Chapter 8
Thioamide-Containing Peptides and Proteins

Taylor M. Barrett, Kristen E. Fiore, Chunxiao Liu and E. James Petersson

Abstract Thioamidation of the peptide backbone can have both subtle and profound effects on peptide and protein properties. Oxoamide-to-thioamide substitutions can alter hydrogen bonding networks, metal interactions, peptide folding, and photophysical properties. Thioamides are found in a small number of natural products with intriguing antibiotic and anticancer activities. A thioamide residue is also found in a natural protein that is essential to biological methane metabolism. Recent genetic scanning has shed light on the biosynthesis of these molecules and indicated that many other thioamide-containing peptides and proteins exist. Thioamide modifications have been installed synthetically to investigate the biosynthesis and biological activity of these thioamide natural products, as well as to serve as biophysical probes or to enhance the stability and activity of medicinally relevant peptides. The synthesis of these molecules has required the development of methods for the incorporation of thioamides by solid-phase peptide synthesis and through native chemical ligation of protein fragments. The expanding number of known thioamide natural products, the ability to gain detailed insights into protein folding mechanisms, and recent demonstrations of valuable in vivo activity for thioamide-modified peptides highlight the impact of thioamides in peptide and protein chemistry.

Keywords Thioviridamide · Methyl-coenzyme M reductase · Closthioamide · Native chemical ligation · Thioprotein

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8.1 Introduction

8.1.1 Scope and Nomenclature

The modification of the peptide backbone has long been of interest to biological and medicinal chemists. Numerous methods for backbone modification with peptide bond analogs exist, including esters, thioesters, *N*-methyl amides, thioamides, selenoamides, alkenes, and fluoroalkenes, along with many others [1]. Chalcogen amide modification is of particular interest as they are nearly isosteric with the native oxoamide and retain many key features, including hydrogen bonding donor and acceptor capabilities. However, due to the instability of seleno- and telluroamide modifications, thioamides are by far the most widely studied of these chalcogen backbone modifications.

It should be noted that there is considerable variability in the nomenclature surrounding thioamides. In the literature, thioamides have been referred to as thionoamides, thioxoamides, endothiopeptides, mercaptoamides, thiopeptide units, thiodepsipeptides, and thiocarboxamides. The suggested IUPAC nomenclature is thioxoamide, along with the ψ[C(=S)–NH] symbol [2]. However, our laboratory prefers to refer to them as thioamides, for the sake of brevity and for consistency with the traditional organic chemistry literature. Also, we and others use the superscript S (^{S}) rather than ψ[C(=S)–NH] to indicate the position of the thioamide, as this is much less cumbersome, particularly when describing a peptide with multiple thioamide substitutions. In our earlier publications, we used the prime (′) symbol to denote the thioamide, but we have since abandoned this nomenclature, since it may lead to confusion when discussing protease substrates, where the prime symbol is used to indicate a residue's location in relation to the scissile bond. Finally, we note that the term thiopeptides has long been used to refer to natural products that contain thiazole and thiazolene functional groups, such as thiostrepton (**66**; Fig. 8.15). Recent discoveries of the similarity of the biosynthetic mechanisms for the installation of thiazoles, thiazolenes, and thioamides, as well as the discovery that "thiopeptides" such as saalfelduracin (**5**; Fig. 8.2) include all three types of modifications, make it difficult to devise a comprehensive nomenclature.

In this review, we will discuss the work that our group and others have done to synthesize and incorporate thioamides into peptides and proteins. We will also describe the recent elucidation of the biosynthetic pathways for the installation of thioamides into naturally occurring peptides and proteins. Finally, we will discuss the usage of thioamides as spectroscopic and proteolysis probes, along with their usage in injectable peptides for therapeutic and diagnostic applications.

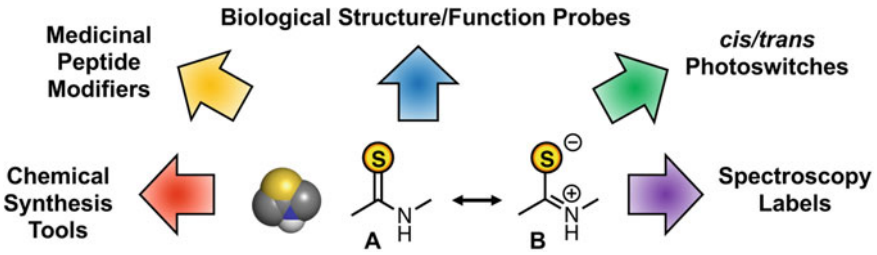


Fig. 8.1 Applications of thioamides

8.1.2 Properties of Thioamides

While the thioamide substitution is appealing as an oxoamide isostere, it does have different physiochemical properties, many of which can be understood by considering that the thioamide populates resonance structure **B** in Fig. 8.1 to a greater degree than resonance structure **A**. The increased single bond character in the thiocarbonyl results from a poorer orbital overlap with the carbonyl carbon's π bonding orbitals, making the thiocarbonyl bond slightly longer than the oxocarbonyl bond, with a lower bond dissociation energy [3]. The increased C=N double bond character explains the higher rotational barrier for the thioamides, and the resulting quaternary ammonium character explains the larger dipole moment of the thioamide as well as the lower N–H pK_a of the thioamide [4, 5]. The decrease in pK_a correlates with stronger N–H hydrogen bond donation by the thioamide [6, 7]. While one might think that the larger dipole moment of the thioamide would make it a better hydrogen bond acceptor as well, the larger van der Waals radius of sulfur makes the partial negative charge more diffuse and again reduces orbital overlap, significantly weakening the hydrogen bond accepting ability of thioamides [8]. Thioamides exhibit higher reactivity as both nucleophile and electrophile [9, 10]. Thioamides are also known to have greater affinities than oxoamides for soft metals, such as Cu(I), Ag(I), Hg(II), and others [11]. In addition to these effects on covalent and non-covalent interactions, thioamidation red-shifts the $\pi \rightarrow \pi^*$ absorption and decreases the oxidation potential of an oxoamide [4, 12]. These two properties, respectively, allow thioamides to participate in Förster resonance energy transfer (FRET) and photoinduced electron transfer (PeT) mechanisms when combined with appropriate donor fluorophores [13]. All of these properties are summarized in Table 8.1 and inform our understanding of the roles of thioamides in natural peptides as well as the use of thioamides as synthetic probes.

Table 8.1 Physical properties of thioamides

Property	Oxoamide	Thioamide
vdW radius (Å) [182]	1.40	1.85
C=X length (Å) [3]	1.23	1.71
Electronegativity [183]	3.44	2.58
C=X···H–N BE (kcal mol^{-1}) [7]	6.1	4.8
N–H pK_a [4]	17	12
$\pi \rightarrow \pi^*$ absorption (nm) [12]	200	270
E_{Ox} (V vs. SHE) [4]	3.29	1.21

Abbreviations: vdW radius refers to the van der Waals radius. BE refers to bond energy. E_{Ox} is the oxidation potential versus a standard hydrogen electrode (SHE)

8.2 Chemical and Biological Synthesis of Thioamides in Peptides and Proteins

8.2.1 Thioamides in Natural Peptides and Proteins

Although relatively rare, several thioamide-containing natural products have been isolated and characterized. In 1997, cycasthioamide (**1**), a tripeptide, was isolated from the seeds of the cycad *Cycas revoluta* (Fig. 8.2) [14]. A combination of high-resolution mass spectrometry and NMR studies confirmed the structure of cycasthioamide, which remains the only thioamide-containing natural product isolated from plants. However, no subsequent studies have revealed its biosynthetic pathway or biological activity.

In contrast, the polythioamidated natural product closthioamide (**2a**) has been extensively studied by Hertweck and coworkers (Fig. 8.2). Closthioamide is a secondary metabolite from the anaerobic bacterium *Ruminiclostridium cellulolyticum* (previously known as *Clostridium cellulolyticum*) [15]. At first, isolation was challenging because expression of the closthioamide biosynthetic gene cluster was silenced under normal laboratory conditions. In 2012, an aqueous soil extract was identified that, when added to the culture, induced natural product and secondary metabolite biosynthesis [16]. The main resulting metabolite was closthioamide **2a**, which was found to have promising antibiotic bioactivity against methicillin-resistant *Staphylococcus aureus* (MRSA), as well as vancomycin-resistant enterococci [17]. The potential to strengthen the antibiotic arsenal led Hertweck and coworkers to characterize the biosynthetic mechanism and the structure–activity relationship of closthioamide using synthetic analogs, as described below. Other closthioamides, including compounds **2b**-**2f**, have been observed in cultures, but their biological activities have not been well characterized [18].

Three types of thioamide-containing ribosomally synthesized and post-translationally modified peptides (RiPPs) have been identified (Fig. 8.2), which

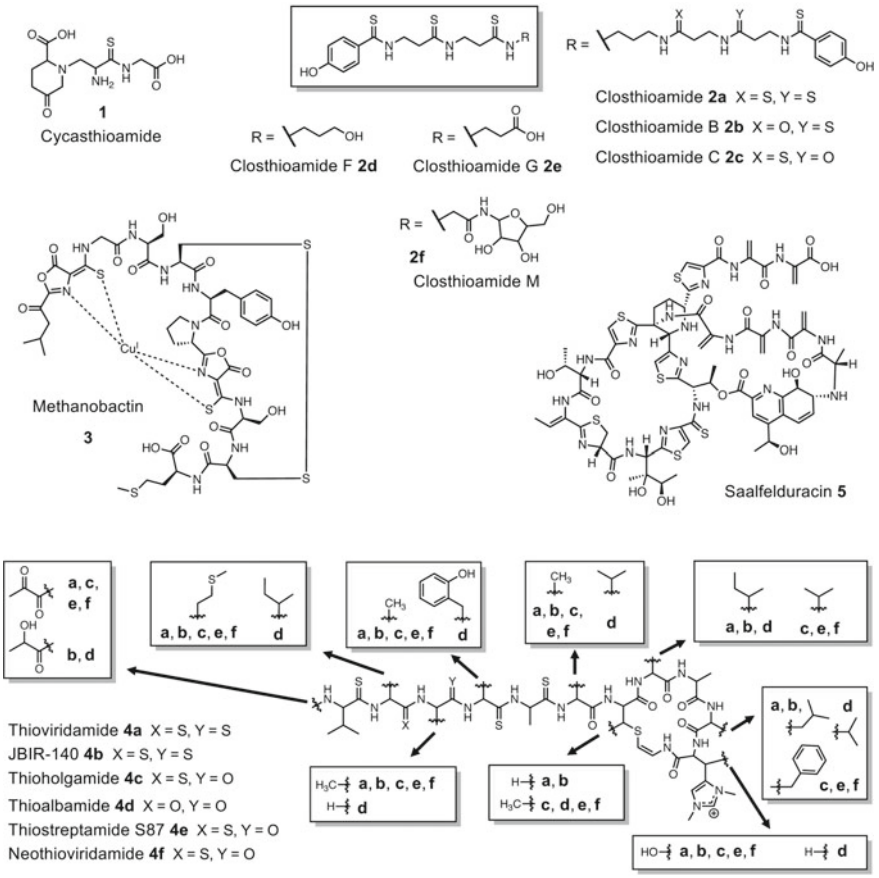


Fig. 8.2 Structures of peptides with naturally occurring thioamides. Cycasthioamide (**1**), closthioamide and closthioamides B, C, F, G, and M (**2a-f**), methanobactin (**3**), thioviridamide (**4a**), JBIR-140 (**4b**), thioholgamide (**4c**), thioalbamide (**4d**), thiostreptamide S87 (**4e**), neothioviridamide (**4f**), and saalfelduracin (**5**)

include methanobactin (**3**), thioviridamide, and related molecules (**4a**-**f**), as well as saalfelduracin (**5**). RiPPs often contain a diverse assortment of posttranslational modifications (PTMs). RiPPs are classically translated as a precursor peptide which consists of an N-terminal leader sequence and a C-terminal core region. Enzymatic machinery then recognizes and modifies the C-terminal core region to generate the desired PTMs [19]. The RiPP methanobactin (**3**) is produced by methanotrophs. Methanobactin is classified as a RiPP because deletion of the gene encoding its precursor peptide, *mbnA*, results in no production of methanobactin [20]. Methanotrophs metabolize methane using methane monooxygenase (MMO) enzymes. Since particulate methane monooxygenase (pMMO) is copper-dependent, there is a cellular need for copper that is fulfilled by methanobactin, a copper-scavenging peptide. The crys-

tal structure of methanobactin isolated from *Methylosinus trichosporium* was solved in 2004 [21]. This structure revealed two thiocarbonyls coordinated to the Cu(I) ion in a distorted tetrahedral arrangement.

The second identified thioamide-containing RiPP is thioviridamide (**4a**), which selectively induces apoptosis in tumor cells expressing E1A-like oncogenes [22]. This peptide was first isolated by Hayakawa et al. in 2006 from an actinomycete, *Streptomyces olivoviridis*, and NMR analysis confirmed the presence of five thioamide moieties [23]. The biosynthetic gene cluster for thioviridamide was elucidated in 2013, and since then multiple thioviridamide-like structures have been isolated, including JBIR-140 (**4b**) [24], thioholgamide A (**4c**) [25], thioalbamide (**4d**) [26], and thiostreptamide S87 (**4e**) [26]. A recently published structure, neothioviridamide (**4f**) [27], is fairly similar to thioholgamide, but the peptides have differing N termini. This discrepancy has been addressed by the work of Truman and coworkers, who determined that the N terminus reported in the original structure of thioviridamide is an artifact of acetone extraction [26]. They instead suggest that the true structure of thioviridamide has an N-terminal pyruvyl moiety, as shown in Fig. 8.2.

To date, a single protein has been identified that has a thioamide PTM. This protein, methyl-coenzyme M reductase (MCR), catalyzes the reversible oxidation of methyl-coenzyme M (CoM) and coenzyme B (CoB) to release methane (Fig. 8.3) [28]. The enzyme is 300 kDa in size and is organized in an $\alpha_2\beta_2\gamma_2$ configuration [28]. Tryptic digest of MCRs from various methanogenic archaea (*Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum*, and *Methanopyrus kandleri*) confirmed that all α subunits contained a thioglycine residue as well as a number of other PTMs [29]. It has been suggested that the thioglycine positioning between the nickel cofactor, CoM, and CoB facilitates the transport of a single electron from a disulfide radical to the nickel [30]. Another possibility is that the thioglycine positions an asparagine residue so that it can hydrogen-bond with the sulfhydryl group of CoB. This decreases the pK_a of the sulfhydryl group, thereby assisting in deprotonation of CoB [30]. Other data from studies of the α subunit of MCR from *Methanosarcina acetivorans* (McrA) suggest that the thioglycine increases its thermostability due to its higher *cis*/*trans* rotational barrier [31]. Since methanogenic archaea can survive in extreme temperature conditions, the stability imparted by the thioglycine modification could have been important in the evolution of the clade.

8.2.2 Biosynthesis of Thioamides in Peptides and Proteins

Recently, significant effort has been devoted to discovering the enzymatic machinery that installs thioamides, as well as the biosynthetic gene clusters that encode these enzymes. One class of enzymes that installs the PTMs on RiPPs is the YcaO superfamily. This superfamily utilizes ATP to phosphorylate and activate the carbonyl of the peptide backbone for the generation of PTMs such as azoline heterocycles and azole PTMs [32]. It is also responsible for the thioamidation of McrA and thioamide-containing thiopeptides, as well as thioviridamide and its associated molecules.

