Biosynthesis and Chemical Applications of Thioamides

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Abstract

Thioamidation as a posttranslational modification is exceptionally rare, with only a few reported natural products and exactly one known protein example (methyl-coenzyme M reductase from methane-metabolizing archaea). Recently, there has been significant progress in elucidating the biosynthesis and function of several thioamide-containing natural compounds. Separate developments in the chemical installation of thioamides into peptides and proteins have enabled cell biology and biophysical studies that advance the current understanding of natural thioamides. This review highlights the various strategies used by Nature to install thioamides in peptidic scaffolds and the potential functions of this rare but important modification. We also discuss synthetic methods used for the site-selective incorporation of thioamides into polypeptides with a brief discussion of the physicochemical implications. This account will serve as a foundation for further study of thioamides in natural products and their various applications.

Keywords

Thioamide = an analog of the amide bond in which the carbonyl oxygen has been replaced by a sulfur atom

Thionucleoside = a nucleic acid analog in which a carbonyl oxygen has been replaced by a sulfur atom

Closthioamide = a thioamide-containing non-ribosomally synthesized peptide exhibiting antibiotic activity

RiPP = ribosomally synthesized and posttranslationally modified peptide

Thioviridamide = a representative of a class of thioamide-containing RiPPs that also includes thioholgamide A/B and thioalbamide

Methanobactin = a thioamide-containing RiPP that functions in copper acquisition by methanotrophs

Methyl Coenzyme M Reductase (MCR) = the only currently known thioamide-containing natural protein, catalyzes the reversible conversion of methyl-coenzyme M (CoM) and coenzyme B (CoB) to methane and a CoB-CoM heterodisulfide

Thiopeptide = a class of RiPPs containing thiazole, thiazoline, and/or thioamide modifications; also sometimes used to refer to any peptide with a thioamide modification of the peptide backbone

Introduction

Prior to their discovery in natural molecules, thioamides were considered as a synthetic isostere for amides in peptide backbones[1] and have been employed by medicinal chemists for improving thermal/proteolytic stability and the pharmacokinetic properties of amide-containing compounds.[2,3] In some cases, the thioamide-modified peptides exhibited improved bioactivity as well.[4,5,6,7] However, while there are pharmaceutical compounds containing thiocarbonyl groups (e.g. thyroid medications methyl- and propylthiouracil, anti-tuberculosis drug thioacetazone, androgen receptor agonist enzalutamide, and antimetabolite tioguanine), to date, no marketed therapeutics contain a peptidic thioamide.[8] Biophysical chemists have used thioamides for perturbing protein folding or introducing spectroscopic probes.[9] The discovery of thioamides in several natural products and in a single protein provides new perspective and prompts investigations of the role of the thioamide as well as a search for additional natural thioamide-containing molecules.

In this review, we have summarized the current state of knowledge of Nature's biosynthetic strategies for generating thioamide-containing polypeptides. The overall understanding of the biosynthesis pathways and the function of this posttranslational modification (PTM) is still rather limited, but rapidly growing. In addition, we have summarized the synthetic methods developed for site-selective incorporation of thioamides into peptides and proteins. These methods can be used to prepare substrates to investigate biosynthetic mechanisms or analogs to investigate natural product bioactivity, as well as thioamide variants of peptides and proteins which may have improved properties relative to the all-amide congeners. Recent progress in the elucidation of

thioamide biosynthetic pathways and methods for their chemical installation has positioned the field for new breakthroughs.

Thioamide Properties

The potential benefits of thioamide incorporation in both natural and synthetic compounds result from the at once subtle and dramatic changes to amide interactions that can come from this single atom substitution. Thioamides are more reactive with both nucleophiles and electrophiles than amides,[10,11] with a weaker carbonyl bond (130 vs. 170 kcal/mol),[12] and therefore have been used as chemical synthesis intermediates. Thioamides also have greater affinity for certain metals over amides. For instance, the natural product methanobactin (**Figure 2**) exhibits extremely high affinity copper binding,[13] and selective, silver-catalyzed transformations have been used to convert thioamides into other carbonyl derivatives.[14] The differences in amide and thioamide geometry govern many of the non-covalent interactions exhibited by thioamide-containing peptides. The thioamide C=S bond is 1/3 longer than the amide C=O bond (1.71 vs. 1.23 Å)[15] and sulfur has a 1/3 larger van der Waals radius compared to oxygen (1.85 vs 1.40 Å).[16] Peptide conformational changes can result from the elongated C=S bond and the higher rotational barrier for the C-N bond (~5 kcal/mol),[17] which reduces conformational flexibility. Additional altered physiochemical properties include: *(i)* thioamide N-H groups are more acidic (ΔpK_a = -6) than the corresponding amide,[18] *(ii)* thioamide N-H groups are better hydrogen bond donors,[19] and (*iii*) the sulfur lone pairs of thioamides are weaker hydrogen bond acceptors relative to oxygen lone pairs in amides.[20] Therefore, thioamides are suitable to evaluate the contribution of single hydrogen bonds to protein folding/stability. Substitution of an amide with a thioamide also imparts significant spectroscopic and electrochemical changes. The thioamide C=S bond has an UV absorption maximum at 265

(±5) nm and an IR stretch at 1120 (±20) cm^{-1}, compared to 220 (±5) nm and 1660 (±20) cm^{-1}, respectively, for the amide C=O bond.[21] Moreover, the ^{13}C NMR chemical shift of the thioamide carbonyl is found 30 ppm downfield (200 – 210 ppm) of the corresponding amide resonance.[22] The oxidation potential of a model thioamide (1.21 eV) is significantly lower than that of the amide (3.29 eV),[23] which has prompted speculation about a potential role in electron-transfer in biological settings.

