethylfluorescein), a fluorescein derivative, initially used as a fluorescent probe for thiols [118], was shown not to react with thiols at pHs <7 (Scheme 10). Consequently, when performed at a pH 5.8 in the presence of DTT, this assay can be used for rapid identification and quantification of known and unknown selenoproteins,

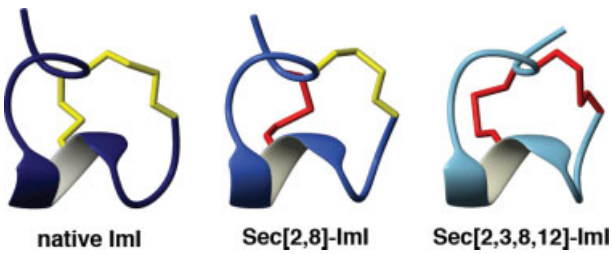

Figure 2 Ribbon representations of the NMR minimum energy structures of native Iml and its selenium analogues [14,104].

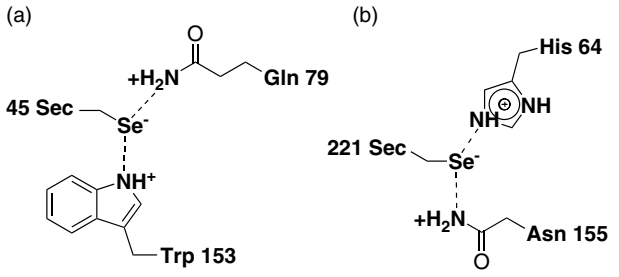

Figure 3 (a) Selenolate stabilisation in the catalytic triad of glutathione peroxidase. (b) Selenolate stabilisation in the catalytic triad of selenosubtilisin.

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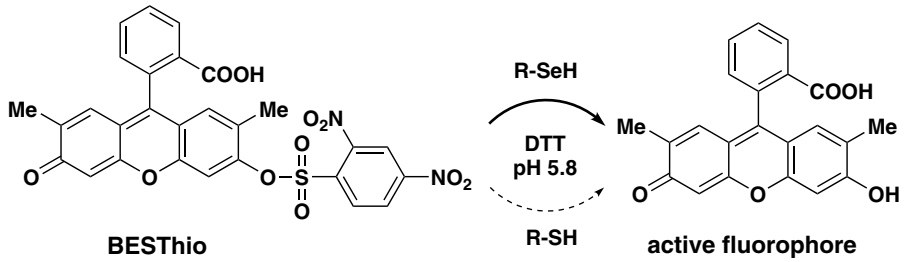


Scheme 10 Fluorogenic reaction of BESThio with selenols and thiols.

which was demonstrated on the selenoproteins glutathione peroxidase and thioredoxin reductase [119].