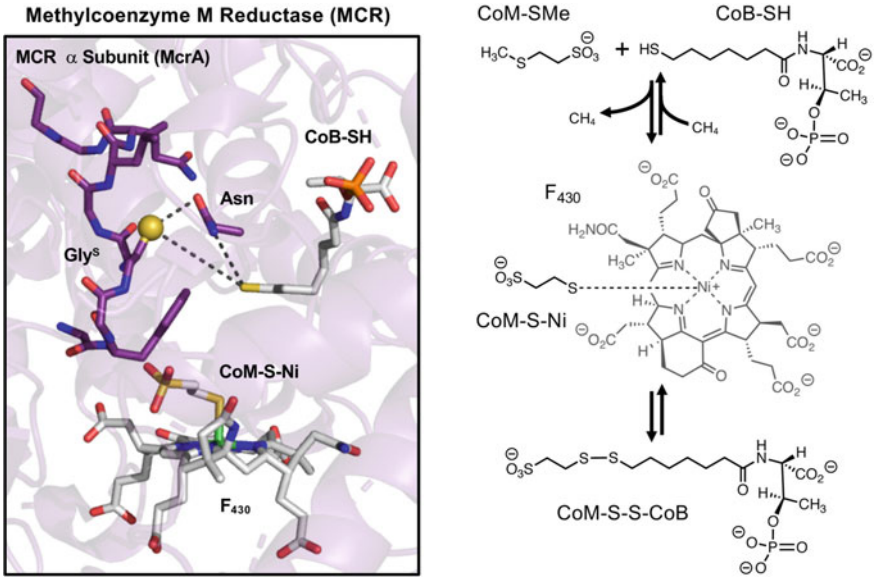


Fig. 8.3 Methyl-coenzyme M reductase (MCR) structure and function. MCR is the only known protein with a thioamide. (A) This enzyme catalyzes the reversible reduction of methyl-coenzyme M (CoM) and coenzyme B (CoB) to release methane in methanogenic archaea. The reaction is catalyzed by the F_{430} cofactor. The thioglycine residue is essential, but its role has not been defined. Left: The active site of the MCR α subunit (McrA) from the X-ray crystal structure (PDB ID: 1MRO) [28]. Right: A minimal reaction scheme showing the Ni-bound CoM observed in the structure

Discovery of the biosynthetic gene cluster for thioviridamide demonstrated that the *tvaA* gene encodes the precursor peptide and the rest of the cluster consists of ten genes (*tvaC-tvaL*) downstream of a regulatory *tvaB* gene (Fig. 8.4). TvaH, which displays homology to the YcaO superfamily, was hypothesized to control thioamide formation [33]. Adjacent to TvaH in the biosynthetic gene cluster is tvaI, which was annotated as TfuA-like. TfuA is involved in the biosynthesis of trifolitoxin, a RiPP antibiotic [34]. Genomic comparison of YcaO domains identified multiple YcaO proteins with TfuA-like partners, defining a class of TfuA-associated YcaOs [35].

Although McrA is not a RiPP, knockout of the *tfuA-ycaO* gene in *M. acetivorans* by Mitchell and Metcalf resulted in no thioglycine generation in McrA (Fig. 8.4) [31]. This association between YcaO-TfuA and thioamidation was further supported when Mitchell showed that purified YcaO and TfuA from *M. acetivorans* could install a thioamide in vitro on a peptide mimicking the native site of thioamidation in McrA [36]. Thioamidation was only possible when both ATP and Na_2S were supplied to the reaction mixture along with TfuA and YcaO. Biophysical analysis with ^{31}P NMR supported the proposed mechanism that YcaO phosphorylates and activates the peptide backbone with ATP for thioamidation (Fig. 8.4). The role of TfuA in thioamidation is currently unknown, as well as how the native sulfide

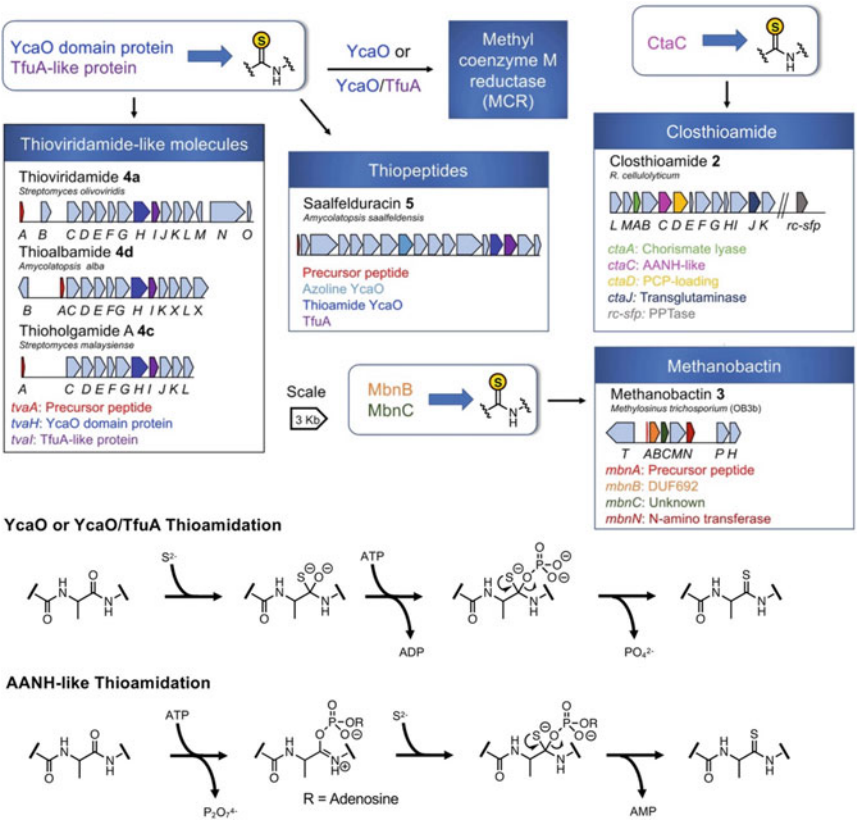


Fig. 8.4 Thioamide genetics and biosynthesis. Based on the known biosynthetic gene clusters for thioamide-containing peptides and protein, there are three enzymatic routes for thioamidation. YcaO and TfuA generate the thioamide in thioviridamide and related molecules, as well as the thiopeptide, saalfelduracin. The thioglycine in MCR is enzymatically installed by either a YcaO/TfuA pair or a TfuA-independent YcaO [31]. The oxazolone–thioamide pair in methanobactin is generated by MbnB and MbnC [39]. The thioamides in closthioamide are proposed to be installed by an α-adenine nucleotide hydrolase (AANH)-related enzyme, which is the same enzymatic machinery that generates the thioamide in 6-thioguanidine and thio-tRNA biosynthesis [18]. Bottom: Proposed mechanism for thioamidation by the YcaO superfamily, which utilizes ATP to phosphorylate the peptide backbone and facilitate the generation of the thioamide, as well as other peptide backbone PTMs. Also shown is the potential mechanism for thioamidation by AANH-like CtaC, which differs in that ATP activation is known to precede sulfur attack in AANH-like enzymes [181]

source is delivered. It is hypothesized that TfuA could allosterically activate YcaO for thioamidation.

Interestingly, TfuA is not required for thioamide generation by YcaO; this was demonstrated by the installation of the thioamide on the same McrA peptide mimic with two TfuA-independent YcaOs from *M. kandleri* and *Methanocaldococcus jannaschii*. A structure of the *M. kandleri* YcaO has been published (PDB ID: 6CIB) and shares homology with LynD, an ATP-dependent cyclohydrase for cyanobactin biosynthesis, but has a single Mg^{2+} ion rather than two [36]. Due to protein instability, a crystal structure for a TfuA-dependent YcaO is currently lacking, and this has prevented structural comparison between the two enzymes [36].

The extent to which the YcaO-TfuA pair is associated with thioamidation was further tested in another class of RiPPs, thiopeptides. Thiopeptides are macrocyclic peptides characterized by the presence of combinations of thiazoline, thiazole, dehydroalanine, and dehydrobutyrine residues. A [4+2] cycloaddition between two dehydroalanine residues and the amide backbone forms the characteristic pyridine lynchpin of the polymacrocyclic structure. The bioinformatics algorithm Rapid ORF Description and Evaluation Online (RODEO), developed by Mitchell, was used to search for YcaO-TfuA pairs in thiopeptide biosynthetic clusters. This method permitted the discovery of a new thioamide-containing thiopeptide, saalfelduracin (**5**; Fig. 8.2) in *Amycolatopsis saalfeldensis* [37]. The connection between YcaO-TfuA and thioamidation in thiopeptides was further confirmed after observing thioamide incorporation in thiostrepton (**66**; Fig. 8.15) following constitutive expression of a noncognate YcaO-TfuA pair from *Micromonospora arborensis* [37].

Unlike the previously mentioned YcaO-TfuA-facilitated thioamidation, the thioamides in methanobactin are generated by a different enzymatic system. Genome mining of *mbn*-like operons based on the presence of the precursor, *mbnA*, determined that *mbnB* and *mbnC* are also always present (Fig. 8.4) [38]. In vitro, data suggest that MbnB and MbnC form a heterodimer that with the presence of reduced iron and O_2 will install the N-terminal oxazolone and thioamide PTM on the precursor peptide [39]. It remains unknown how the N-terminal leader peptide is cleaved or the mechanism by which the second oxazolone–thioamide pair is generated.

The thioamides in closthioamide (**2a**) are also not generated by a YcaO enzyme. Recently, the CTA gene cluster that is responsible for closthioamide production was identified via genome mining, genome editing, and heterologous expression [18] (Fig. 8.4). Unlike thioviridamide and saalfelduracin, which are RiPPs, closthioamide is a non-ribosomal peptide (NRP). Knockout of a gene encoding for a phosphopantetheinyl transferase (PPTase) in the *R. cellulolyticum* genome resulted in the loss of closthioamide production. This demonstrated that synthesis is "thiotemplated" and therefore proceeds through peptidyl carrier protein (PCP)-mediated elongation, characteristic of the synthesis of an NRP. However, none of the non-ribosomal peptide synthetase (NRPS) gene clusters in the *R. cellulolyticum* genome resembled an assembly that could produce closthioamide. Identification of the biosynthetic gene cluster was achieved following mining for a homolog of the enzyme chorismate lyase, which was hypothesized to produce the aromatic building block of closthioamide, *p*-hydroxybenzoate (PHBA). Knockout of the corresponding gene, *ctaA*, resulted

in no production of closthioamide or associated analogs unless PHBA was supplemented in the culture. The *ctaA* gene was then used to identify the neighboring gene cluster, including *ctaA*-*ctaM*. The gene *ctaC* is believed to encode the thionating enzyme, as it has homology to alpha-adenine nucleotide hydrolase (AANH). AANH is known to thionate 6-thioguanine and thioamidated tRNA [18], and hence thionation of closthioamide by the CtaC gene product is plausible. Inactivation of the genes *ctaC* and *ctaD* (PCP-loading enzyme) resulted in no production of closthioamide or associated analogs. The *ctaJ* gene product has homology to the transglutaminase protein family, and it is hypothesized that ctaJ cross-links the diaminopropane linker to the growing precursors. As a result of these findings, Hertweck and coworkers have proposed a mechanism for the biosynthesis of closthioamide (Fig. 8.11, inset). The heterologous production of closthioamide in *Escherichia coli* was attempted, but only thioamide-containing precursors were isolated. Although the full-length product was not produced (for reasons unknown), this was the first occurrence of a thioamide-containing natural product being expressed in *E. coli*.

These advances in genome mining have also allowed for the identification of multiple thioviridamide-like molecules. Since thioviridamide has antiproliferative activity, the discovery of thioviridamide-like molecules is valuable for its potential medicinal purposes. Genome mining for thioviridamide-like molecules has identified 13 homologous biosynthetic gene clusters in actinobacteria (*Streptomyces* spp., *Amycolatopsis alba*, *Micromonospora eburnea*, and *Nocardiopsis potens*), as well as another in cyanobacteria (*Mastigocladus laminosus*) [25, 26]. Homologs of the *tvaA* through *tvaI* (except *tvaB*) genes are shared in all 13 biosynthetic gene clusters, and *tvaJ* through *tvaL* are located in all except for one (Fig. 8.4). Although similar, the precursor peptide sequence differs in these organisms, resulting in analogs of thioviridamide. Further characterization and isolation identified thioholgamide, **4c**, from *Streptomyces malaysiense* [25], as well as thioalbamide, **4b**, from *A. alba* (Fig. 8.2) [26]. Similar to thioviridamide, both molecules demonstrated antiproliferative bioactivity against cancerous cell lines. These findings, as well as Mitchell and Metcalf's observation that YcaO-like enzymes occur widely in microorganisms [31], raise the intriguing possibility that natural thioamide-containing peptides and proteins are more widespread than previously appreciated and may harbor interesting thioamide-dependent activity. This expansion of known thioamide-containing natural products prompts renewed interest in methods for incorporating them at specific sites in peptides and proteins.

8.2.3 Chemical Synthesis of Thioamides in Peptides

The first reported synthesis of a thioamide was performed in 1815 by Gay-Lussac, where cyanogen and hydrogen sulfide were used to form cyanothioformate and dithiooxoamide [40]. However, the products of this reaction are not very useful for the synthesis of thioamide functionalized molecules. One of the first methods used to create a variety of different thioamides was the Willgerodt–Kindler

reaction, which allowed thioamides to be synthesized from ketones, aldehydes, isothiocyanates, and amides [41]. A key development in the field was the advent of Lawesson's reagent (**6**, 2,4-Bis-(4-methoxyphenyl)-1,3,2,4-dithiadiphosphatane-2,4-dithione) and the use of tetraphosphorous decasulfide (P_4S_{10}), which have since served as standard reagents for synthesizing thioamides (Fig. 8.5) [42, 43]. Recently, interest in creating thioamides for uses as biological probes, metal frameworks, and other applications has led researchers to investigate newer, easier methods to synthesize thioamides. These include methods for synthesizing thioamides from methylarenes, aldehydes, ketones, thiols, alkynyl bromides, oximes, and carboxylic acids [44–50]. There has also been an effort to investigate milder conditions to form thioamides, by decreasing the reaction temperature, removing metals, and performing the reactions in different solvents [51–53]. Methods that utilize Lawesson's reagent, elemental sulfur, and P_4S_{10} produce toxic by-products, so there have been several efforts to create newer, safer thionating reagents (Fig. 8.5). To this end, Bergman and coworkers were able to isolate and use a pyridine-P_4S_{10} complex in acetonitrile to thionate a variety of scaffolds [54]. Interestingly, Yadav and coworkers were able to thionate aldehydes and ketones using *O,O*-diethyl dithiophosphoric acid (**7**) [50]. Similarly, Kaboudin and coworkers reported a method that utilizes ammonium phosphorodithioates (**8**) in the direct conversion of carboxylic acids to thioamides [45]. Due to the difficulty in purifying by-products from reactions with Lawesson's reagent, Ley et al. developed a solid-phase thionating reagent (**9**) that is able to convert secondary and tertiary amides to thioamides [55]. This solid-phase thionating reagent can be removed from the reaction mixture using a simple fritted funnel, which makes purification significantly easier. In an alternative approach, Kaleta and coworkers have created fluorinated derivatives of Lawesson's reagent (**10**) that can be removed from the reaction by extraction [56, 57].

For the incorporation of thioamides into peptides, the use of reagents that are compatible with standard peptide synthesis methods is highly desirable. To our knowledge, the first synthesis of a thiopeptide was completed in 1911 by Johnson and Burnham, where they produced thioimidated glycine derivatives by reactions of nitriles with hydrogen sulfide [58]. However, it was not until 1973 that the first thioamide derivative of a complex peptide was made, when du Vigneaud and coworkers synthesized a thioamide analog of oxytocin (**49**; Fig. 8.12) [59]. They first synthesized thioglycinamide by thionating *N*-benzoyl-glycinamide with P_4S_{10}. The thioglycinamide was then incorporated into a tripeptide fragment using solution phase peptide synthesis. Finally, this tetrapeptide was coupled to the cyclic core of oxytocin. The incorporation of thioamides into short peptide fragments for later inclusion into a larger peptide paved the way for the rational synthesis of thiopeptides.