The origin of the higher C-N rotational barrier in thioamides has been examined by NMR[17] and *ab initio* calculations.[24] It was found that the amino group of thioformamide is more conformationally rigid than in formamide. Additionally, the change in charge density at sulfur upon rotation of the amino group in thioformamide is greater than that at oxygen in formamide due to a predominant bipolar amide resonance form. The small difference in electronegativity between carbon and sulfur (Pauling electronegativities for C=2.55 and for S=2.58), along with the larger size of sulfur, are the predominant factors that allow charge transfer from nitrogen to sulfur in thioamides. A special feature of the thioamide bond arises from the red-shifted absorption bands and the higher barrier for *cis-trans* isomerization. This property allows access to either the *cis* or *trans* isomer by irradiation with the appropriate UV wavelength. The photoinduced isomerization is efficient (30%) and fast (<600 ps), whereas thermal relaxation is comparatively slow (>10 min).[21] Hence, thioamide-containing peptides are good candidates for fast photo-switches in proteins, either to regulate activity, or to initiate conformational transitions for time-resolved studies.[25] The above properties, summarized in **Table 1**, allow thioamide modification to affect biomolecule function in valuable ways which have led to the evolution of several distinct mechanisms for thioamidation in natural products.

Thioamides in Nature: Biosynthesis, Structure, and Function

Thioamides are exceptionally rare in biology. Most reported natural thioamides are of bacterial origin, except for the plant-derived cycasthioamide (**Figure 1**). Among these are the ribosomally synthesized and posttranslationally modified peptide (RiPP) natural products thioviridamide and its analogs, methanobactin, and thioamidated thiopeptides as well as closthioamide, which is a non-ribosomal natural product (**Figures 1 and 2**).[26,27,28,29] Known thioamide-containing nucleotides include thiouridine, thiocytidine, and thioguanine (**Figure 1**).[30,31] Recent identification of the proteins responsible for thioglycine formation in the active site of methyl-coenzyme M reductase (MCR), the only known thioamidated protein, further adds impetus to this area.[32,33] On the other hand, a distinct biosynthetic mechanism has been identified for closthioamide,[34] which shares features of thionated nucleoside biosynthesis,[30] as well as for methanobactin.[35] Collectively, these revelations suggest that natural thioamidation pathways arose through multiple different independent routes.[32,33,34]

Non-ribosomal natural products

Cycasthioamide

Cyscasthioamide is a thioamide-containing, non-proteinaceous amino acid obtained from the plant *Cycas revoluta* Thunb with unknown biological function (**Figure 1**).[36] In addition, another thioamidated compound is reported from the tunicate *Polycarpa aurata*; however, it is believed to be an artifact of product isolation or degradation of the major alkaloid compound polycarpine.[37]

Closthioamide

Closthioamide, a polythioamide compound, produced by *Ruminiclostridium cellulolyticum* (previously *Clostridium cellulolyticum*), a strictly anaerobic, Gram-positive, soil-dwelling bacterium (**Figure 1**).[27] Until recently, no *R. cellulolyticum* natural products were isolated from standard laboratory cultivation. Hertweck and co-workers mimicked the natural environment by adding soil extracts to the culture which led to the production of closthioamide.[27] Closthioamide is a symmetrical hexathioamide and displays a central diaminopropyl group with four β-alanyl extender units and two terminal *p*-hydroxybenzoyl groups. Closthioamide is growth-suppressive towards several human pathogens, including *Staphylococcus aureus*, *Enterococcus faecalis*, and *Neisseria gonorrhoeae*.[27,38,39] Replacement of the thioamides with amides abolished the antibacterial activity of closthioamide implicating the importance of thioamide moieties in its biological activity.[27]

Studies using whole cell-based assays showed that closthioamide inhibits the ATPase activity of DNA gyrase and topoisomerase IV, thus effectively blocking DNA replication.[40] Closthioamide also inhibits the relaxation activity of DNA gyrase, which does not require ATP hydrolysis and thus may allosterically, rather than directly, interfere with the ATPase activity of gyrase. Notably, this mode of action differs from that of the other DNA gyrase inhibitors such the fluoroquinolones and aminocoumarins.[40] The discovery of closthioamide from an underexplored anaerobe with a new mode of action holds promise that additional, novel antibiotics might be discovered by parallel methods.

Manipulation of the regulatory elements involved in the global activation of secondary metabolism in *R. cellulolyticum* sustained the production of closthioamide and allowed isolation of seven

congeners which showed varied levels of bioactivity.[41] Evaluation of the antibacterial activity of the congeners demonstrated the importance of all six thioamide moieties, terminal aromatic residues, the modular arrangement of the β-thioalanyl units, and the distinct length of spacer units for antibacterial activity.[41,39] Moreover, it was also found that closthioamide is a selective Cu(I) chelator akin to methanobactin and forms a compact symmetrical dinuclear copper complex.[13,42]