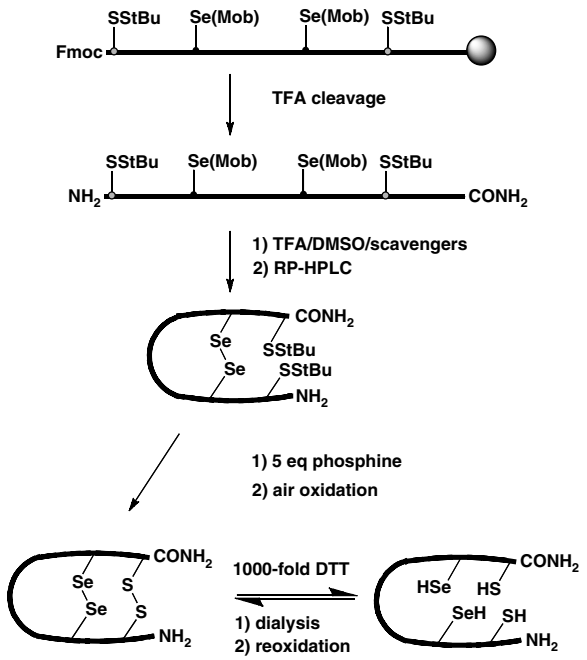
The Redox Potential of Selenocysteine

The redox potentials of thiols and selenols in peptides and proteins are usually extracted from the equilibrium constants of exchange reactions with reference redox systems such as DTT or glutathione. For this purpose, apparent redox potentials of −323 mV for DTT [120,121] and −240 mV for glutathione [122] are generally used for calculating the apparent redox potentials by the Nernst equation. Studies on aliphatic and aromatic compounds as well as on peptides showed that the reduction of the diselenide bond is possible in the presence of $NaBH_4$, tris (2-carboxy ethyl)phosphine (TCEP), tri-n-butylphosphine (TBP) or DTT, while monothiols are not as efficient and higher concentrations are necessary [78,84,110,123–125]. Excess of TCEP has been observed to lead to deselenation, similar to the well-established desulfurisation reaction of disulfides in the presence of basic tris(ethylamine)phosphine [126,127]. Cyclic voltammogram studies of cystine and selenocystine showed a striking difference in redox potential between them [59]. The highly negative redox potential of selenocystine (−488 mV) compared to that of cystine (−223 mV) [128] suggested a very different behaviour in redox reactions, which has captured the interest of various research groups in recent years. Reinvestigation of the redox potential of selenocystine led to a value of −386 mV as electrode potential and −388 mV against DTT at pH 7 [129], which is almost identical to that determined for an unstructured linear bis-Sec-peptide (−381 mV at pH 7.0 and 25 °C) using DTT (−323 mV) as reference redox system (Table 6) [78].

In general, the proximity of neighbouring Cys and Sec residues and overall structural differences influence the redox potential significantly (Table 6). In the case of Cys residues it can vary from approximately −125 mV for *Escherichia coli* disulfide bond promoting product of gene dsbA (DsbA) [133] to −270 mV for thioredoxin [132]. This diversity in potentials is also reflected in the various functions that these thiol/disulfide oxidoreductase proteins play *in vivo*, ranging from protein reduction to disulfide bond formation [59]. Further comparative studies on diselenide, selenylsulfide and disulfide bonds in linear unconstrained glutaredoxin fragments as well as in folded glutaredoxin 3 analogues were conducted, providing more relevant values for thiol/selenol oxidation [13,78]. The observed difference in redox potentials (111–166 mV) of the disulfide and the diselenide bond (Table 6) in combination with the higher nucleophilicity of selenium suggest a highly favoured diselenide or selenylsulfide bond formation over disulfide bond formation. This hypothesis was experimentally confirmed on selenium analogues of native and non-native disulfide/diselenide analogues of the bee venom toxin apamin [17]. Regioselective deprotection and oxidation of the Sec(Mob) followed by the Cys(StBu) residues resulted in correct selenocysteine and cysteine pair bond formation for all three isomers (Scheme 11). Upon complete reduction of the correctly folded isomers followed by reoxidation by air-oxidation, no mixed selenylsulfide bonds were observed [17,29].

Selenocystine in Reducing Environments

In stability studies, the globular selenium analogues of α-conotoxin ImI were exposed to various reducing



Scheme 11 Regioselective selenocysteine and cysteine pairings in Sec [1, 11]-apamin [17,29].

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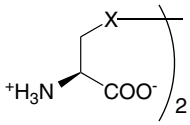
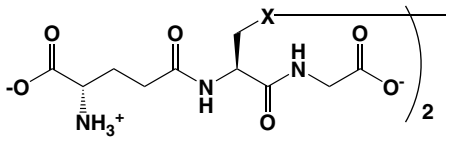
Table 6 Redox potentials of cysteine, related peptides and proteins, and their selenocysteine analogues