Direct thionation of peptides has also been reported as a method for incorporation of thioamides into the backbone of peptides [3, 60]. In these examples, short peptides containing α-aminoisobutyric acid (Aib) or β-amino acids are directly thionated with **6**. However, in some cases, a mixture of thionated products is observed. Additionally, Heimgartner et al. reported the synthesis of thionated dipeptides containing Aib by the "azirine/thiazolone" method, which incorporates thioamino acids into a peptide through a ring opening reaction [61]. Initially, epimerization of the α-carbon

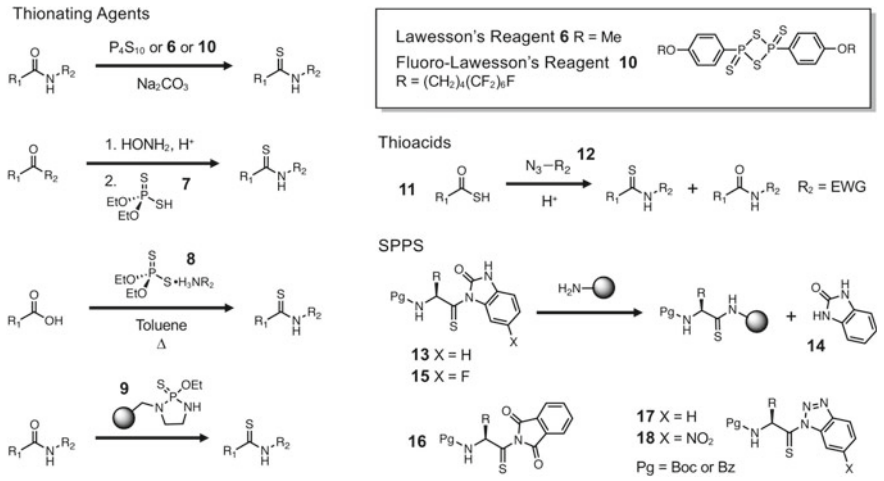


Fig. 8.5 Previous routes to thioamides. Left: Thionating agents to generate thioamides in small molecules. Right: Thioamide incorporation into peptides using thioacids and azides (EWG: electron-withdrawing group) or activated thiocarbonyl solid-phase peptide synthesis (SPPS) reagents

of the amino acid selected for thionation made this method problematic. Later, it was reported that the use of HCl/$ZnCl_2$ during the migration of the thioamide from Aib to the $n + 1$ residue abolished epimerization, leading to stereochemically pure thiopeptides [62]. However, the need for including Aib in the peptide sequence still limited the utility of this method.

Thioacids (**11**) have also been used to install thioamides into peptides, both using peptide coupling reagents and reactions with electron-poor azides (**12**). Studies by Williams and coworkers elaborated the mechanistic pathways leading to either oxoamide or thioamide incorporation through reactions with azides [63]. More recently, Hackenberger's group determined that the ratio of oxoamide-to-thioamide peptides could be controlled through optimization of the azide moiety and the pH of the reaction [64]. It was found that electron-poor azides and acidic reaction conditions gave the best conversion to the desired thioamide peptide, but that selectivity was still limited to 11:1 (Fig. 8.5). Thioacids have also been activated for peptide incorporation using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and bis(2-oxo-3-oxazolidinyl)phosphinic chloride coupling reagents [65, 66]. Activation with PyBOP was performed on fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, which enabled easy integration with common solid-phase peptide synthesis (SPPS) procedures, unlike the previous methods. However, it was found that these coupling procedures must be optimized for each amino thioacid, due to the potential for epimerization.

In order to efficiently incorporate thioamides into peptides made by SPPS, Belleau and coworkers established a method using thioacyl-benzimidazolones (**13**) as chemoselective thioacylating agents that were stable and storable (Fig. 8.5) [67].

While this was a valuable first account of a chemoselective thioacylating agent, there were a few drawbacks to this method, including a small loss (2%) of enantiometic purity and a reactive benzimidazole by-product (**14**), reducing the overall yield to 20%. After this report, several others reported similar thioacylating reagents such as fluorobenzimidazolones (**15**), thioacyl-*N*-phthalimides (**16**), benzotriazoles (**17**), and nitrobenzotriazole (**18**) derivatives (Fig. 8.5) [68, 69]. The nitrobenzotriazole thioacylation method reported by Rapoport and coworkers is now by far the most widely used route for high yielding syntheses of thioamide precursors that can be stored and coupled with no observable epimerization [68].

We and others have successfully synthesized Fmoc thioacylbenzotriazole monomers (**22a**/**22b**) of Ala, Ile, Leu, Phe, Pro, and Val utilizing *N*-Boc-1,2-phenylenediamine (**20a**) and L- and D-Ala, Arg, Asp, Cys, L- and D-Glu, Gly, Ile, Leu, Lys, L- and D-Phe, Pro, Ser, L- and D-Tyr, and Val, as well as D-Trp and L-4-hydroxyproline (Hyp), utilizing 4-nitro-1,2-phenylenediamine (**20b**) as amidating reagents (Fig. 8.6) [70–81]. Fmoc protection of the α-amine was used due to its compatibility with SPPS methods that do not require HF for sidechain deprotection and resin cleavage. These thioamino acid monomers are stable to storage conditions for several months and can be used without further purification after the ring closing step. Incorporation follows standard Fmoc SPPS procedures, excepting that no additional coupling reagent is required for addition of the thioacylbenzotriazole monomer. Typical yields of thioamide-containing peptides are about 25–50% of the corresponding oxoamide sequence. While this is undesirable, the methods are sufficiently robust that they have been used to synthesize a variety of thiopeptides of up to about 40 amino acids in length, including yeast transcription factor GCN4-p1, villin head piece variant HP35, glucagon-like peptide 1 (GLP-1), gastric inhibitory peptide (GIP), and various β-hairpins [73, 75, 82–84]. To our knowledge, the longest peptide sequence synthesized by SPPS is the B1 fragment of protein (GB1, 56 amino acids) synthesized by the Horne Laboratory, but this was produced in only 1.2% yield [85].

The lower yields of thioamide-containing peptides are caused by side reactions that can arise during SPPS due to the fact that thioamides are more reactive than oxoamides with both nucleophiles and electrophiles. Specifically, in the acidic conditions of resin cleavage, an Edman degradation-like reaction can cause backbone cleavage at the $n + 1$ position (Fig. 8.7). To avoid this, one must consider shortening cleavage times, which must be balanced with a need to fully deprotect all acid-labile protecting groups. Another side reaction that can occur is a S-to-O exchange when water and base are present (Fig. 8.7). In order to avoid this, our laboratory and others have used anhydrous methylene chloride in the coupling step of the thioamino acid precursor [72]. Finally, epimerization of the α-carbon of the thioamide residue is a serious issue that can arise during the Fmoc deprotection steps subsequent to incorporation (Fig. 8.7). This epimerization is possible due to the lower pK_a (~13) of the thioamide α-carbon (see a detailed analysis in the Supporting Information of Szantai-Kis, [85]) [86]. In order to decrease the amount of epimerization, Chatterjee and coworkers decreased the concentration of piperidine used in their Fmoc deprotection solution and shortened the reaction time [79]. While this led to a noticeable decrease in epimerization of the thioamide, they also noted that the yield of their

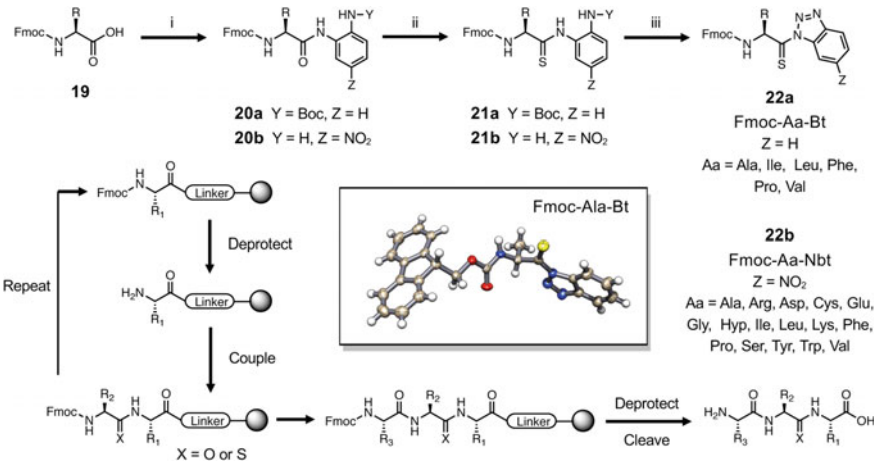


Fig. 8.6 Thioacylbenzotriazole monomers for solid-phase peptide synthesis (SPPS). Top: Synthesis of benzotriazolide and nitrobenzyltriazolide thioamino acid precursors: (i) 1. *N*-methylmorpholine, isobutyl chloroformate, THF, −10 °C, 2. *N*-Boc-1,2-phenylenediamine or 4-nitro-1,2-phenylenediamine; (ii) Na_2CO_3, P_4S_{10}, THF; (iii) for benzotriazolide: Trifluoroacetic acid (TFA), then $NaNO_2$, 5% H_2O/AcOH, 0 °C; for nitrobenzyltriazolide: $NaNO_2$, 5% H_2O/AcOH, 0 °C. Bottom: SPPS with thioamides. Deprotect: 20% piperidine is used in deprotections prior to thioamide incorporation, and 2% DBU is used following thioamidation to decrease the amount of epimerization. Couple: For thioamides, Fmoc-Aa-Bt (**22a**) or Fmoc-Aa-Nbt (**22b**); for other amino acids, Fmoc-Aa-OH, (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIPEA). Cleave: TFA and other additives, TFA concentrations limited to reduce Edman degradation-like reactions

peptide synthesis decreased significantly due to incomplete Fmoc deprotection. Our laboratory has also investigated methods for decreasing the epimerization of the thioamide. Specifically, we have used a 2% solution of a more hindered base, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), in our deprotection solutions for removal of all Fmoc groups subsequent to thioamide incorporation [86]. When treated for a shorter time with the DBU solution, there is a significant decrease in the amount of epimerization of the thioamide.

Due to the difficulty that can come with incorporating thioamides by SPPS, a few groups have studied incorporating thioamides enzymatically. Unverzagt and coworkers utilized chymotrypsin in the enzymatic incorporation of thiodipeptides into growing peptide chains [87]. While this work was only limited to the synthesis of short peptides, it could be applicable to longer sequences upon optimization of the enzyme and protecting groups that are utilized. More recently, Mitchell and coworkers have examined the usage of thiazole/oxazole-modified microcin (TOMM) cyclodehydratases to install thioamides (and other amide modifications) into the backbone of peptides and proteins, a method they call azoline-mediated peptide backbone labeling [88]. Through the use of the Balh cyclodehydratase, Cys, Ser, and Thr residues can be cyclized onto the carbonyl of the n-1 residue to form an

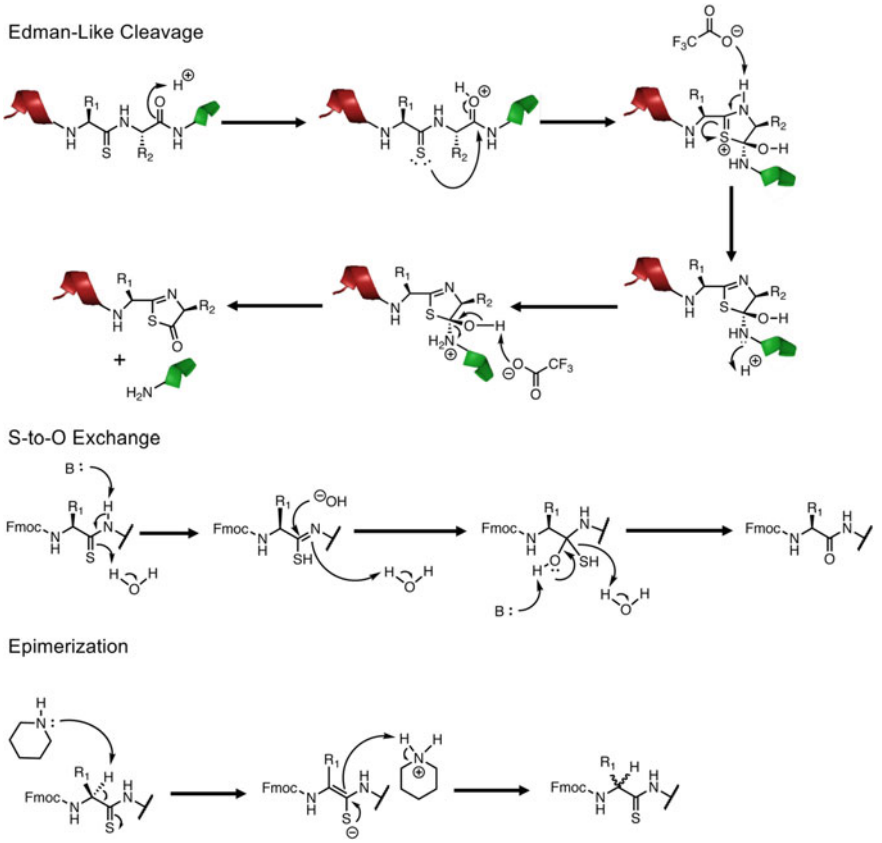


Fig. 8.7 Side reactions of thioamides. A cleavage reaction similar to Edman degradation occurs through reaction of the thioamide with the $n + 1$ amide in acidic conditions (i.e., during resin cleavage). S-to-O exchange can occur in basic aqueous conditions due to the lower N-H pK_a of the thioamide. Epimerization can occur with piperidine during Fmoc deprotection due to the decreased C_α-H pK_a. of the thioamide

azoline. After treatment with KHS, the azoline ring is opened to afford a thioamide N-terminal to the Cys, Ser, or Thr residue. While this reaction is limited in sequence scope, the elucidation of thioamide biosynthesis pathways may lead to more practical enzymatic methods for installing thioamides, for example by use of YcaO enzymes (see above). At this time, SPPS methods remain the most practical way of installing thioamides into peptides of up to about 40 amino acids and coupling of peptide segments must be used to form longer sequences.

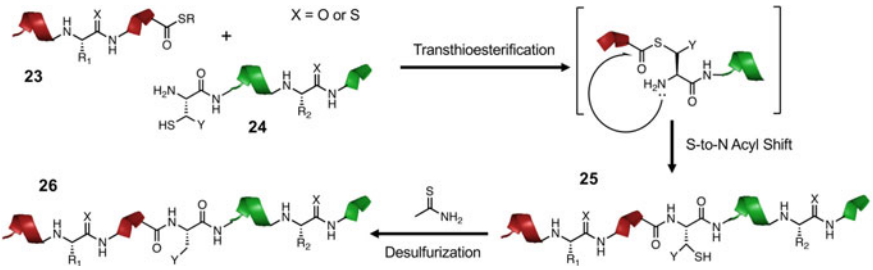


Fig. 8.8 Native chemical ligation (NCL). NCL generally requires a peptide with a C-terminal thioester (**23**) and a peptide and an N-terminal Cys (**24**) or Cys analog (Y = amino acid sidechain). Thioamide incorporation is compatible with both the C-terminal thioester portion and the N-terminal Cys portion. In the first step, a transthioesterification reaction between the C-terminal thioester and the N-terminal Cys generates an initial covalent intermediate species. Next, an intramolecular S-to-N acyl shift forms the ligated product (**25**) with a "native" amide bond at the Cys ligation site. Finally, the Cys can be desulfurized to form Ala in the final product protein (**26**). Cys analogs are desulfurized to form the corresponding natural amino acid. In the presence of a thioamide, desulfurization can be performed with the use of organic radical initiator VA-044 and the use of thioacetamide as a sacrificial additive to prevent S-to-O exchange of the thioamide

8.2.4 Thioamide Incorporation into Synthetic Proteins

While thioamides can be incorporated into peptides through SPPS, there is currently no way to incorporate backbone thioamides into full-length proteins ribosomally (see Sect. 8.4 for discussion of thioamide dipeptide incorporation by Hecht). Therefore, thioamides must be incorporated into proteins through the use of native chemical ligation (NCL) or expressed protein ligation (EPL) [89]. Briefly, NCL refers to the reaction of a peptide fragment containing a C-terminal thioester (**23**) with a peptide fragment containing an N-terminal Cys or Cys analog (**24**) to yield a peptide product linked by a "native" amide bond. The key step in the reaction is the initial engagement of the two fragments through transthioesterification, as shown in Fig. 8.8. While this step is reversible under the reaction conditions, the subsequent S-to-N acyl shift is not, and the ligated product (**25**) can then be desulfurized to form the final synthetic protein (**26**) if a Cys is not desired at the ligation site. Desulfurization of Cys provides Ala, and a variety of other β- or γ-thiol analogs can be used to produce other amino acids, so that one's choice of ligation site is not significantly limited when designing a protein retrosynthesis [90]. EPL allows one to produce one of the two fragments by expression and purification from cells to avoid unnecessary synthesis of long stretches of natural amino acids. When we began our study of the applicability of NCL to synthesize thioamide-containing proteins, there were no previous published efforts, and in fact Fischer and coworkers had stated that "the presence of thioxo-peptide bonds is not compatible with the subsequent synthesis of the thioester which is necessary for the ligation procedure," presumably referring to HF-based SPPS methods [91]. Thus, much of our early effort was devoted to finding NCL conditions, whereby thioamides would not undergo significant side reactions.