Through synthesis and application of deuterium- and fluorine-labeled probes (see below), initial insights into closthioamide biosynthesis were obtained, which predicted the involvement of an unusual non-ribosomal peptide synthetase (NRPS).[22,41] A very recent study reported the closthioamide biosynthetic gene cluster (BGC) in *R. cellulolyticum* and demonstrated that closthioamide biosynthesis involves a novel thiotemplated peptide assembly line that differs from known NRPSs.[34,43] Extensive genome editing and isolation of intermediates from the knock out mutants revealed that the closthioamide BGC consisted of genes *ctaA-M* along (**Figure 3A**) and a preliminary biosynthetic pathway has been proposed (**Figure 3B**).[34] First, *p*-hydroxybenzoic acid (PHBA) is synthesized from chorismate by chorismate lyase CtaA (NCBI accession number: WP_015926607.1) and loaded onto a peptidyl carrier protein (PCP), either CtaE (WP_015926603.1) or CtaH (WP_015926600.1). [44] This step is probably catalyzed by either CtaD (WP_015926604.1) or CtaI (WP_015926599.1), as both enzymes are members of PCP-loading protein families (ATP-grasp and AMP-dependent ligase, respectively).[45] The second PCP would be loaded with two molecules of β-alanine by the iterative action of CtaD/I, which itself could be biosynthesized from L-aspartate by CtaF (decarboxylase, WP_015926602.1). Then, the PHBA thioester is proposed to be loaded onto this PCP-bound β-alanyl dipeptide. CtaG (WP_015926601.1), whose N-terminus shows homology to an amide synthase, BtrH from

butirosin biosynthesis, is the likely candidate.[46] The resultant PCP-bound product would be converted to the corresponding polythioamide intermediate by CtaC (WP_015926605.1), which shows homology to the alpha-adenine nucleotide hydrolase (AANH) superfamily.[28] Finally, it would be coupled to diaminopropane (DAP) by CtaJ (amide synthase, WP_015926598.1) and thionated by CtaC to yield mature closthioamide.[34] The biosynthetic intermediate, DAP, could be biosynthesized from L-aspartate by the sequential action of CtaK (WP_015926597.1), CtaB (WP_015926606.1), and CtaF.[47] Currently, the source of sulfur is not known.[34] This is the first example of a thioamidated compound that employs a novel NRPS biosynthetic assembly line.[34]

Thioamide-containing nucleosides

6-Thioguanine (6TG)

6-Thioguanine (6TG) is a highly potent cytotoxin developed in the 1950s by Elion and Hitchings, who received the Nobel Prize for discovering antimetabolite drugs (**Figure 1**).[48] 6TG exerts its cytotoxicity by *in situ* transformation to 6-thioguanine nucleosides and subsequent incorporation into DNA.[49] As a result, a mismatch repair pathway triggers cell-cycle arrest and apoptosis.[50] 6TG is used for the treatment of several ailments such as leukemia, psoriasis, and inflammatory bowel disease; however, it is known to exhibit some deleterious side effects.[51,52,53]

Naturally produced 6TG plays a critical role in the pathogenesis of *Erwinia amylovora*, the causative agent for fire blight, a devastating disease that affects apple and pear trees, causing immense ecological and economic damage.[54,31,55] Functional gene analyses revealed that the biosynthesis of 6TG is encoded by a small gene locus (*ycf*ABCD), as shown by successful heterologous expression of the *ycf* gene cluster in *E. coli* (**Figure 4A**).[31] YcfA (WP_004156383.1)

is homologous to proteins of the diverse AANH-like superfamily, which display a conserved ATP-binding αβα fold and includes enzymes responsible for sulfur incorporation on tRNA nucleobases.[56] YcfB (WP_004156386.1) is a homolog of NUDIX hydrolase while YcfD (WP_004156390.1) is a transporter, and thus, neither were implicated directly in thioamidation chemistry. YcfC (WP_004156388.1) is distantly related to pyridoxal phosphate (PLP)-dependent transferases. It was also demonstrated that an *E. coli* strain expressing only *ycfA* and *ycfC* produces 6TG equivalent to the one that has *ycfABC*,[56] indicating that YcfA and YcfC are necessary and sufficient for heterologous 6TG biosynthesis. Besides these, YcfR (WP_004156381.1) has been identified as the major transcriptional regulator of 6TG biosynthesis and related pathogenicity.[55]

A recent *in vitro* study using purified enzymes revealed that 6TG thioamidation is achieved by a bipartite enzyme system consisting of YcfA and YcfC with cysteine as the sulfur source.[31] Guanine nucleotides were shown to be the substrates, indicating that thioamide formation occurs prior to cleavage of the glycosidic bond. Detection of AMP and pyrophosphate as reaction byproducts and mutational analysis of YcfA provided evidence for an adenylation mechanism.[56] According to the current mechanism (**Figure 4B**), YcfA activates guanosine nucleotides by adenylating the carbonyl oxygen. Then, YcfC provides the sulfur nucleophile from cysteine that is transferred onto YcfA, most likely to Cys113 in the form of a persulfide, which in turn, would attack the activated carbonyl group.[31] AMP would be released after sulfur insertion yielding 6TG. This study highlights the distinct mechanism that Nature employs for thioamidation of nucleosides.

4-Thiouridine (s^4U)

4-Thiouridine (s^4U) is a modified nucleobase in tRNA of some prokaryotes and is proposed to serve primarily as a photosensor (**Figure 1**).[57,58] When the organism is exposed to UV light, s^4U

(position 8 in *E. coli* tRNA) undergoes photoinduced crosslinking with nearby cytidine 13. This stalls protein synthesis and triggers a controlled growth arrest, allowing the organism time to repair the UV-damaged DNA with growth resuming after the UV exposure ends.[30]