Compounds	Potential (mV)	Reference system	Structure/active motif	Reference
Cystine	−233	NHE		59
Cystine	−223	GSSG[a]		128
Selenocystine	−488	NHE		59
Selenocystine	−388	DTT[b]		129
Glutathione	−240	Lipoic acid		122
Selenoglutathione	−407	DTT[c]		12
Glutaredoxin 1 (Grx1)	−233	Trx[e]	-CPYC-	130
Glutaredoxin 3 (Grx3)	−198	Trx[e]	-CPYC-	130
[C^{11}, C^{14}]-Grx (10–17)	−215	GSSG[a]	Ac-GCPYCVRA-NH_2	131
[U^{11}, C^{14}, K^{19}]-Grx (10–17)	−219	GSSG[a]	Ac-GCPYCVKA-NH_2	12
	−235	GSSG[f]	Ac-GCPYCVKA-NH_2	
[U^{11}, C^{14}, K^{19}]-Grx (10–17)	−326	DTT[d]	Ac-GUPYCVKA-NH_2	78
[U^{11}, U^{14}, K^{19}]-Grx (10–17)	−381	DTT[d]	Ac-GUPYUVKA-NH_2	78
[U^{11}, U^{14}]-Grx3	−309	Trx[e]	-UPYU-	13
[U^{11}, C^{14}]-Grx3	−260	Trx[e]	-UPYC-	13
[C^{11}, U^{14}]-Grx3	−275	Trx[e]	-CPYU-	13

[a] Determined with $E'_0 = -240$ mV for GSSG.
[b] With $E'_0 = -332$ mV for DTT [129].
[c] With $E'_0 = -327$ mV for DTT.
[d] With $E'_0 = -323$ mV for DTT [121].
[e] With $E'_0 = -270$ mV for Trx [132].
[f] With $E'_0 = -256$ mV for GSSG.

conditions (blood plasma, glutathione, albumin, thioredoxin) in several biological systems. While the *all*-Cys-peptide scrambled (Scheme 12) into a mixture of ribbon and globular isomers within 24 h in all the conditions applied, the selenium analogues retained their three-dimensional structure and no diselenide/disulfide shuffling was detected. In addition, it seems that substitution of only one disulfide bond by a diselenide bond is sufficient to achieve complete structural and bioactive integrity in two-disulfide bond containing peptides [14].

Selenocysteine and its Role as a Mechanistic Probe

Selenocysteine plays an important role in the elucidation of structural, mechanistic and functional features in many biomacromolecules. Functional information can be obtained by replacement of active-site Cys residues by Sec based upon the differences in redox properties and nucleophilicity demonstrated with selenosubtilisin [31,37], metalloselenonein [75], interleukin-8 [10], and seleno-glutaredoxin 3 [13]. Incorporation of selenocysteine into proteins for the purpose of X-ray crystallography significantly facilitates the phasing problem, and the lengthy and problematic heavy-atom screening procedure can be avoided [6–9,123]. The anticipated difference in bond length between Se–C and S–C does not affect the properties of proteins or peptide analogues since the structures generally retain sufficient plasticity and flexibility to accommodate the selenium residues within the geometries of the wild type (Figure 4).

Selenium has six isotopes: ^{74}Se (0.87%), ^{76}Se (9.02%), ^{77}Se (7.58%), ^{78}Se (23.52%), ^{80}Se (49.82%) and ^{82}Se (9.19%). Only one of them, ^{77}Se, has a nuclear spin quantum number of $I = 1/2$ and can be employed in high-resolution NMR spectroscopy [2,5]. Uniform mutation of Cys residues by Sec should theoretically allow specific resonance assignment and conformational analysis of unknown disulfide bond connectivity in Cys-rich peptides and proteins by ^{1}H-^{77}Se-correlated NMR experiments [4]. However, ^{77}Se[^{1}H], ^{1}H-HMBC experiments performed on an oxidised diselenide glutaredoxin fragment at natural ^{77}Se abundance did not allow for assignment of the diselenide connectivity [134] and reports on the use of this methodology are generally very rare. Recent advances in synthetic access to the rather expensive ^{77}Se-selenocysteine building

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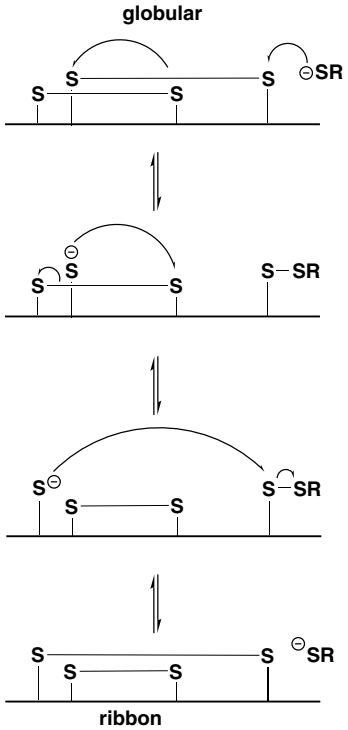


Scheme 12 Possible disulfide bond scrambling in presence of free thiols.