We synthesized short peptides for test ligations where Leu^S was placed in either the N or the C terminus of fragments with a variety of sequences and lengths [71]. In general, we observed that thioamides were well tolerated in both the C-terminal thioester and the N-terminal Cys containing peptide for NCL, with only a few sequence-specific issues. For example, we found that thioamides are not tolerated at the n-1 position in relation to the C terminus of the fragment due to cyclization caused by the increased electrophilicity of the thioester. Side product formation was observed when Cys disulfides were reduced with *tris*(2-carboxyethyl)phosphine (TCEP), but this was shown to be avoidable upon sparging solvents with argon in order to prevent prior disulfide formation. No side reactions were observed when thiol-reducing agents were used. Thus, we found that one of the most significant barriers to overcome was the need for a robust method for the synthesis of thioamide-containing thioester fragments.

Our initial investigations utilized PyBOP to form the C-terminal thioester (**27**; Fig. 8.9), which requires that the sidechain-protecting groups remain intact for activation. This can lead to decreased solubility and epimerization of the C-terminal residue at long reaction times, limiting the sequences and sizes of thioester peptides that can be made using this method. To avoid this issue, we investigated using a method developed by Kawakami and MacMillan that would form the thioester through intramolecular cyclization (Fig. 8.9) [92]. In this method, a tripeptide sequence containing Csb-Pro-Gla (**28**, where Csb is a *t*-butyl thiol-protected cysteine and Gla is glycolic acid) is appended to the C terminus of the peptide of interest through SPPS on Rink amide resin [92, 93]. Upon cleavage of the peptide from resin and subsequent treatment with TCEP, the free Cys residue undergoes an N-to-S acyl shift with the backbone carbonyl of the n-1 residue, yielding a free amine that is able to attack the *O*-ester of the Gla residue to form a diketopiperazine thioester (**29**). This thioester either can be used directly in a ligation reaction or can undergo transthioesterification with other thiols to yield a different thioester. We utilized this method to create a hexameric peptide containing Leu^S with the Csb-Pro-Gla sequence that, upon cleavage from the resin, could be used in a one-pot Csb deprotection, cyclization, and ligation reaction with very little side product observed [71].

Our group has also examined the utility of Dawson's *N*-acyl benzimidazolinone (Nbz) method to synthesize a thioamide with a C-terminal Nbz group (**31**; Fig. 8.9) [70, 94]. 3,4-Diaminobenzyl (Dbz, **30**) resin was used to synthesize a Val^S containing peptide fragment. After peptide synthesis, 4-nitrophenylchloroformate was used to form the *N*-acylurea of the Nbz group. When treated with an N-terminal Cys containing peptide, ligation proceeded with 90% conversion. Despite the high conversion, we were only able to obtain an isolated yield of 15% of the desired peptide. This is likely due to the side reactivity that the Dbz resin can undergo at its secondary amino group. In order to address this issue, many reports suggest that the usage of allyloxycarbonyl (Alloc) protection of this amine can avoid branching and by-products. However, the removal of Alloc groups requires Pd^0, which desulfurizes thioamides [95, 96]. We investigated a variety of other Fmoc SPPS thioester generation methods, including the use of Botti and Muir's α-hydroxy Cys analog (Chb), [97, 98] which performed very well in ligations, but was less desirable because the Chb required a

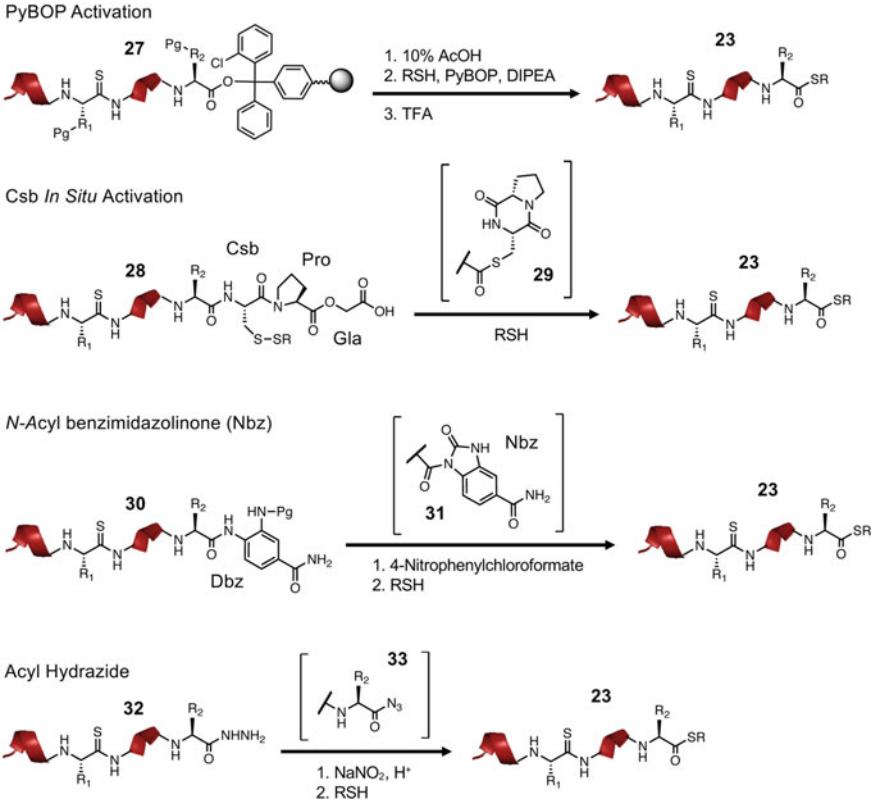


Fig. 8.9 Thioamide-containing peptide thioester synthesis. PyBOP activation: After cleavage of a peptide (**27**) from 2-chlorotrityl resin with mild acid, leaving sidechain-protecting groups intact, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) can be used to activate the carboxy terminus to form the thioester (**23**) for use in ligation. Csb in situ activation: Using a Csb-Pro-Gla linker (**28**, where Csb is *t*-butyl thiol-protected cysteine and Gla is glycolic acid), after disulfide reduction, the free Cys is able to attack the Gla ester linkage to form a diketopiperazine thioester (**29**) for use in ligation. The diketopiperazine thioester can be exchanged by treatment with a thiol additive. *N*-Acyl benzimidazolinone (Nbz, **31**): Using resin with a cleavable 3,4-diaminobenzyl (Dbz, **30**) linker, the peptide C terminus can be transformed into an *N*-acyl benzimidazolinone upon treatment with 4-nitrophenylchloroformate. Nbz can be displaced by treatment with a thiol. Acyl hydrazide: After synthesis on hydrazine-loaded 2-chlorotrityl resin and cleavage from the solid phase, the C-terminal acyl hydrazide (**32**) can be reacted with sodium nitrite in acid to form the C-terminal acyl hydrazide (**33**), which can be displaced by treatment with a thiol to form the C-terminal thioester (**23**)

five-step synthesis before resin loading [99]. Therefore, we were intrigued by Liu's 2011 report of the use of C-terminal acyl azides as latent thioesters [100].

We investigated the compatibility of thioamides with C-terminal acyl azides (**33**) in the synthesis of GB1, a 56 amino acid protein that is frequently used as a model system in protein structure investigations. The synthesis of C-terminal acyl azides starts by treating 2-chlorotrityl resin with a 5% hydrazine in DMF solution. This forms a C-terminal hydrazide (**32**) which is stable during the entirety of the peptide synthesis and cleavage. Prior to ligation, the C-terminal hydrazide can be converted to the acyl azide through treatment with $NaNO_2$ in acidic buffer. Then, this acyl azide can be converted to the thioester in situ using a thiol (often 4-mercaptophenylacetic acid) in a neutral to a basic buffer solution (Fig. 8.9) [100]. We successfully utilized this method in the ligation of GB1 constructs containing thioamides at positions 5, 6, and 7 [85]. Utilizing the C-terminal acyl azides to access the C-terminal thioester, we were able to synthesize thioamide-containing GB1 constructs with 30–40% yields, after purification.

Our laboratory has also investigated the compatibility of thioamides with methods that are used for multi-part ligations. For a central fragment that will undergo two ligations, the N-terminal Cys needs to be protected so that it will not react with C-terminal thioesters in an intramolecular fashion to cyclize or in an intermolecular fashion to oligomerize. In 2006, Kent and coworkers described a protection method where the Cys could be reversibly masked as a thiazolidine (Thz; Fig. 8.10) [101]. Once the C-terminal thioester has been ligated, the N-terminal Thz can be treated with methoxylamine to reveal an N-terminal Cys that can be used in another NCL reaction. There was some concern that there might be cleavage of the peptide at the thioamide bond due to treatment with a nucleophile like methoxylamine (see Sect. 8.3.5 below). However, we were able to demonstrate that Thz protection was compatible with the three-part ligation of a thioamide-containing peptide with no observable thioamide backbone cleavage [71].

In the pursuit of chemically synthesized proteins, EPL has been an important advance, utilizing the expression of large portions of proteins that can be used in NCL with shorter, synthetic peptides [102]. We have successfully used EPL to incorporate thioamides into α-synuclein (αS), calmodulin (CaM), and GB1, with 99% retention of the thioamide [77, 85, 103]. However, we are also interested in double-labeling proteins with thioamide–fluorophore pairs for FRET studies. In order to achieve this, we have used unnatural amino acid mutagenesis to incorporate cyanophenylalanine (Cnf) into $\alpha S_{9\text{-}140}$ expressed as in *E. coli*. To obtain an N-terminal Cys, we can express the protein with an N-terminal polyhistidine tag for purification by Ni^{2+} affinity chromatography. After this step, the His tag can be removed using the appropriate protease, to reveal an N-terminal Cys for ligation. We have also had success utilizing the endogenous methionine aminopeptidase in *E. coli* to cleave Met from an appended N-terminal Met-Cys sequence. The N-terminal Cys reacts with endogenous aldehydes to form Thz (**34**; Fig. 8.10). Deprotection with $MeONH_2 \cdot HCl$ reveals the N-terminal Cys on the protein for ligation. After purification of the deprotected expressed fragment (**35**), a short peptide containing Phe^S was ligated for 24 h, resulting in double-labeled protein. However, this method still required the instal-

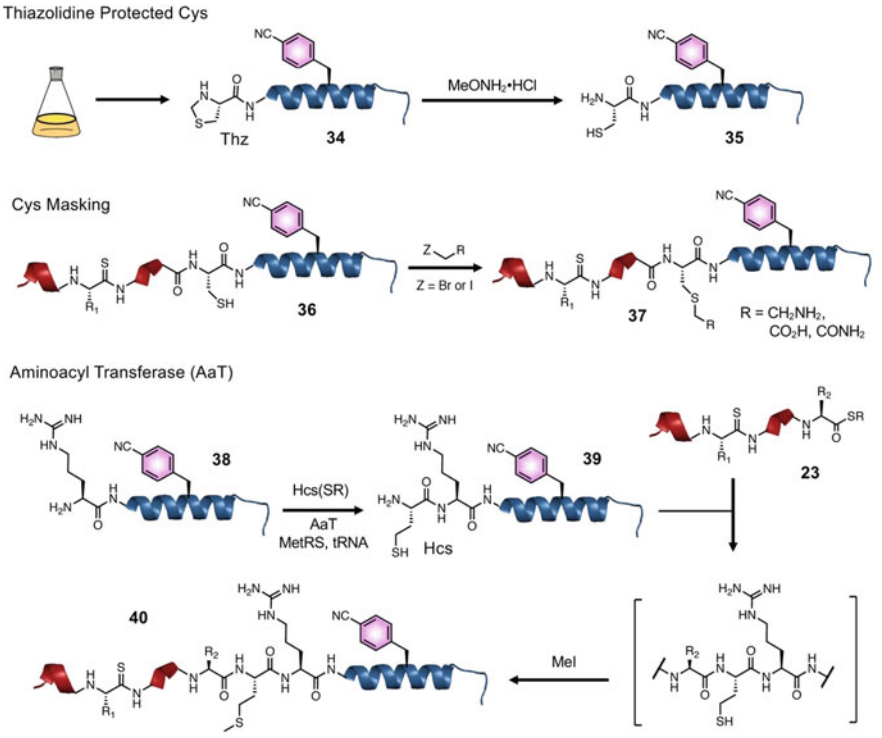


Fig. 8.10 Thioamide expressed protein ligation (EPL). Thiazolidine protected cys. Proteins expressed with an N-terminal Met-Cys sequence and an unnatural amino acid (**34**) are obtained with the Met residue cleaved by methionine aminopeptidase and the N-terminal Cys converted to a thiazolidine (Thz) by reaction with endogenous aldehydes. Treatment with methoxylamine opens the Thz ring, exposing the Cys residue for ligation (**35**). Cys masking: After ligation (**36**), Cys can be masked as mimics of other amino acids by reaction with alkyl halides (**37**); bromoethylamine yields a Lys mimic, iodoacetic acid, or iodoacetamide yields a Glu or Gln mimic, respectively. Aminoacyl transferase (AaT): AaT and a Met tRNA synthetase (MetRS) can be employed to append an *S*-protected homocysteine (Hcs) to the N terminus of an expressed protein with a terminal Arg (**38**) or Lys (not shown) residue. This segment (**39**) can be used in a ligation reaction with a thioester (**23**) and then masked with methyl iodide to form a Met residue at the ligation site in the product protein (**40**)

lation of a non-native Cys residue in order to perform the chemistry required for ligation. Our group has also shown that C-terminal thioesters can be obtained from expressed proteins by simply incubating an intein-containing protein with MESNa (2-mercaptoethanesulfonate), which will cleave the intein leaving a C-terminal thioester for ligation.

Requirement of a ligation site Cys in order to perform NCL or EPL was a significant initial limitation of the methods which has been largely eliminated through methods for desulfurization of Cys or Cys surrogates after ligation. It has been shown that β- or γ-thiol analogs of many of the natural amino acids can be used in NCL

reactions, with selective desulfurization following, in order to obtain a traceless ligation [104]. The Raney Ni conditions used in early NCL desulfurization reactions were not compatible with thioamides, leading to desulfurization and backbone cleavage [105]. The organic radical initiator VA-044 (2,2′-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride) developed by Danishefsky was reported to be selective for desulfurization at Cys, even in the presence of possibly reactive PTMs [106]. Our group was able to demonstrate that thioamides are also compatible with the usage of VA-044 for selective desulfurization, provided that thioacetamide is used as a sacrificial scavenger (Fig. 8.8) [105]. This was then successfully applied to the synthesis of a thioamide-containing analog of GB1.