Biosynthesis of s^4U in *E. coli* tRNA is accomplished by two enzymes, IscS (named for its role in iron sulfur cluster assembly, WP_001295373.1)[59] and ThiI[60] (an enzyme involved in thiamin biosynthesis, NP_414957.1) with the sulfur donor being L-cysteine.[61,62] Like YcfC in 6TG biosynthesis, IscS is a PLP-dependent cysteine desulfurase which transfers the sulfur atom of cysteine to generate a persulfide (R-SSH) group on an active site cysteine residue.[63] The terminal sulfur of the persulfide is then transferred to ThiI (s^4U synthetase) where it also resides on an active site cysteine in what is referred to as a rhodanese homology domain (RHD, Cys456 in *E. coli* ThiI).[64,65] The N-terminal portion of ThiI, which is composed of three domains (ferredoxin-like, THUMP, and pyrophosphatase), is responsible for tRNA-binding and activating uridine-8 using ATP while the C-terminal portion delivers sulfur to the adenylated uridine in tRNA.[66,67] Two mechanisms were proposed for the formation of s^4U (**Figure 5**).[30,65,68,69] In one, the persulfide attacks the activated uridine to release AMP and generate a disulfide bond that links ThiI to the tRNA. A second active site cysteine (Cys344 in *E. coli* ThiI) then attacks the ThiI-tRNA disulfide bond to liberate s^4U in tRNA and make a disulfide bond (Cys344-Cys456), reduction of which regenerates ThiI for another catalytic cycle (**Figure 5A**). In the second proposed mechanism, Cys344 attacks the persulfide on Cys456 to form the same disulfide but instead generate bisulfide nucleophile ($^-$SH) that displaces AMP from the adenylated uridine (**Figure 5B**). Both the Cys456 persulfide and the Cys344-Cys456 disulfide bond have been observed.[69] However, it remains unclear whether persulfide or bisulfide acts as a nucleophile.[30]

The mechanism of s^4U biosynthesis shows striking differences based on the organism. It was demonstrated that ThiI from *Bacillus subtilis*, which lacks a RHD (WP_003229326.1), strictly depends on a sulfurtransferase, NifZ (WP_072589459.1), which transfers the sulfhydryl group onto a yet-unidentified cysteine.[70] RHD-deficient ThiI from *Methanococcus maripaludis* (WP_011171298.1) showed as well that s^4U biosynthesis involves a conserved CXXC motif within the N-terminal pyrophosphatase domain that forms the persulfide and disulfide using the active site cysteines (Cys265 and Cys268) with bisulfide as a potential sulfur source.[71] This is supported by the fact that *Methanococci* are well adapted to live in sulfide-rich environments and also use free sulfide, not cysteine, for Fe-S cluster biosynthesis.[72] In addition, recent structural studies of ThiI from *Bacillus anthracis* (WP_011053248.1) and *Thermotoga maritima* (WP_010865383.1) further define substrate specificity during s^4U biosynthesis.[67,73]

2-Thiouridine (s^2U)

Several derivatives of 2-thiouridine (s^2U) are found in the anticodon loop of tRNAs (at wobble position 34) which are specific for glutamate, glutamine, and lysine (**Figure 1**).[74] This modification stabilizes anticodon structure, confers ribosome binding ability to tRNA, and improves reading frame maintenance.[74] In *E. coli*, seven proteins have been identified which are responsible for generation of 5-methylaminomethyl-2-thiouridine (mnm^5s^2U): IscS, a modification enzyme (MnmA) and three persulfide carriers (TusA, TusBCD complex, and TusE).[75,76] Using this sulfur relay system (**Figure 6**), the sulfur atom of cysteine is first activated by IscS to form an enzyme-bound persulfide. The group is then transferred to the small sulfur-carrier protein TusA (NP_417927.1), which, in turn, passes it on to TusD in the $\alpha_2\beta_2\gamma_2$ TusB (NP_417802.1)/TusC

(NP_417803.1)/TusD (NP_417804.1) hexamer for delivery to TusE (NP_415489.4), and then finally to tRNA bound to MnmA (NP_415651.4).[78] Biochemical studies revealed that the conserved cysteines in TusA, TusD, and TusE are essential for sulfur relay.[76] MnmA is a N-type ATP-pyrophosphatase with two catalytic cysteines (Cys102 and Cys199).[77] Mechanistic insights were obtained from a MnmA-tRNA complex which revealed that MnmA binds the anticodon arm and D-stem regions of tRNA and activates the C2-position of the uracil ring at position 34 as an acyl-adenylated intermediate. Thioamidation is then achieved through nucleophilic attack by the MnmA persulfide (**Figure 6**).[79] Bioinformatics revealed that IscS, TusA, and MnmA are mostly conserved among bacteria, while TusBCD and TusE homologs are relatively rare, suggesting there may be alternative s^2U biosynthetic pathways.[80,81,82]

2-Thiocytidine (s^2C)

2-Thiocytidine, s^2C (position 32 of *E. coli* tRNA), is another modified tRNA base in the anticodon loop and plays a critical role in maintaining translational fidelity and efficiency (**Figure 1**).[75] There are two pathways for thioamidation of the four nucleosides in *E. coli* tRNA.[58] First, an Fe-S cluster-independent pathway leads to the formation of s^4U (position 8) and mnm^5s^2U (position 34) which uses the persulfide (IscS-SSH) intermediate.[30] The second pathway is Fe-S cluster-dependent and carries out the biosynthesis of s^2C (position 32) and *N*-6-isopentenyl-2-methylthioadenosine, ms^2i^6A (position 37).[58] In addition to IscS, a major scaffold protein involved in the biosynthesis of Fe-S clusters, IscU (WP_000331707.1), is required for the second pathway. Biosynthesis of ms^2i^6A requires a radical *S*-adenosylmethionine enzyme, MiaB (WP_000162740.1), that contains two [4Fe-4S] clusters which participate during methylthiolation of *N*-6-isopentenyl adenosine, i^6A.[83,84]