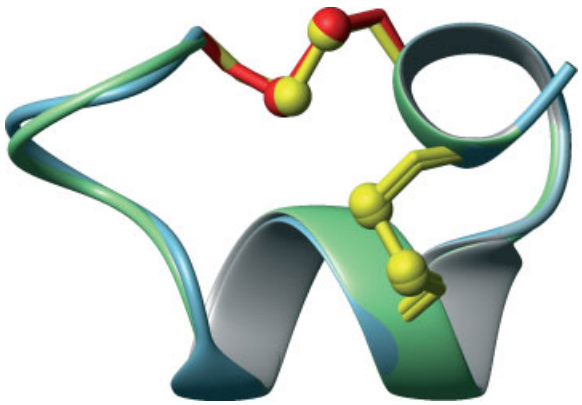


Figure 4 Superimposition of the crystal structures of PnIA and Sec[3,16]-[A10L]-PnIA illustrating the isosteric character of the diselenide bond in red and with the disulfide bond in yellow [27].

blocks [4,91] should facilitate the labelling process, as ^{77}Se can now readily be incorporated into peptides and proteins by synthesis and native chemical ligation.

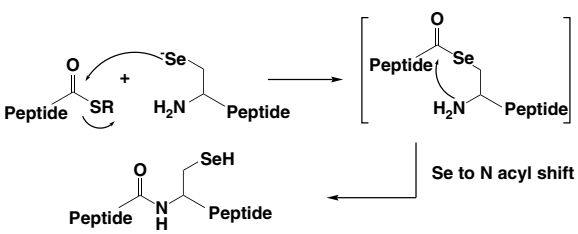
REACTIONS WITH SELENOCYSTEINE

Selenocysteine-mediated Native Chemical Ligation

Native chemical ligation [1] has proven to be a very attractive approach to the synthesis and semisynthesis of a wide range of proteins, and it is now possible to study selenoproteins by selenocysteine-mediated native chemical ligation. The native peptide bond is formed between a *C*-terminal peptide thioester and another peptide containing an unprotected Sec residue at its *N*-terminus when mixed together in presence of a reducing agent. Without a reducing agent no ligation takes place supporting the mechanism of initial attack of the selenolate on the thioester to give a selenoester, followed by acyl migration from the Se- to the *N*-terminus to yield the thermodynamically more stable product (Scheme 13) [39,80].

The first example of a Sec-mediated ligation was achieved with two peptides related to ribonucleotide reductase [79]. Although a faster and more efficient reaction was anticipated due to the higher nucleophilicity, the lower pK_A of selenocysteine and the faster aminolysis of the selenoester, the ligation only proceeded slowly and with low yields. The rate of the ligation seems to depend on the equilibrium between the reactive selenolate and its oxidised diselenide or selenylsulfide form. The use of only weakly reducing thiophenol is most likely the reason for slow reaction times as only a small quantity of the selenolate will be present for the nucleophilic attack due to the higher stability of diselenide and selenylsulfide bonds. Indeed, in model studies on ligation of acetyl-glycine thioesters with Cys and Sec it was shown that once the selenolate was generated (in this case with the stronger and irreversible reducing agent TCEP), the ligation proceeded faster than with cysteine. At a pH of 5 a 10^3-fold faster rate of product formation was observed, indicating that Sec-mediated chemical ligation can be chemo-selective [23]. A drawback of using excess of TCEP can be deselenation which leads to monoselenide formation, similar to the well established desulfurisation reaction of disulfides in the presence of basic tris(ethylamine)phosphine [126,127]. An overview of native chemical ligation reactions incorporating a selenocysteine is given in Table 7.