In addition to desulfurization, reactions which convert Cys to mimics of other amino acids are also valuable ways of masking the ligation site Cys. Previously, it has been reported that Cys can be reacted with bromoethylamine in order to yield a Lys mimic [107, 108]. In this reaction, the protein is dissolved in pH 8.6 Tris buffer and reacted with bromoethylamine in order to obtain the alkylated Lys mimic (**37**, R=CH_2NH_2; Fig. 8.10). In our hands, this reaction has proven to be compatible with thioamides in the semi-synthesis of GB1 constructs. Cys can also be masked to produce Gln/Glu mimics at the site of ligation. In our synthesis of thioamide-containing CaM constructs, a Gln was mutated to a Cys to allow for ligation to occur. After ligation, this free Cys containing CaM was treated with iodoacetamide in order to yield the Gln mimic [85]. Similarly, iodoacetic acid can be used in order to create a Glu mimic at the ligation site [109]. Our group has observed that these masking techniques are compatible with thioamide inclusion provided that Cys alkylation times are limited. These findings allow for a larger sequence space of thioproteins to be synthesized using ligation techniques.

Finally, we have shown that some Cys analogs can be enzymatically transferred to the N terminus of proteins, used in ligation, and then converted to other amino acids (Fig. 8.10). Homocysteine (Hcs) can be used in this manner, as treatment with CH_3I upon completion of protein synthesis converts it to Met [110]. In order to also expand the scope of possible EPL reactions, our group developed a method to enzymatically incorporate Hcs into proteins [111]. Utilizing the *E. coli* enzyme leucyl/phenylalanyl amino acyl transferase (AaT), a protein with N-terminal Lys or Arg (**38**) can be functionalized with *S*-(thiomethyl)homocysteine (Hcm) [112–114]. After transfer, the Hcm can be converted to Hcs through TCEP deprotection (**39**), which allows for subsequent reaction with a C-terminal thioester (**23**). Hcs can then be converted to Met with CH_3I (**40**). We have shown that this can be used with unnatural mutagenesis in the expressed protein fragment to generate Cnf-labeled αS containing an Asp^S using Met_5 as the ligation site. AaT can also be used to transfer selenocysteine (Sec) to proteins with N-terminal Lys or Arg. [105] Sec can be used in NCL reactions and selectively deselenized to Ala using TCEP without desulfurizing Cys residues in the ligated protein. Sec can also be converted to Ser [115]. These experiments demonstrate the value of being able to functionalize protein N termini with traceless ligation handles. Our laboratory has investigated mutation of AaT to remove the limitation of specificity for N-terminal Lys or Arg (Unpublished Results), and recently Rozovsky and Wang have developed a tRNA synthetase for

Sec incorporation, which provides a more general method for Sec insertion at ligation sites [116].

Taken together, these developments have enabled the synthesis of large proteins containing thioamides in combination with other unnatural amino acid modifications. We are interested in using these methods to synthesize a variety of thioamide-containing proteins in order to get structural data, such as 2D NMR spectra or a crystal structure, to determine the effect of this substitution on the global structure of the system within which it is placed. Our laboratory is also interested in determining ways to incorporate sidechain thioamides into peptides and proteins as a way to utilize thioamides as biophysical probes with less potential for disrupting the secondary structure of the protein. This method is particularly interesting as there is the possibility that sidechain thioamides can be incorporated ribosomally into the protein of interest [117].

8.3 Applications of Synthetic Thioamides

8.3.1 Introduction to Applications

The ability to chemically synthesize thioamides enables their site-specific installation to study natural systems and to be used as probes in artificial peptides and proteins. Thioamides have been used to investigate the biosynthesis and the structure/function relationships of natural thioamide-containing peptides. They have also been incorporated to alter the chemical properties of antibiotic peptides in order to better understand their bioactivity, to optimize potency, and to serve as synthetic intermediates for other transformations. Their subtle, yet significant, differences from the native amide bond have been used to probe protease mechanisms and to stabilize peptides for in vivo applications by preventing proteolysis and restricting peptide conformations.

8.3.2 Using Synthetic Thioamide Probes to Study Natural Systems: Closthioamide

The most extensive work to date in studying a thioamide natural product has been done by Hertweck and coworkers. Striving to complete a more thorough analysis of the secondary metabolites from *R. cellulolyticum*, they cultured a mutant that overexpressed the antiterminator gene, *nusG* [17]. Closthioamide and hydroxybenzoate were isolated, as well as various analogs of closthioamide. To determine if the analogs were produced as biosynthetic side products or by degradation of closthioamide, deuterium-labeled closamide (the all oxoamide analogs of closthioamide) and fluorinated closthioamide (**46**) were supplemented in the cultured *R. cellulolyticum*

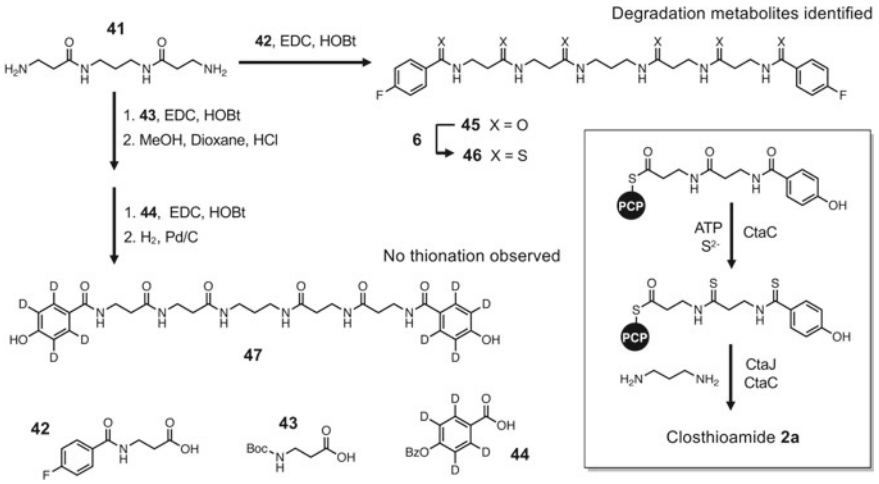


Fig. 8.11 Thioamide studies of closthioamide. Prior to the identification of the CTA cluster (Fig. 8.4), these closthioamide analogs were used to analyze the biosynthetic pathway [119]. The presence of certain fluorinated fragments after addition of **46** confirmed which of the observed were degradation fragments of closthioamide. Slow S-to-O exchange of **46** to form **45** was also observed. The absence of thionation of deuterated analog **47** demonstrated that biosynthesis of closthioamide did not occur by production of the full oxoamide compound, followed by thioamidation. These data agree with the proposed mechanism of the identified CTA biosynthetic gene cluster, in which CtaC acts on a phosphopantetheinyl-linked tripeptide intermediate on a peptidyl carrier protein (PCP). The union of two tripeptides with a diaminopropane linker occurs concurrently with another thioamidation step to form the final closthioamide (**2a**) product

(Fig. 8.11) [17]. The fluorinated analog was generated by the coupling of a *p*-fluorobenzamide/β-alanine unit (**42**) to a central bis-amide structure (**41**), consisting of two β-alanine units connected by a diaminopropane linker. Treatment of **45** with Lawesson's reagent (**6**) in pyridine was used to generate the final fluorinated closthioamide analog (**46**). The closamide core was made by coupling Boc-protected β-alanine (**43**) to the central bis-amide structure (**41**). Following deprotection, amide coupling was used to introduce a benzyl-protected [D_4]-*p*-hydroxybenzoyl (**44**). The final deuterium-labeled closamide (**47**) was achieved following ultrasound-mediated hydrogenolysis. It was determined that some of the previously observed analogs were indeed degradation fragments because fluorine-labeled versions of these analogs were detected after addition of **46**. Since no thionation of **47** occurred, Behnken et al. concluded that thionation occurred during biosynthesis [17]. Considering these results, it was hypothesized that closthioamide is synthesized by the joining of hydroxybenzoate with one, two, or three β-alanine units, while thionation occurs alongside elongation. Two of these units are then linked together by diaminopropane to form the closthioamide product [17]. This hypothesis was further supported when knockout studies revealed the NRPS-type biosynthetic genes responsible for closthioamide production (Fig. 8.11, inset) [18].

Synthetic closthioamide derivatives with single thio-to-oxoamide substitutions did not display the same bioactivity as closthioamide, confirming that the thioamides are vital for antibiotic activity [16]. Although closthioamide has been shown to chelate copper in an elegant fashion, this is not believed to be relevant to its bioactivity [118]. In order to determine which chemical features are crucial for the antibacterial and antiproliferative activity of closthioamide, various analogs were synthesized and assayed for bioactivity [119]. It was found that (1) the six-membered aromatic ring was not essential, but did contribute to antibiotic activity; (2) any analog with a small electron-withdrawing *p*-substituent on the PHBA portion displayed bioactivity; (3) the length of the diamine linker is vital; and (4) the thio-PHBA and two β-thioalanine units are sufficient for bioactivity, and therefore the symmetric structure of closthioamide is not crucial. Later, they used a combination of synthetic and genetic studies to identify the target of closthioamide. In Chiriac et al., they reported that closthioamide impaired DNA replication and inhibited DNA gyrase activity, in particular the ATPase function of gyrase and topoisomerase IV [120].

8.3.3 Incorporation of Thioamides to Optimize Bioactivity

In addition to studying their roles in natural peptides, thioamides have been ectopically introduced into peptide natural products as part of structure–activity relationship studies. As noted above, the first example of this type of study was performed by Vincent du Vigneuad in his 1973 analysis of the hormone, oxytocin (**48**; Fig. 8.12). At that time, it was understood that the C-terminal amide of the terminal glycine residue was important for oxytocin bioactivity. The decreased bioactivity of [1-deamino,9-thioglycine]oxytocin (**49**) implicated the importance of hydrogen bond accepting ability over hydrogen bond donation at this position [59].

Similar research has been completed with peptide antibiotics. Lantibiotics are a class of RiPPs classified by cyclic motifs with thioether linkages, but are not known to include natural thioamides. Nisin is a well-studied lantibioitic which is active against MRSA and *Listeria monocytogene*. Nisin binds to the pyrophosphate on lipid II of bacterial cell walls through a series of hydrogen bonds, including one with the N-H of dehydroalanine (Dha) on the A ring fragment (**50**; Fig. 8.12). To increase the hydrogen bonding propensity and further study this binding interaction, the nisin A ring was synthesized with a thioamide in this location (**51**) [121]. The method for solid-phase peptide synthesis of this fragment has been determined, but NMR studies of the structural impact of thioamide incorporation are yet to be published.

Thioamide scanning was performed on the helical lipopeptaibiotic [Leu^{11}-OMe] trichogin GA IV at locations terminal and internal to the peptide helix, with the goal of studying the structural implications of thioamide incorporation into various peptide antibiotics that target membranes (**52**; Fig. 8.12) [122]. All thioamide-containing peptides formed right-handed, mixed 3_{10}/α-helixes. The membrane permeability of the thiopeptides was 30–50% lower than that of the corresponding oxopeptides. The antibacterial activities were similarly limited, and the analogs were active against

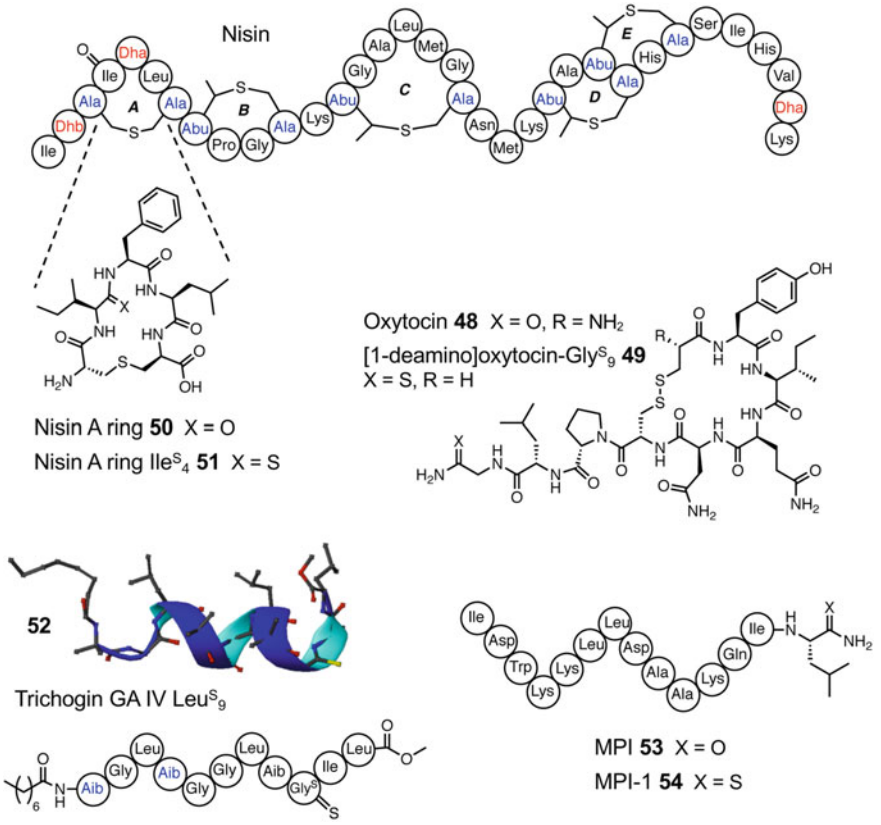


Fig. 8.12 Thioamides enhancing medicinal peptides. The following are peptide antibiotics of which thioamide-containing derivatives have been synthesized to attempt and increase bioactivity. Nisin: The A ring fragment of nisin (**50**), a lantibiotic food preservative, is essential for bioactivity. At physiological pH, the Dha_5 residue is prone to hydrolysis. To better study the effect of Dha_5 on the structure of the A ring, various analogs were synthesized, including one with a thioamide (**51**). Oxytocin: Vigneaud and coworkers synthesized oxytocin (**48**) with a thioamide at the C-terminal glycine (**49**), which is important for bioactivity. The derivative was less bioactive, but this demonstrated the importance of the Gly amide as a hydrogen bond acceptor. Trichogin GA IV: For helical lipopeptaibiotic [Leu^{11}-OMe] trichogin GA IV, thioamide-containing analogs such as trichogin GA IV Leu^{S}_{9} (**52**) exhibited decreased membrane permeability and antibacterial activity [122]. MPI: A peptide antibiotic active against cancerous cell lines, polybia-MPI (**53**), was synthesized with a C-terminal thioamide (**54**) to decrease enzymatic hydrolysis [124]. The derivative was more hydrophobic, which increased its cell lysis potency, but decreased its toxic side effects *in vivo*. Trichogin GA IV structure reproduced with permission from Zotti et al. [122] licensed under CC BY 2.0—published by Beilstein-Institut

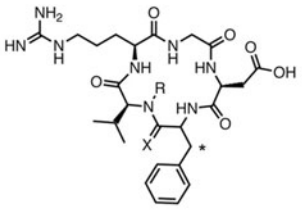
fewer bacterial species. Therefore, the introduction of the thioamide did not perturb the helical structure, but it slightly reduced bioactivity.

Another peptide with potential therapeutic value is polybia-MPI (MPI; **53**; Fig. 8.12), a short cationic α-helical amphipathic peptide isolated from the venom of social wasp *Polybia paulista*. This peptide exhibited promising in vitro activity against cancer cell lines [123]. In order to study its activity in vivo, the C-terminal amide was replaced with a thioamide (MPI-1, **54**) to prevent enzymatic hydrolysis [124]. This resulted in a minimal change to the helical structure, but greatly increased the hydrophobicity. Treatment of cancerous cells with MPI and the more hydrophobic MPI-1 both resulted in the swelling and bursting of the cells. This was further evidenced that these peptides associate within the lipid bilayer and destabilize the membrane. The in vitro anticancer and hemolytic activity of MPI-1 exceeded that of MPI, whereas in vivo studies revealed that MPI-1 was less toxic than MPI. The increased hydrophobicity was hypothesized to result in MPI-1 binding to serum proteins, such as albumin, thereby reducing toxicity. Not only did the introduction of a thioamide develop a new anticancer therapeutic, but it also demonstrated that backbone modification can reduce the general cellular toxicity of antimicrobial peptides.