TtcA (WP_001157406.1), responsible for s^2C biosynthesis, is intriguing as it contains the characteristic nucleotide-binding motif and ATPase activity as in ThiI and MnmA (Fe-S cluster-independent pathway), but also TtcA contains a MiaB-like motif that comprises a [4Fe-4S] cluster.[85,86] TtcA is the first example of an Fe-S cluster- and ATP-dependent enzyme that thioamidates tRNA via a non-radical mechanism.[86] Based on several lines of evidence, it was proposed that the [4Fe-4S] cluster participates in sulfur transfer. The bisulfide group is transferred from the IscS-SSH (obtained from Cys/IscS) to an Fe of the cluster, forming an Fe-SH intermediate.[84] Subsequently, a nucleophilic attack on the activated cytidine is proposed that would release AMP and form s^2C.[82,86] These studies on nucleoside thioamidation indicate that analogous mechanisms of sulfur transfer might be operating in the thioamidation of some peptidic scaffolds as well.

YcaO-independent RiPP pathways:

Methanobactin

Methanotrophic bacteria, obligate anaerobes that oxidize methane, produce a small copper-chelating molecule called methanobactin (Mbn).[13] Mbn is a RiPP natural product (**Figure 2**), first discovered in *Methylosinus* (*Ms.*) *trichosporium* OB3b which binds Cu(I) with high affinity (or Cu(II) via a reductive process)[87,26] Methanotrophs use the Cu-dependent particulate methane monooxygenase or, when starved for copper, the Fe-dependent soluble methane monooxygenase for oxidation of methane. Mbns play a critical role in their metabolism by participating in Cu uptake, transport, and homeostasis.[88,89] The high affinity of Mbns for Cu(I) is conferred by a pair of bidentate ligands, each comprising of a nitrogen heterocycle (primarily an oxazolone ring) and an adjacent thioamide or enethiol, which chelate Cu(I) in a distorted tetrahedral geometry.[88]

The Mbn BGC was first identified in *Ms. trichosporium* OB3b and is present in a range of methanotrophic bacteria.[88,90] Mbn operons consist of biosynthesis genes *mbnABC* along with other genes involved in secondary modification, regulation, and transport (**Figure 7A**). The precursor peptide, MbnA (WP_003614758.1), consists of an N-terminal leader peptide, which is cleaved in the mature Mbn (**Figure 7B**), and a C-terminal core peptide (L**C**GSCYP**C**SCM), in which thioamide modifications are installed at two cysteines (Cys21 and Cys27 in *Ms. trichosporium* MbnA).[88,90,91] For the structurally characterized Mbns, there are two heterocycle-thioamide moieties in the final compound, with the second heterocycle uniformly being an oxazolone. Formation of each oxazolone-thioamide group requires a net four-electron oxidation which suggested that the modification enzymes contained redox cofactors.[35 88]

MbnB (WP_065083569.1) belongs to an uncharacterized protein family (DUF692) and is predicted to be an Fe-containing enzyme based on distantly related enzymes of the triose phosphate isomerase (TIM) barrel family 15.[92] The crystal structure from a related homolog from *Haemophilus somnus* 129Pt revealed a diiron cluster at the center of the TIM barrel fold, based on which a model for the MbnB active site was generated.[35] MbnC (WP_003614727.1) is a protein of unknown function, sometimes found separately from *mbnB* as in *Pseudomonas* sp.[35] MbnB/C does not contain a RiPP precursor peptide recognition element (RRE), a ~90 amino acid structurally conserved domain which is present in many leader peptide-dependent modifying enzymes from several RiPP families.[26,93]

Rosenzweig and co-workers recently showed that purified MbnA and MbnBC from *Ms. trichosporium* OB3b form a heterotrimeric complex[35] and spectroscopic studies revealed that all the Fe(II) was associated with MbnB. MbnBC reacts with MbnA in the presence of O_2, to yield a MbnA-related product that is 4 Da lighter and absorbs strongly at 335 nm. The mass loss was localized to the N-terminal Cys21, which is the position of the N-terminal oxazolone ring in the final Mbn. MbnBC did not modify the core peptide alone and truncation of the leader peptide resulted in diminished activity. This indicates that RRE-independent MbnBCs possess an alternate way of engaging the MbnA leader peptide.[35] In the mature Mbn, the leader peptide is cleaved, and the N-terminal primary amine is converted to a carbonyl group by the PLP-dependent aminotransferase MbnN (WP_051418802.1),[35,94] an enzyme previously purported to be responsible for N-terminal oxazolone formation.[95] This modification extends the conjugation of the N-terminal oxazolone ring, accounting for the bathochromic shift of the mature Mbn to 392 nm. On the other hand, C-terminal oxazolone moieties in all Mbns lack such extended conjugation and their absorption fall within 325-345 nm (**Figure 7B**).[35]

The formation of the oxazolone thioamide moiety is dependent on the presence of the predicted multinuclear Fe (di/tri) cofactor.[35] Substitution of the proposed iron ligands in *Ms. trichosporium* OB3b MbnB model structure diminished or abolished activity. The absolute requirements for Fe(II) and O_2 suggest that MbnBC is an oxidase that, possibly via the Fe center, activates O_2 for the cleavage of the three aliphatic C-H bonds on Cα and Cβ of Cys21 of MbnA to catalyze the four-electron oxidation to form the oxazolone-thioamide group.[35] MbnA processing could be initiated by abstraction of a hydrogen atom from the Cβ of the Cys21 by a superoxo (di/tri) Fe(III) intermediate.[96] However, how MbnBC proceeds to the second C-terminal site on MbnA (Cys27),

remains unclear. Leader peptide removal also remains enigmatic. Nonetheless, the formation of the oxazolone thioamide moiety in Mbn using a metalloenzyme-mediated radical mechanism presents a novel way for carrying out this chemistry.[35,89]