Sec-mediated native chemical ligation was applied to the synthesis of a selenium analogue of the three-disulfide bond BPTI. The peptide fragments BPTI(1–37)-thioester and [C38U]-BPTI(38–58) were synthesised by Fmoc/tBu synthesis on solid phase and ligated in presence of TCEP (1 equiv.) and 3% (v/v) thiophenol to reduce the selenylsulfide bond and to initiate the



Scheme 13 Selenocysteine-mediated native chemical ligation.

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Table 7 Overview of native chemical ligation reactions incorporating selenocysteine

Ligation reaction	Reactants	Yield %	Reference
Ac-LVPSI$\overline{\text{C}}$DDG-SBzl + UESGACKI → Ac-LVPSI$\overline{\text{C}}$DDGUESFACKI	4% PhSH, 6 M GnHCl	60	79
Ac-LVPSI$\overline{\text{C}}$DDG-SBzl + CESGAUKI → Ac-LVPSI$\overline{\text{C}}$DDGCESFAUKI	4% PhSH, 6 M GnHCl	76	92
Ac-LVPSI$\overline{\text{C}}$DDG-SBzl + UESGAUKI → Ac-LVPSI$\overline{\text{C}}$DDGUESFAUKI	4% PhSH, 6 M GnHCl	48	92
LYRAG-SEt + selenocystine → $(LYRAGU)_2$ diselenide dimer	3% PhSH, 1.7 equiv. TCEP 6 M GnH CL	NA	80
BPTI(1–37)-SEt + [C38U]-BPTI(38–58) → [C38U]-BPTI	3% PhSH, 1 equiv. TCEP 6 M GnHCL	NA	80
[C1 1X]-[C14X]-Grx3(1–37)-MPAL-thioester + [A38C]-Grx3(38–82) → [C1 1X]-[C14X]-[A38C]-Grx3 with X = U/C	1.5% PhSH 3 mM peptide	40–50	13
14P-Set + selenocystine → 15SeP diselenide dimer	5% PhSH, 6 M GnHCL	25	18
UYAVTGRGDSPAASSG-SEt → c[UYAVTGRGDSPAASSG] + [c[—UYAVTGRGDSPAASSG]$]_2$	3% PhSH	Quant	39

ligation by generating the reactive selenolate. [C38U]-BPTI was then subsequently folded to its fully active native structure. CD spectra confirmed the nearly identical conformation and the selenium analogue-inhibited trypsin and chymotrypsin with unaltered affinity, providing further support for the versatile use of selenocysteines in the future of rational peptide and protein design [80]. Another example is the synthesis of a 15-mer selenopeptide (15SeP) by ligating the fragment 1–14 thioester to selenocystine under reducing conditions yielding the 15SeP diselenide dimer [18]. Standard native chemical ligation in presence of fully oxidised fragments (diselenide and selenylsulfide) was shown to be possible in the synthesis of the complete set of selenocysteine variants of glutaredoxin 3, which provided insights into the catalytic machinery of selenoenzymes [13]. Intramolecular Sec-mediated ligation can be used for *N-C* terminal backbone cyclisation for the formation of stable cyclic peptides. A linear 16-mer-diselenide dimer was cyclised in 0.1 M phosphate buffer at pH 7.5, containing 3% thiophenol (v/v). The reaction was complete after 3 h, resulting in a mixture of the diselenide dimer and the mixed selenylsulfide with thiophenol (Scheme 14) [39].

Dehydroamino Acids – Versatile Precursors

The α,β-unsaturated amino acid, dehydroalanine, is often found in biological polypeptides and natural products [135,136], and from a synthetic point of view represents a useful electrophilic precursor for preparation of peptide conjugates such as glycopeptides and lipopeptides. Introduction of dehydroamino acid can be achieved by various methodologies [137] with the most common ones being the activation and elimination of serine residues or Hoffmann elimination of 2,3-diaminopropionic acid [138]. These methods might be useful for specific peptides, but lack overall sufficient chemoselectivity. A more versatile approach is the oxidative elimination of cysteine derivatives, but that precludes other protected cysteine residues. With the selenocysteine residue being a better leaving group than the cysteine residue it enables selective access to dehydroalanine and other non-natural amino acids in a facile and convergent way. Selenocysteines can either be introduced site-specifically as phenylselenocysteine during the synthesis or by choosing the ligation site utilising selenocysteine-mediated chemical ligation. It can then either be converted to dehydroalanine by mild oxidative elimination with hydrogen peroxide or sodium periodate [38,40], or directly transformed into an alanine residue by reduction of the relatively weak Se–C bond under hydrogen atmosphere using Raney nickel and TCEP (Scheme 15) [39].