Recently, Chatterjee and coworkers demonstrated that a thioamide-modified superactive antagonist of pro-angiogenic αvβ3, αvβ5, and α5β1 integrins—which are responsible for cancer cell proliferation and survival—showed better efficacy in inhibiting the pro-angiogenic integrins than the drug candidate cilengitide, and suggested the promise of thioamides in markedly improving the affinity, efficacy, and pharmacology of peptide macrocycles (Fig. 8.13) [81, 125]. Verma et al. showed with several NMR experiments that the introduction of a thioamide into a peptide macrocycle restricted conformational flexibility. They used this effect to stabilize active conformations of derivatives of cilengitide (**55**), an *N*-methylated cyclic peptide that failed in Phase III clinical trials against glioblastoma [126]. Intriguingly, thioamide derivatives **57** and **59**, which differ in stereochemistry at residue 4, both were potent against MDA-MB-231 cancer cells. However, the L-Phe$^{S}_{4}$ containing macrocycle **59** was also active against U-87 MG cells, while the D-Phe$^{S}_{4}$ macrocycle **57** was not, in spite of greater similarity to cilengitide. The corresponding oxopeptides **56** and **58** have no significant activity against either cell line. Computational docking of the solution NMR structures of **57** and **59** aligned well to a bound cilengitide molecule in an integrin receptor co-crystal structure [127]. These docking studies allowed them to identify the basis for increased affinity in the optimal thioamide RGD macrocycle as arising from stabilization of this ring conformation. The potential for broader application of this strategy is very exciting as macrocyclic peptides are the subject of much recent attention in the pharmaceutical industry [128].

8.3.4 Thioamides in Peptidomimetic Systems

There has been a limited exploration of potential applications of thioamides in structures related to peptides and proteins that are made using similar synthetic methods,



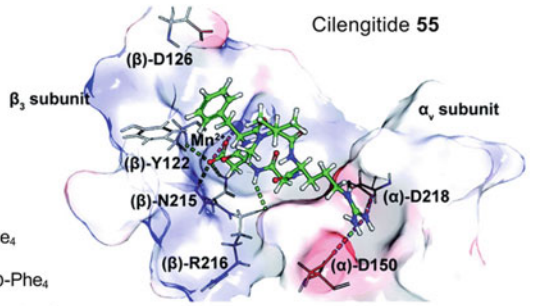
Cilengitide **55** X = O, R = Me, *D-Phe$_4$

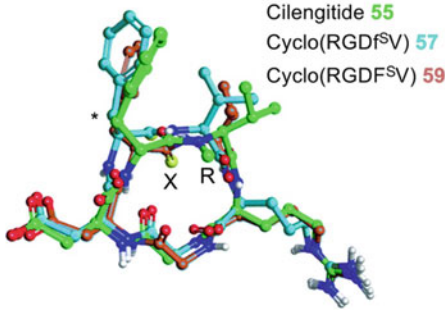
Cyclo(RGDfV) **56** X = O, , R = H, *D-Phe$_4$

Cyclo(RGDf^{S}V) **57** X = S, , R = H, *D-Phe$^S_4$

Cyclo(RGDFV) **58** X = O, , R = H

Cyclo(RGDF^{S}V) **59** X = S, , R = H





	MDA-MB-231	U-87 MG
Cilengitide (**55**)	1.2 ± 1	3 ± 2
56	>10^5	>10^5
57	4 ± 2	326 ± 97
58	>10^5	>10^5
59	1 ± 0.1	1.6 ± 0.6

Fig. 8.13 Thioamide analogs of cilengitide. Top left: Chemical structures of cilengitide (**55**), macrocyclic thioamide analogs **57** and **59**, and the respective oxoamide analogs **56** and **58**. Top right: Structure of cilengitide bound to αvβ3 integrin from X-ray crystal structure (PDBID: 1L5G). Bottom right: Overlay of integrin bound cilengitide (green) structure docked with NMR structures of **57** and **59**. Bottom left: Cell viability data (IC_{50} in nM) demonstrating thioamide-dependent cytotoxicity of **57** against MDA-MB-231 and cytotoxicity of **59** against both MDA-MB-231 and U-87 cancer cells [81]. Figures reproduced with permission from Verma et al. [81] licensed under CC BY 3.0—published by the Royal Society of Chemistry

such as β-peptides and peptoids. Seebach and coworkers studied the effect of incorporating one, two, or three thioamides into the β-peptide H-β^3-HVal-β^3-HAla-β^3-HLeu-β^3-HVal-β^3-HAla-β^3-HLeu-OH (β^3-HXxx refers to the homoligated β^3-amino acid of the corresponding α-amino acid Xxx) which has a known left-handed, (*M*)-3_{14}-helix (Fig. 8.14) [3]. Thioamides were incorporated into the β-peptides using thioamide dipeptide building blocks prepared using Lawesson's reagent, and there were no reported complications that were new to β-thiopeptides. Incorporation of N-terminal β-thioamides did not change the overall 3_{14}-helix of the peptide. Sifferlen et al. were able to show that β-thiopeptides can switch conformations, like their α-thioamide counterparts, upon photoirradiation. Olsen and coworkers showed that thioamide incorporation into peptoids (Fig. 8.14) led to thioamide–aromatic interactions that stabilized some of the β-thiopeptoids in the *cis* conformation, likely due to C^2_{sp}–H···S_{amide} "hydrogen bonds [129]." This was surprising to them as they anticipated seeing $n \rightarrow \pi^*$ interactions of the backbone carbonyls based on previous work by Raines with thioamides in polyproline II helices and model systems [130,

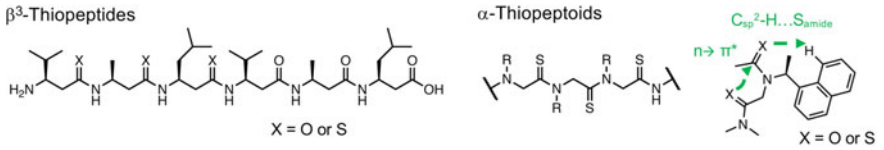


Fig. 8.14 Thioamide peptidomimetics. β^3-Thiopeptides: Seebach and coworkers synthesized and characterized thioamide analogs of β^3-peptides, showing that properties (including photoswitching) observed in thioamide α-amino acid peptides were also observed in β^3-peptide. α-Thiopeptoids: Olsen and Gorske have shown thioamides to be compatible with α-peptoids as well as β-peptoids. Crystal structure analysis of α-dipeptoids was used to investigate the role of $n \rightarrow \pi^*$ carbonyl and aromatic interactions by Olsen

131]. However, crystal structure analysis revealed that there were no substantial differences with oxoamides in the backbone interactions, but rather in the aromatic interactions. Inspired by this work in β-peptoids, Gorske and coworkers examined the effect of thioamide incorporation on α-peptoids [132]. They discovered that this $n \rightarrow \pi^*_{Ar}$ interaction can indeed stabilize the *cis* conformation in α-peptoids as well and is enhanced when an electron-poor aromatic sidechain is present. In all of these works, the authors utilized Lawesson's reagent to selectively thionate the amide bond over the ester bonds present. These studies highlight the potential value of thioamide effects outside of strictly α-amino acid peptides.

8.3.5 Thioamides as Synthetic Intermediates

Thioamides have also been used as synthetic intermediates in the generation of other amide analogs for the study of natural products. This is exemplified in Boger's study of vancomycin (**60**; Fig. 8.15), a glycopeptide antibiotic for bacterial infections [133–136]. Originally discovered in 1954 by Eli Lilly and Co., vancomycin is often considered the "antibiotic of last resort," used in hospitals to treat MRSA infections. The emergence of vancomycin-resistant strains has prompted interest in the rational design of vancomycin derivatives. Vancomycin binds to the *N*-acyl-D-Ala-D-Ala (**64**) terminus of peptidoglycan cell wall precursors (D-Ala is D-alanine). Resistant strains utilize *N*-acyl-D-Ala-D-Lac instead (**65**, where Lac is lactate). It is believed that the repulsive lone pair interaction between the carbonyl (Y=O in **60**) of vancomycin and the ester oxygen of *N*-acyl-D-Ala-D-Lac disrupts the binding affinity. The challenge for the rational design came with synthesizing a derivative that could bind both the D-Ala and D-Lac containing substrates.

The [Ψ[CH_2NH]Tpg^4]vancomycin aglycon derivative (**63**), in which the carbonyl was synthesized as a methylene group (Y=H_2), had a 40-fold increased binding affinity for D-Ala-D-Lac, along with a 35-fold decreased affinity for D-Ala-D-Ala [134]. In order to increase affinity for D-Ala-D-Ala, an amidine derivative (**62**, Y=NH) was synthesized in order to remove the repulsive lone pair interaction, as well as

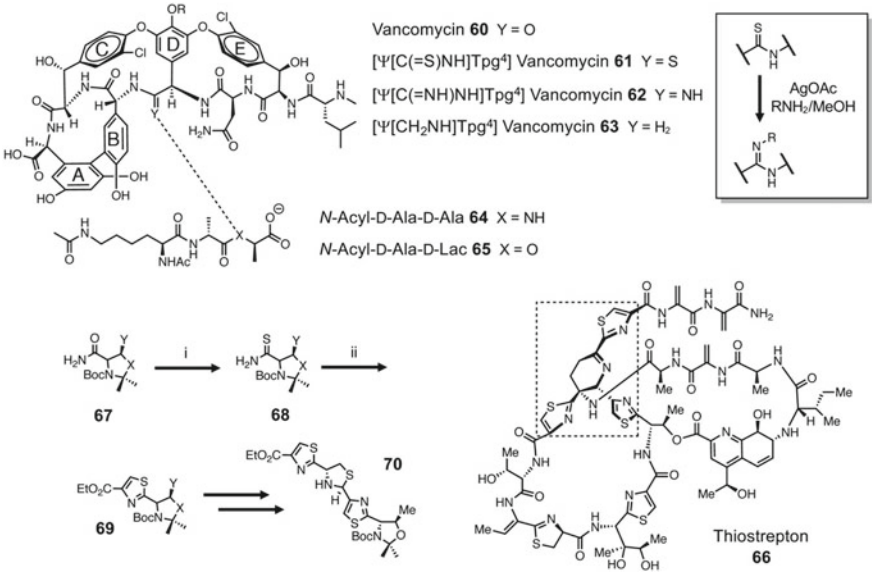


Fig. 8.15 Thioamides as synthetic intermediates. Top: Vancomycin (**60**, Y=O) binds to the *N*-acyl-D-Ala-D-Ala (**64**) peptidoglycan cell wall precursor, thereby inhibiting bacterial growth. Resistant strains utilize *N*-acyl-D-Ala-D-Lac (**65**), to which vancomycin cannot bind. Synthesized derivatives such as [Ψ[CH_2NH]Tpg^4]vancomycin aglycon (**63**, Y=H_2) exhibited increased binding to *N*-acyl-D-Ala-D-Lac **65**, but decreased binding to *N*-acyl-D-Ala-D-Ala **64**. An amidine derivative (**61**, Y=NH) bound well to both **64** and **65** due to its ability to function as both hydrogen bond donor and hydrogen bond acceptor. In order to synthesize this amidine derivative, Okano et al. used a thioamide-containing (**62**, Y=S) intermediate which could be converted to other carbonyl derivatives in the final stages of the synthesis through highly selective, silver-catalyzed reactions [133]. The thioamide derivative exhibited decreased binding to both substrates. Bottom: In the total synthesis of thiostrepton (**66**), Nicolaou and coworkers utilized thioamide precursors (**68**) to synthesize the thiazole rings (**69**) of the central dehydropiperidine core (**70**) [138]. The thioamide precursors were generated by treatment of the L-cysteine (Y=H, X=S)/L-threonine (Y=Me, X=O) derivatives (**67**) using Lawesson's reagent (**6**) as follows: (i) **6**, Na_2CO_3, toluene, reflux; (ii) $BrCH_2COCO_2Et$, $KHCO_3$/$NaHCO_3$, trifluoroacetic anhydride

potentially introduce a hydrogen bond donor to the Lac ester oxygen [136]. This derivative had 600-fold increased binding to D-Ala-D-Lac in comparison with vancomycin aglycon and displayed only twofold decreased binding to D-Ala-D-Ala. The derivative was 1000-fold more active than vancomycin against resistant bacteria in comparison with vancomycin aglycon and vancomycin [137].

In order to synthesize **62**, the authors developed a total synthesis for a thioamide-containing vancomycin derivative (**61**, Y=S) that could then be converted to the amidine in a single step with AgOAc and ammonia in methanol [136]. Lawesson's reagent (**6**) was used to generate the thioamide in an intermediate containing the B, C, and D rings in which all of the alcohols were protected as either methyl or *t*-butyldimethylsilyl (TBS) ethers. Attempts to thionate B/C/D ring intermediates

also containing the A ring or the entire A/B ring macrocycle resulted in much lower yields. The thioamide-containing derivative was also tested for binding affinity and bioactivity, but did not display any potent antibacterial abilities. It was hypothesized that the larger van der Waals radius and the longer C=S bond length prevented binding of the thioamide derivative to the D-Ala-D-Ala substrate. Further research in the Boger group has utilized the thioamide as an intermediate to prepare various vancomycin analogs with Ag(I)-prompted reactions [133, 135, 137].

Thiopeptides are peptide antibiotics that generally inhibit protein biosynthesis in bacteria. The total synthesis of a thiopeptide, thiostrepton (**66**), was completed by Nicolau and coworkers [138]. Taking inspiration from its biosynthesis, Nicolau et al. decided to synthesize the dehydropiperidine core using a Diels–Alder dimerization. Generation of the precursor thiazolidine was achieved by utilizing thioamide-containing precursors (**68**) to generate the surrounding thiazole rings (Fig. 8.15). On L-cysteine and L-threonine derivative **67**, Lawesson's reagent (**6**) was used to establish the thioamide, following which, treatment with ethyl bromopyruvate in basic conditions with $KHCO_3$, followed by trifluoroacetic acid-assisted dehydration with pyridine, yielded the desired thiazole (**69**). These two fragments were condensed in EtOH with $KHCO_3$ to produce the desired thiazolidine ring (**70**, 1:1 diastereomers). The thiazolidine fragment was converted to the azadiene with Ag_2CO_3 and DBU in pyridine at −12 °C, following which the Diels–Alder dimerization occurred to produce the desired dehydropiperidine core. Addition of benzylamine to the reaction mixture reduced production of an aza-Mannich rearrangement by-product. This once again demonstrated the value of thioamides for a variety of carbonyl transformations in the presence of many other functional groups.

8.3.6 Applications of Thioamides as Spectroscopic Probes

Due to the changes in physical properties upon sulfur substitution, thioamides can be used in a number of applications as spectroscopic probes. Specifically, we will discuss the usage of thioamides as circular dichroism (CD) and fluorescence probes, as well as their applications as photoswitches. More detailed discussion of these applications is available in Walters et al. [139] as well as Petersson et al. [13].

Thioamides can serve as site-specific CD probes due to the redshift in their $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions relative to oxoamides. The thioamide $n \rightarrow \pi^*$ signature is shifted by 120–140 nm, placing it at about 350 nm [140]. This makes thioamides usable as CD probes where their absorbance can be distinguished from normal amide resonances at 200–220 nm and even from Trp or Tyr bands at 270–280 nm (Fig. 8.16). In studies by Hollosi and coworkers, the utility of thioamides as CD probes was extensively characterized in different conformational contexts. It was observed that thioamides are compatible with γ-turn conformations, as well as type II β-turn conformations. Overall, they determined that the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ signatures that are seen reflect the local conformation of the amino acid residue that precedes the thioamide [140]. In order to determine the compatibility of thioamides

with other secondary structures, Miwa and coworkers incorporated a thioamide into the turn of a β-hairpin peptide that had been designed by Stanger and Gellman [83]. Utilizing CD and NMR, it was observed that the peptide had characteristics of a β-sheet, specifically a strongly negative $\pi \rightarrow \pi^*$ thioamide band, indicating that the thioamide was participating in a β-hairpin structure. More recently, thioamides were used by Raines and coworkers to examine the backbone of collagen. In this work, thioamides were incorporated into either the Yaa-Gly or the Gly-Xaa motif of the collagen triple helix repeat (Xaa-Yaa-Gly) in order to examine the $n \rightarrow \pi^*$ interactions of the collagen backbone [131]. Environmental effects on thioamide absorption were observed, as both the Gly^S-Xaa and the Yaa^S-Gly peptides had minima at 265 nm, while the Gly^S-Xaa peptide also had a maximum at 287 nm. This work was the first example of a backbone modification that did not negatively impact the thermal stability of the collagen triple helix and showed the utility of thioamides as probes for polyproline type II structures. Lastly, our own group has used CD to examine the stability of proteins containing thioamides in the context of α-helices, β-sheets, and polyproline II helices. Through CD and thermal denaturation, Walters et al. were able to show that thioamides can be tolerated in some portions of α-helices and polyproline II helices, along with being moderately perturbing to β-sheet structures [85].