YcaO-TfuA dependent RiPP pathways:

Thioviridamide and related compounds

Thioviridamide (and its derivative JBIR-140) is a RiPP natural product obtained from *Streptomyces olivoviridis* NA05001 which exhibits activity in several cancer cell lines as well as antibiotic properties.[97,98] Thioviridamide features an N-terminal pyruvyl group, a β-hydroxy-*N*1, *N*3-dimethylhistidinium (hdmHis) residue, and a *S*-(2-aminovinyl)-cysteine (AviCys) residue that forms part of a macrocycle (**Figure 2**).[99,100,101] Thioviridamide also has five thioamide groups in place of backbone amide groups. The thioviridamide (*tva*) BGC was identified from *S. olivoviridis* and confirmed by heterologous production of thioviridamide in *Streptomyces lividans* TK23.[102] This demonstrated the ribosomal origin of this molecule, which derives from a 12 amino acid core peptide at the C-terminus of the TvaA precursor peptide (BAN83916.1). An additional 11 proteins encoded by this gene cluster (TvaB-TvaL) are predicted to be involved in the maturation of the precursor peptide into thioviridamide, although virtually nothing has been reported regarding the individual steps (**Figure 8 A,B**).[102] Two proteins encoded by the thioviridamide BGC have plausible roles in thioamide synthesis. TvaH is a member of the YcaO superfamily (BAN83923.1),[103] while the second, TvaI, is annotated as a "TfuA-like" protein (BAN83924.1).[102] Biochemical characterization of YcaO-family proteins illustrates that they catalyze ATP-dependent cyclodehydration of cysteine, serine, and threonine residues to the corresponding thiazoline, oxazoline, and methyloxazoline (**Figure 9**) in the biosynthesis of various RiPP natural

products.[26,103] Characterized YcaOs often require a partner protein for efficient cyclodehydration.[104,105] Two types of such partner proteins are reported so far, with one type resembling E1-ubiquitin activating like enzymes while the other is referred to as an "ocin-ThiF" protein.[104,106] These partner proteins harbor N-terminal RREs[93] which bind mostly the N-terminal leader peptide region. Once the substrate peptide is bound, the YcaO performs modifications in the C-terminal core region.[103] In contrast, two recently characterized YcaOs from bottromycin biosynthesis can catalyze the formation of thiazoline and macrolactamidine moieties independently of a partner protein.[103,107,108]

Based on sequence similarity, the TfuA protein encoded adjacent to the YcaO in the thioviridamide BGC and in numerous other genomic contexts,[32] is predicted to assist in the thioamidation reaction, although this proposal awaits biochemical validation in thioviridamide biosynthesis. However, an exogenous source of a sulfide equivalent will be required for thioamide formation (**Figure 9**). Recently, genetic deletion studies in *Methanosarcina acetivorans* corroborated with *in vitro* studies on thioglycine formation, a universal PTM in MCR, provide the first evidence for this proposal.[32,33] Potential roles for TfuA include allosteric activation of the YcaO and/or delivery of sulfur equivalents, possibly in collaboration with sulfurtransferases.

Recent genome-mining efforts led to the discovery of several thioviridamide-like compounds with improved bioactivities (**Figure 2**). Truman and co-workers reported three such analogs, thioalbamide (from *Amycolatopsis alba* DSM44262), thiostreptamide S4 (from *Streptomyces* sp. NRRL S-4), and thiostreptamide S87 (from *Streptomyces* sp. NRRL S-87).[100] Of these, thioalbamide possesses nanomolar antiproliferative activity with about 6-fold selectivity for cancer

cells.[100] Around the same time, the Müller and Koehnke groups reported the discovery of thioholgamides A/B from *Streptomyces malaysiense* (**Figure 2**).[109] Thioholgamide A showed markedly increased activity compared to thioviridamide with submicromolar activity against several cancer cell lines (30 nM against HCT-116 cells) and 10-fold higher potency than thioholgamide B.[109] The minimal set of responsible biosynthetic genes have been identified, however, biochemical evaluation of the pathways has yet to be achieved.[109]

Thioamidated thiopeptides

Thiazolylpeptides (or thiopeptides) are RiPP natural products with many displaying nanomolar growth suppression activity towards Gram-positive human pathogens.[110,111] Thiostrepton is used as a topical agent in veterinary medicine, while LFF571, a semisynthetic analog of the thiopeptide GE2270A, displayed promising efficacy in human clinical trials against *C. difficile* infections.[111,112] Nosiheptide has been widely used in animal feed as a growth promoter. Thiopeptides exert their antibiotic activity by inhibiting protein translation, with the exception of cyclothiazomycins (reported as RNA polymerase inhibitors) and lactazoles (no reported activity).[111,113,114]