Once electrophilic dehydroalanine is obtained, modification can take place by nucleophilic attack or other compatible chemo-selective reactions. The oxidation

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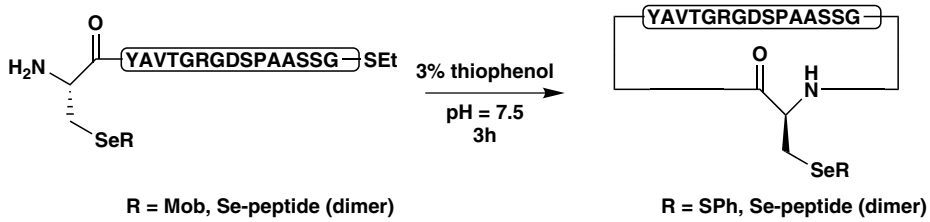
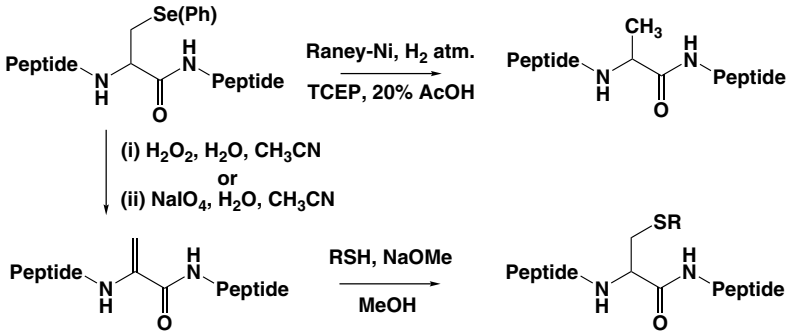
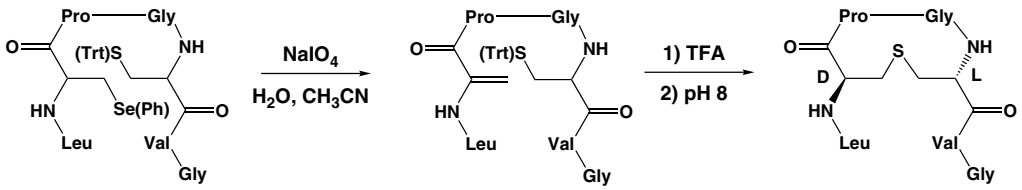


Scheme 14 Selenocysteine-mediated backbone cyclisation.



Scheme 15 Sec(Ph) – A versatile precursor.



Scheme 16 Lanthionine formation via intramolecular Michael addition of a cysteine onto a dehydroalanine.

conditions tolerate functionalities such as tryptophan and methionine and are compatible with thiol protection such as the trityl and *tert*-butylthio group. One application for the electrophilic handle is the intramolecular Michael addition of a trityl-deprotected cysteine to generate the lanthionine (Scheme 16). Interestingly, the formation of only a single diastereoisomer was observed [40]. The incorporation of dehydroamino acid into peptides serves as a complementary chemoselective ligation approach and enables the access of highly complex biomolecules containing multiple site-specific modifications.

CONCLUSION

Without a doubt, selenium can be seen as the most conservative substitution for sulfur and its properties may be exploited as spectroscopic and mechanistic probes. When incorporated into biomolecular structures, the resulting changes in catalysis, hydrolysis and redox properties can be utilised in a variety of ways (see Table 1). Examples of future applications might be found in drug scaffolds, folding pathway and enzymatic reaction design.

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