Thioamides also have utility as photoswitches due to their red-shifted $\pi \rightarrow \pi^*$ absorption band, which allows them to be selectively excited to cause *cis/trans* isomerization through rotation about the C–N bond (Fig. 8.16). Oxoamides can also undergo photoisomerization, but this requires irradiation at 190 nm, which degrades peptides [91]. In studies by Frank and coworkers, the photo-controlled *cis*/*trans* isomerization of thioamide-containing endomorphins was characterized, with eventual application in studying isomer-specific interactions with the opioid μ-receptor [141]. In this report, it was shown that the thiopeptides remained intact after multiple rounds of excitation, illustrating that this application can be performed without degrading the peptide sample. Similarly, Zhao and coworkers showed that secondary thiopeptide bonds can undergo *cis*/*trans* isomerization in both directions (*trans* to *cis* and vice versa) with a re-equilibration rate that is much slower than the thermal re-equilibration rate of oxopeptides [142]. In one of the first reported applications of thiopeptide *cis*/*trans* isomerization, Fischer and Kiefhaber modified RNase S with a thioamide in order to monitor the change in enzyme activity upon isomerization [91]. A thionated Ala residue was incorporated into the S peptide of RNase S, far from the active site, which allowed the enzyme to retain activity in the ground state. Photoisomerization of the thioamide led to a drastic decrease in the enzyme efficiency, which increased again upon thermal re-equilibration of the system. In another interesting application of thioamide photoswitches, Huang and coworkers used thioamides to examine the cockroach hindgut myotropic activity of both the *cis* and the *trans* conformations of the insect kinin peptide core. Previously, insect kinin analogs with a significant 1-4 *cis*-Pro type VI β-turn population had been shown to be very active, with the *cis* population believed to be the contributing factor. Due to the long re-equilibration half-life of the thioamide derivative of the insect kinin active core, Huang and coworkers were able to turn on myotropic activity upon irradiation to

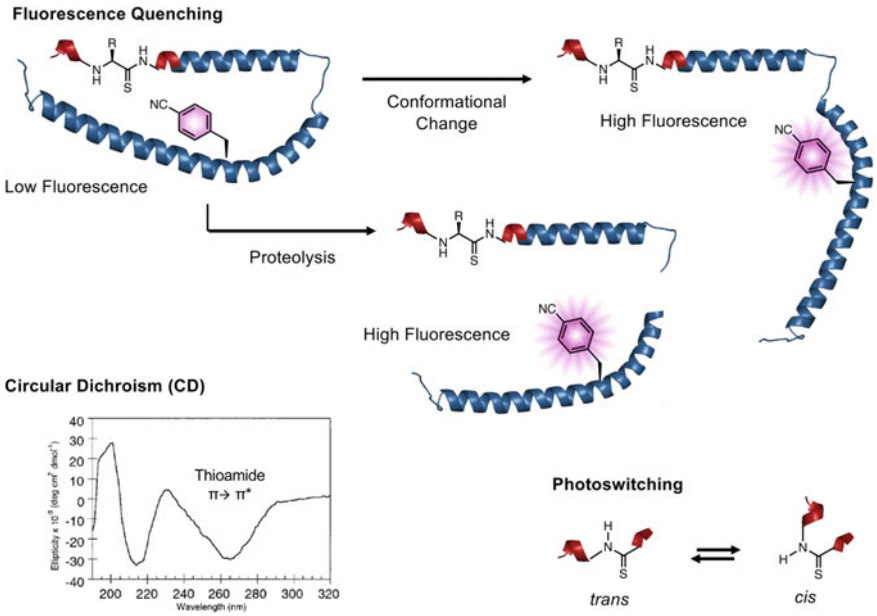


Fig. 8.16 Thioamides as probes of biological structure/function. Fluorescence quenching: The Petersson laboratory has used thioamides as fluorescent probes, both to study conformational changes in proteins and to monitor proteolysis of a variety of substrates. In a compact conformation, the thioamide quenches the fluorescence of the fluorescent amino acid. Upon refolding or proteolysis, the thioamide and fluorophore move apart, allowing for a turn-on in fluorescence of the fluorophore. Circular dichroism (CD): Thioamides have successfully been employed as circular dichroism probes to examine how they affect the global structure of small, structured systems. In this example from Miwa and coworkers, the thioamide serves as a probe for the environment of a turn in a β-sheet structure [83]. Photoswitching: Thioamides can be irradiated to convert to the *cis* conformation and thus used as a photoswitch to trigger larger conformational changes in proteins. CD figure reprinted (adapted) with permission from Miwa et al. [83], Copyright (2001) American Chemical Society

demonstrate that the *cis* conformer is much more active than the *trans* conformation of the peptide [143]. This work is an elegant example where thioamides were used to interrogate how specific conformations of peptides affect their activity.

Thioamides can be utilized as fluorescent probes due to their red-shifted $\pi \rightarrow \pi^*$ absorption, which gives them spectral overlap with a number of fluorophores. The first characterization of a thioamide in a FRET pair was reported by Wiczk and coworkers, which described a thioamide–Trp pair in a 4mer peptide [144]. This FRET pair was determined to have a Förster radius (R_0, a characteristic distance of half-maximal energy transfer) of 16.9 Å in propylene glycol (the R_0 for Trp in water is much shorter) [76]. Our laboratory has had significant success in using thioamides in different FRET and PeT applications. In 2010, we examined the utility of a Cnf–thioamide FRET pair. It was determined that this FRET pair has a theoretical R_0 of ~15.6 Å, which correlated very well with the observed changes in

fluorescence using rigid polyproline spacers to vary the distance between the two fluorophores [73]. The temperature-dependent unfolding of Villin HP35 with a Leu^S and Cnf FRET pair was also observed, with fluorescence changes correlating well with measurements of unfolding by CD (Fig. 8.16). Our group has shown that the thioamide/Tyr and thioamide/Cnf FRET pairs can be used to characterize the binding of CaM to a known peptide binding partner, pOCNC [76, 77]. Moreover, we have been able to use this pair to characterize the aggregation kinetics of αS, an intrinsically disordered protein that has been associated with Parkinson's disease [71]. This fluorescence quenching is complementary to the thioflavin T assay, which is typically used to characterize the kinetics of aggregation, and can give insight into oligomerization pathways that are not detected through ThT fluorescence. Similarly, our group has used the thioamide/Cnf FRET pair to characterize the compaction of αS in the presence of trimethylamine oxide (TMAO), an osmolyte that is typically used to compact proteins. Through these experiments, we were able to see the N terminus of αS compact with higher concentrations of TMAO, which is consistent with what others have seen with other methods [77, 78]. Our group has also characterized the interaction of thioamides with various fluorophores through a photoinduced electron transfer (PeT) mechanism. We have shown that thioamides can quench 7-azatryptophan, 7-methoxycoumarin-4-yl-alanine (Mcm), 5-carboxyfluorescein, and acridon-2-yl-alanine through a PeT mechanism [72, 75]. Specifically, our group has used this quenching to study the unfolding of Villin HP35, the cleavage of a variety of protease substrates, and the compaction of αS in the presence of TMAO [72, 75]. Recently, our group has become interested in the implications of utilizing two thioamides in a FRET or PeT pair. Huang, et al. showed that increasing the number of thioamides increases the fluorescence quenching effect that they have in a FRET or PeT pair [145]. In 2018, our group has also demonstrated that the increased fluorescence quenching of dithioamide incorporation can be utilized to study CaM binding to a W-pOCNC peptide [103]. Notably, multiple thionations can also improve the thermal stability of these native, functional protein folds while increasing the quenching efficiency in order to better characterize protein interactions.

The use of thioamides as fluorescence quenching probes has been exploited by our group to make sensors for over 15 different proteases (including Unpublished Results) [74, 84, 145]. The basic design relies on placing a selectively excitable fluorophore on one side of the scissile bond and a thioamide quencher on the other side (Fig. 8.16). Thus, proteolysis will separate the thioamide from the fluorophore, leading to a turn-on of fluorescence that can be used to monitor proteolytic activity. We showed that these substrates were cleaved at rates identical to the corresponding oxoamide peptide provided that the thioamide was placed 3 or more amino acids from the scissile bond. Sensors of this design could even be used to monitor cleavage at one of two adjacent proteolytic sites based on the placement of the thioamide. These studies led us to systematically investigate the positional effects of thioamides on proteolysis rates. Such studies have informed the design of sensors by identifying non-perturbing sites for thioamide labeling. The identification of perturbing sites in these studies has the potential for even greater impact, where highly perturbing sites

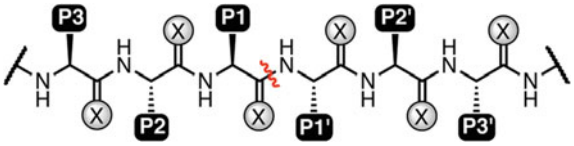


Fig. 8.17 Thioamide proteolysis scheme. Proteolysis occurs at the position of the red slash. Amino acids surrounding the scissile bond are denoted P3, P2, P1, P1′, P2′, or P3′ as shown. Thioamide substitutions at these sites can be used to probe effects on proteolysis. X=O or S

can be used as the basis for the design of protease inhibitors or for stabilizing peptides for in vivo applications.

8.3.7 Thioamide Substitution Effects on Proteolysis

Proteases play an important role in drug development since they are involved in many biological signaling pathways, and underlie a variety of diseases [146]. Inhibitors of well-established protease targets such as angiotensin-converting enzyme and HIV protease have shown significant therapeutic success [147]. Thioamides introduced at the scissile bond of protease substrates or inhibitors have been applied to study catalytic mechanisms of proteolysis with the potential to develop improved protease inhibitors or to stabilize injectable peptides. Throughout our discussion, we will refer to the thioamide position in terms of the common protease nomenclature where P1 is the amino acid at the position of cleavage, or scissile bond, and the other neighboring amino acids in the substrate are designated P3, P2, P1, P1′, P2′, P3′, from the N terminus to the C terminus (Fig. 8.17). Typically, only these 3-4 flanking amino acids are relevant to protease recognition of substrates.

8.3.8 Thioamide Probes of Protease Mechanism

Numerous proteases have been investigated through thioamide modifications, including representatives of the major protease classes—Ser, Cys, metallo-, and Asp proteases—which are categorized based on their catalytic mechanism. The zinc metalloprotease carboxypeptidase A (CPA) is one of the most well-studied proteases to date, in terms of thioamide effects [148–151]. A peptide substrate containing a thioamide linkage at the scissile bond (P1) was found to be less efficiently cleaved by CPA [151]. In addition, thioamide incorporation near the scissile bond of substrates yielded peptides with a similar K_M, but a greater than tenfold decrease in k_{cat} compared to the corresponding oxoamide substrates. These results suggested a mechanism involving a rate-determining step requiring C–N bond rotation, wherein the higher rotational barrier of the thioamide would slow reaction [148, 149]. In a subsequent study,

Table 8.2 Protease inhibition by thioamides at the scissile bond (P1)

Thioamide substrate	Enzyme	Thio/Oxo k_{cat}/K_M^a
Bz-Gly-Gly^S-Phe	Zn(II) CPA	6.00×10^{-4}
Z-Gly^S-Phe		0.18
Z-Gly-Ala^S-Phe		3.67×10^{-3}
Z-Phe^S-Phe		1.82×10^{-3}
Z-Gly-Ala^S-Phe	Cd(II) CPA	0.03
Z-Phe^S-Phe		0.05
Z-Gly-Ala^S-Phe	Mn(II) CPA	9.44×10^{-4}
Z-Phe^S-Phe		3.44×10^{-4}
Z-Lys^S-AIE	Papain	0.18
Z-Phe-Arg^S-AIE		0.21

[a] k_{cat}/K_M value of thioamide substrate versus corresponding oxoamide substrate
Bz: benzoyl; Z: *N*-carbobenzoxy; Gly^S: thioglycine; Ala^S: thioalanine; Phe^S: thiophenylalanine; Lys^S: thiolysine; Arg^S: thioarginine; CPA: carboxypeptidase A; AIE: isophthalic acid dimethyl ester
CPA data from Bond et al. [150]; Papain data from Cho [152]

other metal ions, including Cd(II), Mn(II), Co(II), and Ni(II), were substituted for the native zinc to study their effects on the cleavage of oxoamide and P1 thioamide substrates [150]. It was found that thioamidation led to a large increase in the rate of Cd(II) CPA cleavage relative to the oxoamide substrate, but a large decrease in relative activity was observed with Mn(II) CPA. This was explained in that sulfur and oxygen functional groups are classified as soft and hard ligands, respectively, and similarly Cd(II) and Mn(II) are classified as soft and hard metals. Empirically, hard metals tend to form stable complexes with hard ligands, and soft metals act similarly with soft ligands. Thus, the favorable soft/soft thioamide/Cd(II) combination had a 100-fold increase in k_{cat}/K_M in comparison with Mn(II) CPA (Table 8.2). These results reflect the mechanism for peptide bond hydrolysis in which the metal atom interacts with the substrate C=X group at the scissile bond during catalysis.

Cho studied the ability of papain and trypsin to catalyze the hydrolysis of thioamide and corresponding oxoamide substrates using dipeptides [152]. They found that both the oxo- and thioamide substrates were cleaved by papain, while a thioamide at P1 significantly suppressed the proteolysis rates of trypsin as compared to the oxopeptides. According to Roberts et al., the classical "oxyanion hole" might be destabilized in the case of the tetrahedral intermediate formed during thioamide cleavage due to the longer C=S bond and poorer hydrogen bond accepting ability of the thioamide [153]. Thus, Cho. et al. concluded that the stabilization of the oxyanion plays an important role for trypsin, but not papain, which may reflect broader rules for P1 thioamide peptide cleavage by serine versus cysteine proteases [152]. However, Foje and Hanzlik reported that papain cleavage of Phe-Gly-NH-R dipeptides with a thioamide at P1 depended on the identity of the R group, demonstrating that the active site interactions are more subtle [154].