A few thiopeptides have been reported that bear a thioamide group, but until recently, the origin of this group was unknown (**Figure 2**).[115,116] A recent discovery of a thiopeptide from *Amycolatopsis saalfeldensis* NRRL B-24474 also displays a thioamide moiety.[29] A bioinformatic survey detected a thiostrepton-like BGC which contained a *ycaO-tfuA* gene pair (YcaO: SEO90172.1; TfuA: SEO90188.1) in addition to other thiopeptide biosynthetic genes. Upon isolation and structure elucidation, it was confirmed that the new thiopeptide "saalfelduracin" is a thioamidated thiopeptide as evident from extensive NMR studies (**Figure 10**).[29] The antibiotic

activity of saalfelduracin is approximately equivalent to thiostrepton.[29, 111] This study also provided insight into thioamide biosynthesis for the previously reported thioamidated thiopeptides, which include thiopeptin (from *Micromonospora arborensis* NRRL 8041) and Sch 18640 (*Streptomyces tateyamensis* ATCC 21389).[115,116] The genomes for the thiopeptide- and Sch 18640-producing organisms were sequenced, which upon analysis found *ycaO-tfuA* genes present in a BGC along with other anticipated thiopeptide biosynthetic genes.[29] The function of the YcaO-TfuA proteins was corroborated by functional expression in *S. laurentii* (producer of thiostrepton). Mass spectrometry analysis identified a derivative 16 Da heavier, consistent with thioamidation (formal replacement of oxygen by sulfur, +15.9772 Da, error ~0.5 ppm) as opposed to oxidation or hydroxylation (formal addition of oxygen, +15.9949 Da, error >12 ppm). Further *in vitro* experiments are required to decipher the biosynthetic timing and role of this PTM in thiopeptides.

Thioamide-containing protein: Methyl-coenzyme M reductase (MCR)

MCR is found strictly in methanogenic (methane-producing) and methanotrophic (methane-consuming) archaea and carries out the reversible conversion of methyl-coenzyme M (CoM, 2-methylmercaptoethanesulfonate) and coenzyme B (CoB, 7-thioheptanoylthreoninephosphate) to methane and a CoB-CoM heterodisulfide.[117,118] MCR plays an important role in the global carbon cycle by maintaining steady-state levels of atmospheric methane, a potent greenhouse gas.[119] The 300 kDa enzyme is a heterodimer of three subunits in an $\alpha_2\beta_2\gamma_2$ arrangement and uses a tightly bound, Ni-containing coenzyme F_{430}.[117,120] The Ni(I) oxidation state of this porphinoid cofactor is crucial for catalysis.[121]

Notably, the α subunit of MCR (MCRα, WP_011024419.1) has several PTMs including 3-methylhistidine, S-methylcysteine, 5-methylarginine, 2-methylglutamine, didehydroaspartate, and thioglycine with varying degrees of occurrence.[122,123,124] Thioglycine is present in all the methanogens analyzed thus far and MCR is the only protein known to biology to bear a thioamide PTM.[123] Although there have been proposals for thioglycine in the MCR catalytic mechanism,[125,126,127] recent work has shown that it might instead play a structural role in properly organizing the active site.[32]

Thauer originally proposed that thioglycine formation in MCR could be similar to thioviridamide biosynthesis.[97, 123] Upon elucidation of the thioviridamide BGC, it was then proposed that *ycaO-tfuA* would be involved in MCR thioamidation. Bioinformatics analysis of all available methanogen genomes revealed universal occurrence of *ycaO* with ~90% also encoding a *tfuA* gene.[32] To evaluate any role in thioglycine formation, *ycaO* (WP_011020223.1) and *tfuA* (WP_011020222.1) genes were deleted (individually and together) in *Methanosarcina acetivorans*.[32] MCRα peptide fragments from the wild type and deletion strains were analyzed by mass spectrometry and it was found that each variant (i.e. Δ*ycaO*, Δ*tfuA*, and Δ*ycaO-tfuA*) lacked thioglycine (at Gly465 in MCRα), implicating their importance in thioamide installation.[32] While each variant was viable, they were incapable of growth at higher temperatures and displayed marked growth defects compared to wild type *M. acetivorans*.[32] This could be due to the increased flexibility of the unmodified glycine in the Δ*ycaO-tfuA* strain, which would render the contorted conformation of the peptide backbone, otherwise stabilized by several interactions involving the thioglycine, in the Gly462-Leu469 region of MCRα considerably less stable (**Figure 11**).[32]

This genetic deletion study was further supported by *in vitro* thioamidation using purified YcaO TfuA, ATP, and substrate peptides from *M. acetivorans* MCRα. The requisite bisulfide nucleophile was supplied chemically as sodium sulfide or produced enzymatically from L-cysteine and *M. acetivorans* IscS (AAM06097.1).[33] The residues encompassing Gly465 that are directly engaged by YcaO were evaluated using biochemical and biophysical methods. Structural insights were obtained using thermophilic YcaO homologs from *Methanopyrus kandleri* (AAM01332.1, PDB codes: 6CIB and 6CI7) and *Methanocaldococcus jannaschii* (WP_010870606.1) which confirmed the ATP-binding pocket. Sequence- and structure-guided alanine scanning was performed to elucidate the role of residues involved in ATP/Mg^{2+} and possible peptide binding.[33] According to a mechanistic proposal supported by spectroscopic and isotopic labeling experiments (**Figure 9**), upon substrate binding, an external source of sulfide will attack the target amide bond (in this case, Gly465) generating a tetrahedral intermediate. The amide oxyanion will then attack the γ-phosphate of ATP, releasing ADP and a phosphorylated thiolate intermediate. This thermodynamically favorable step in which ATP cleavage is coupled with C-S bond formation could be concerted or step wise. Finally, the tetrahedral intermediate will collapse by releasing phosphate and the thioamidated peptide.[33] Further studies are needed to elucidate the substrate orientation in the active site as well as the role of TfuA, which is currently proposed to act as a partner to YcaO that may regulate ATP usage or participate in sulfur delivery in collaboration with sulfurtransferases.[33]