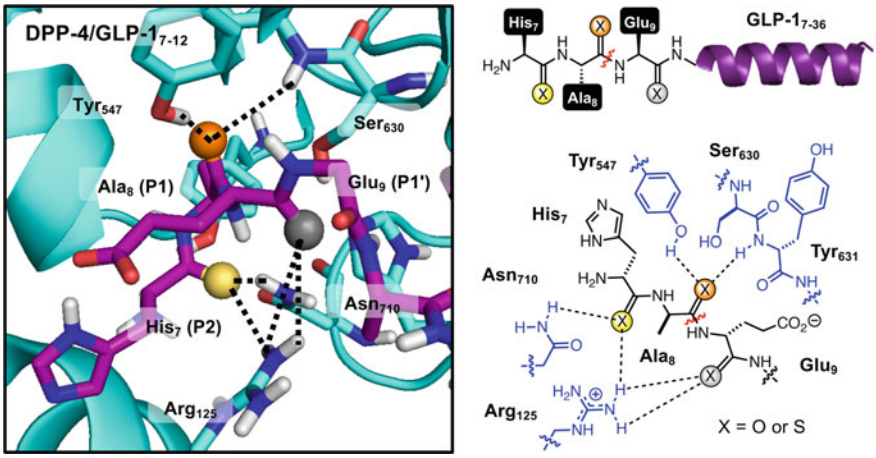


Fig. 8.18 Structural analysis of the impact of thioamide substitution on DPP-4 substrate recognition. Left: An image of the DPP-4 (cyan) active site with a GLP-1 N-terminal fragment (purple) bound, modeled based on the neuropeptide Y bound DPP-4 structure in PDB entry 1R9N [156]. The P2, P1, and P1′ carbonyl oxygens are highlighted as yellow, orange, and gray spheres, respectively. Key interactions with DPP-4 are shown as dashed lines. Right: A schematic representation of the P2, P1, and P1′ binding sites. The DPP-4 cleavage site is shown as a red slash

In another example, Fischer and coworkers investigated dipeptidyl peptidase-4 (DPP-4) using Ala^{S}-Pro-pNA as substrate, where the enzyme hydrolyzes the bond between Pro and *para*-nitroaniline (pNA) [155]. They found a 1,100-fold decrease of k_{cat}/K_m compared to the oxoamide, which they attributed to the decrease of k_{cat}, caused by the increased rotational barrier of the thioamide. Recently, our laboratory built on this observation in studying GLP-1 and GIP [84], natural substrates of DPP-4. A thioamide substitution at either of the two terminal positions increased the peptide half-life in an in vitro proteolysis assay from 2 min to greater than 12 h. Competition experiments with an alternate DPP-4 substrate revealed that thioamide GLP-1 was not a competitive inhibitor, seemingly in conflict with Fischer's finding of a primary k_{cat} effect. This may be due to the fact that the 36 residue GLP-1 peptide cannot be repositioned in the active site to accommodate the thioamide, whereas the Ala^{S}-Pro-pNA can, but in a way that is not optimal for catalysis. Examination of the crystal structure of DPP-4 with a peptide substrate reveals bifurcated hydrogen bonds with the carbonyls of the two N-terminal amino acids (Fig. 8.18) [156]. Thioamidation would disrupt these interactions, preventing productive binding of substrates (although the rotational barrier may still play a role). Our laboratory is currently evaluating thioamide effects on other proteases by scanning thioamide incorporation around the scissile bond, and this systematic approach, coupled with the increased availability of protease crystal structures, should provide additional mechanistic insight.

Table 8.3 Positional effects of thioamides on the inhibition of HIV-1 protease

Thioamide position	IC_{50} (μM)
P3	160
P2	51
P1	18
P1′	>200
P2′	4.5

Data from Yao et al. [163]

8.3.9 Thioamide Inhibitors of Protease Activity

Given the observed resistance to proteolysis for certain thioamide positions, there have been attempts to introduce them into protease substrates or other peptides to create inhibitors of proteases such as papain, a variety of proline-specific peptidases, leucine aminopeptidase (LAP), and HIV-1 protease [157–161]. Most of the thioamide inhibitors were found to be competitive inhibitors with weak binding affinities. Thioamide-modified inhibitors were studied for the first time by Lowe and Yuhavong in 1971 [157]. A papain substrate was modified by introducing a thioglycine at the scissile bond, and weak competitive inhibition was observed, with a K_I 100-fold greater than the K_M of the corresponding oxoamide substrate. Moreover, Stöckel-Maschek characterized amino acid thiazolidides (dipeptides with thiazolidine units) as DP II protease inhibitors and found that a thioamide modification could improve its inhibitory effect [162]. Interestingly, in the thioamide positional scanning from P3 to P2′ of an HIV-1 protease substrate by Chimeleski and coworkers, the thioamide at the P2′ position rather than P1 position produced the most significant inhibition effect (Table 8.3) [163]. Moreover, in the study of the positional effect of thioamide on prolyl oligopeptidase by Schutkowski et al., two series of tetrapeptide-*p*-nitroanilides, Ala-Gly-Pro-Phe-pNa and Ala-Ala-Pro-Phe-pNa, along with all possible thioamide derivatives were examined [164]. They found that a thioamide introduced at the P2 position enhanced k_{cat}/K_M fivefold in the Gly series substrates, while it resulted in a 1.7-fold decrease in the Ala series substrates. Studies such as this highlight the need for a greater mechanistic understanding of thioamide effects to be able to use them rationally in inhibitor design.

8.3.10 Thioamide Modifications to Improve Injectable Peptides

The resistance of thioamide substrates to proteolysis also lends itself to the improvement of peptide therapeutics due to the potential for selective perturbation of proteolysis without altering interactions with a target receptor. There are more than 60 FDA (US Food and Drug Administration) approved peptide drugs on the market

currently. In the USA, over 140 peptide therapeutic candidates are in clinical trials and more than 500 peptide compounds in preclinical development [165, 166]. The global sales of peptide therapeutics increased each year from 2009 to 2011, and the top 25 US-approved products had global sales of US$14.7 billion in 2011. Peptide therapeutics have obvious advantages compared to traditional small molecule drugs or large protein drugs, such as target specificity deriving from the native peptide and lower production complexity than protein drugs [167]. However, there are problems with utilizing native peptides as therapeutic agents, due to their short duration of in vivo activity coming from low stability against proteolysis as well as other factors such as kidney clearance. To overcome stability problems, a variety of strategies have been developed, such as: (1) polyethylene glycol conjugation, which can protect against proteases and solubilize hydrophobic molecules [122, 168–170]; (2) stabilizing secondary structure by "stapling," macrocyclization, or the use of structure inducing modifications [171–174]; (3) unnatural amino acid substitutions or peptide bond mimics, including α,α-disubstitution, *N*-methylation, and D-amino acids [175]; and (4) peptidomimetics such as peptoids [176], urea peptidomimetics [177], and peptide-sulfonamides [178].

Thioamides have significant advantages over these other stabilizing modifications. Since they are a minor modification, they are less likely to disrupt interaction with the target protein or other peptide properties than PEG modification or cyclization. The use of these modifications often involves trial and error testing to find the right place for modification. While the other amino acid modifications discussed are also small, most of them remove one or more of the hydrogen bonding interactions of the peptide bond or introduce significant steric differences around the peptide bond. If the same region of the peptide is necessary for protease recognition and target receptor recognition, use of a D-amino acid may not be a viable option. Thus, thioamides provide a balance of being sufficiently different to suppress proteolysis while not dramatically altering the other peptide properties.

To our knowledge, the idea of using thioamides to stabilize peptides toward proteolysis was first tested by introducing a thioamide at the scissile bond of a CPA substrate, leading to at least 1,000-fold slower turnover compared to its corresponding oxoamide substrate [148, 149, 151]. Substrates of numerous proteases, such as LAP, papain, mammalian membrane dipeptidase, and aminopeptidase P, were stabilized later by introducing a thioamide at the scissile bond [148, 153, 158, 160, 161]. The first tests that included evaluations of biological activity involved the immunostimulant IMREG-1 (Tyr-Gly-Gly) [68]. The half-lives of thionated IMREG derivatives with sequences Tyr^{S-}Gly-Gly and Tyr-Gly^{S-}Gly were determined as 45 min and over 180 min, respectively, compared to the half-life of natural IMREG-1 at only 1 min. Moreover, Tyr^{S-}Gly-Gly was found to display some biological activity as evidenced by mild stimulation of cytotoxic T-lymphocytes and appeared to be well tolerated and devoid of any apparent toxicity. In a study noted above, Zhang et al. introduced a thioamide to the C-terminal residue of MPI (**54**; Fig. 8.12). In addition to changes in membrane binding and activity, they observed a significant enhancement of stability against proteolysis in mouse serum [124]. After 3 h, almost all of the natural MPI was gone, while only 21% of the thio-MPI was degraded. Similarly, the

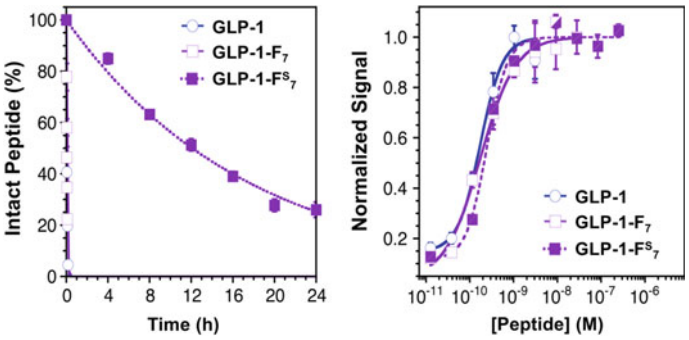


Fig. 8.19 Thioamide substitution stabilizes GLP-1 analogs without disrupting activity. Left: In vitro proteolysis data demonstrating that thioamide GLP-1-F^{S}_{7} is cleaved by DPP-4 more slowly than the corresponding oxopeptide (GLP-1-F_7, a Phe mutant to GLP-1) or native GLP-1. Half-lives: GLP-1, 2 min; GLP-1-F_7, 3 min; GLP-1-F^{S}_{7}, 12 h. Right: Dose–response curves for cAMP stimulation by GLP-1, GLP-1-F_7, and GLP-1-F^{S}_{7} demonstrating that the thioamide does not affect potency. EC_{50}s: GLP-1, 207 pM; GLP-1-F_7, 171 pM; GLP-1-F^{S}_{7}, 244 pM. Bars represent standard error

thioamide RGD macrocycles studied by Chatterjee and coworkers (Fig. 8.13) were all found to be more stable than cilengitide in ex vivo metabolic stability assays in human serum over 72 h [81]. This was independent of the position of the thioamide, suggesting that it was not due to specific hydrogen bonding interactions, but may be related to the overall conformational rigidity of the macrocycles.

Our laboratory is interested in studying positional effects of thioamides to stabilize peptides, as well as to develop protease sensors and protease inhibitors. GLP-1 and GIP, two therapeutically relevant peptides introduced above, were modified by introducing thioamides [84]. In spite of the large perturbation to interactions with proteases noted above, target receptor activation was largely unperturbed, with increases in receptor EC_{50} of threefold or less (Fig. 8.19). Moreover, thioamide-modified GLP-1 was found to improve glycemic control in rats relative to the native GLP-1. We anticipate that thioamides will be highly useful for the stabilization of newly discovered signaling peptides toward key proteolytic events for early in vivo investigations of their signaling activity. In addition, since thioamides increase proteolytic stability without altering physical properties for trafficking or renal clearance, thioamide-stabilized peptides should be excellent for imaging applications, where one wishes for the peptide to be either intact and bound to the target cells (e.g., tumors) or cleared from the bloodstream to eliminate background signal. These types of applications require an understanding of the effects of thioamides on cleavage by various proteases in order to design their placement. Currently, we are using a series of peptide substrates with a thioamide at the P3, P2, P1, P1′, P2′, or P3′ position to study the positional effects on the activity of various proteases. We will use this information to guide the application of thioamides to stabilizing peptides for therapeutic and imaging applications as well as for the development of potential inhibitors.

8.4 Outlook

Recent developments in biological, biophysical, and medicinal chemistry have generated much interest in the study of thioamide-containing natural products and synthetic thioamide modifications of peptides and proteins. Discovery of the biosynthetic gene clusters for thioamide installation has vastly increased the number of putative thioamide-containing RiPPs and may indicate that MCR is not the only thioamidated protein. Thioviridamide and closthioamide have been shown to have promising anticancer and antibiotic activities, respectively. Making thioamidated analogs of natural products or biosynthetic intermediates has enabled the understanding of biosynthetic pathways and the determination of the role of the thioamides in bioactivity. Stabilization of GLP-1, GIP, cilengitide-like macrocycles, and other peptides has provided promise for directed thioamide substitution for in vivo applications. Thioamides can also be used as biophysical probes to study conformational changes in proteins using fluorescence quenching or photoisomerization. Our laboratory has used fluorescence quenching by thioamides in peptides to study the positional effects of the thioamides on cleavage by a wide variety of Ser and Cys proteases. This has informed our design of thioamide modifications of peptides to stabilize them toward proteolysis for in vivo therapeutic and imaging applications. These thioamides can also have useful effects on peptide conformation and binding affinity, leading to alterations in selectivity. We anticipate that the seeds planted by these studies will lead to a dramatic increase in the number of laboratories studying peptidyl thioamides in the coming years.

One of the most significant unanswered questions in the study of thioamide effects in proteins is of the structural basis for thioamide effects on protein stability and protein interactions. To achieve a predictive understanding of thioamide effects will require the ability to easily install thioamides in a variety of protein sequences and to prepare sufficient quantities of proteins for structural and biophysical studies. Although current methods allow one to insert thioamides at specific sites with great generality, the synthesis of proteins through NCL is inherently labor-intensive. Biosynthetic insertion of thioamides by YcaO-type enzymes is unlikely to provide a solution since it will probably not be sufficiently general in sequence to enable the exploration of sequence effects. Thus, one of the outstanding areas of need in the field is for an easily scalable, facile method for genetic thioamide insertion. Hecht and coworkers have published a method for in vitro protein translation using tRNAs acylated with thioamide-containing dipeptides (**73**) which comes close to filling this need, but is limited by the relatively low yields of thioamide-containing protein (**74**) available from in vitro translation and the need to prepare the tRNA acylating precursors (**71**, **72**) as dipeptides (Fig. 8.20) [179]. In spite of some previous reports [180], our own unpublished investigations with chemically thionoacylated tRNAs (**75**) have indicated that S-to-O exchange of the thiocarbonyl (**76**) is a significant concern, providing a challenge to circumventing the use of dipeptide acylated tRNAs for thioamide insertion (Fig. 8.20). If this chemical challenge can be met, the ability to genetically encode thioamides will vastly increase the scope of biological exploration that can be undertaken, from mapping thioamide effects on protein stability

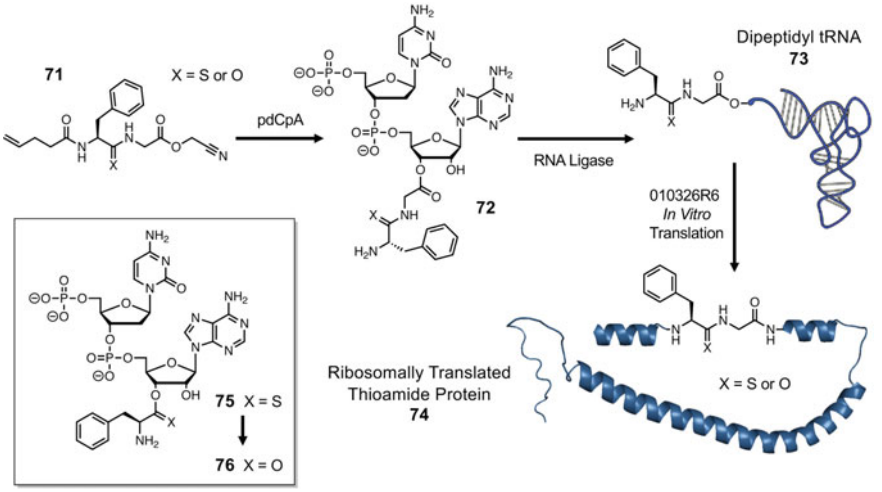


Fig. 8.20 Prospects for ribosomal translation of thioamides. Acylation of the pdCpA dinucleotide with an *N*-protected dipeptidyl cyanomethyl ester (**71**) to give **72**, followed by ligation to a truncated tRNA with RNA ligase and deprotection, gives a dipeptidyl tRNA (**73**). Hecht and coworkers have shown that this can be used in translation with *E. Coli* extracts containing a mutant 23S ribosome (01032R6) to afford proteins with a backbone thioamide (**74**, X=S) [179]. Inset: Unpublished investigations from the Petersson laboratory using thionoacyl pdCpA (**75**) show significant S-to-O exchange in aqueous buffer to generate the acyl pdCpA (**76**)

and binding interactions, to scanning peptide sequences for stabilization effects, and even determining the role of the thioamide in MCR and other natural thioproteins yet to be discovered.

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