Potential for other thioamide natural products

The discoveries of thioamide biosynthesis pathways and the assignments of thioamide-specific functions in polypeptides reveal the potential that this backbone modification might be widely

used in nature to confer evolutionarily valuable properties that presumably cannot be attained through sidechain modification. However, one is then left to wonder why natural thioamides are not more prevalent. Studies on MCR, thioviridamide-like molecules, and thioamidated thiopeptides have identified biosynthetic signatures of such compounds, which surveys predict to be considerably more numerous than currently appreciated.[29,32,33] Indeed, given that the mass for a thioamide-containing peptide is ~16 Da heavier than the corresponding amide, the mass change can easily be misinterpreted as oxidation. This may explain why some thioamide-containing natural products have gone undetected, although thioamidation may still represent a relatively rare PTM. Bioinformatic-driven searches based on the presence of *ycaO* and *tfuA* genes are already aiding in the discovery of thioamide-containing molecules and will undoubtedly help to resolve such mis-assignments. As more thioamide-containing compounds are discovered, the ability to synthetically introduce thioamides will be important to define the functional role of the thioamide. This knowledge can be applied by exogenously introducing thioamides to confer new functions on peptides and proteins.

Synthetic Thioamides in Peptides and Proteins

Studies of the roles of natural thioamides are enhanced by the ability to site-specifically incorporate thioamides in order to generate biosynthetic precursors, probe molecules, or natural product analogs. This is well-illustrated in the above-noted studies of closthioamide by Hertweck and coworkers.[42] [40] In addition to testing the activity of synthetic closthioamide,[128] closthioamide variants were prepared with some or all of the thioamides replaced by amides, variants which had been observed in *R. cellulolyticum* extracts.[42] Using these compounds, amide variants were not converted to thioamide variants, but that thioamide variants were hydrolyzed to form the amide

variants. Thus, it appeared that thioamide incorporation occurred during closthioamide backbone synthesis, not through downstream modification of amides. In the second study, the motifs that crucial to the antibiotic properties of closthioamide were investigated by modulating, amongst other things, the number and position of thioamides.[40] Removal of even one thioamide caused a greater than 100-fold decrease in potency toward methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. As these studies make clear, the ability to insert thioamides at specific locations allows one to interrogate both the biosynthesis and mechanism of action of thioamide-containing natural products.

Chemical synthesis of thioamide-containing peptides

Site-specific installation of a thioamide into a peptide of interest is readily accomplished by incorporating a thioamide precursor during peptide synthesis rather than attempting a selective transformation of a specific amide bond. Various routes have been investigated, including activation of thioacids with phosphonium-based reagents like benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP)[40] or the synthesis of thioacyl-benzimidazolinones.[129] However, these reactions suffered from low yields and racemization of the thioamide residue. An improved route was devised based on thioacyl-benzotriazoles, which are sufficiently reactive that they can thioacylate amines in the presence of base with no other activating reagent.[130] While *tert*-butyloxycarbonyl (Boc)-protected precursors were synthesized in the initial report, the methods have been shown to be compatible with fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids,[131] which are more commonly used. These precursors have become widely used; the route to their synthesis and incorporation is illustrated in **Figure 12**. The synthesis starts with Fmoc-protected amino acids, which are commercially available in most cases, with

typical yields of 40% over the three steps. So far, thioamide versions of 15 of the 20 canonical amino acids (Ala,[132] Arg,[133] Asp,[134] Cys,[133] Glu,[135] Gly,[133] Ile,[136] Leu,[137] Lys,[133] Phe,[138] Pro,[138] Ser,[133] Trp,[133] Tyr,[136] and Val[139]), as well as hydroxyproline,[136] have been reported.

While the aforementioned benzotriazole synthesis can be used for Boc- or Fmoc- based precursors, thioamides are incompatible with the harsh HF cleavage used in Boc-based solid phase peptide synthesis (SPPS). However, thioamides are compatible with the conditions and reagents used in Fmoc-based SPPS, including various activating reagents (for the other amino acids), bases, cleavage additives, capping solutions, and strong acids like trifluoroacetic acid (TFA) that are used in cleavage reactions. Fmoc-based SPPS using thioacyl-benzotriazole building blocks has been used to routinely prepare thioamide-containing peptides of 20-40 amino acids and on the multi-mg scale.[136 4]

There are several side-reactions that must be considered during SPPS and cleavage. First, prolonged exposure (>30 minutes) to strongly acidic conditions (95% trifluoroacetic acid, TFA) typically used during peptide cleavage can lead to an Edman degradation-type reaction that results in backbone cleavage at the n+1 site.[140] Therefore, one must limit TFA concentrations and deprotection times to achieve a delicate balance between full removal of all acid-labile side-chain protecting groups while also maintaining the integrity of the thioamide. Second, thioamide precursors can react with residual amounts of water during coupling reactions, resulting in an S-to-O exchange reaction to yield the corresponding amide. Fortunately, the use of anhydrous methylene chloride during thioamide coupling can greatly reduce this side reaction.[133] Last, thioamides can undergo epimerization during peptide synthesis. While there have been few studies

in peptides, studies of model compounds consistently show that the pK_a of the thioamide Cα proton is about 5 pH units lower than that of the corresponding amide, equating to a pK_a of 12-13 in peptides. A more detailed analysis can be found elsewhere.[141] Therefore, prolonged exposure to base during Fmoc-deprotection steps can lead to peptide epimerization. Although there have been various approaches to this problem,[135] [141] the use of a 2% (*v*/*v*) solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) seems to give the best combination of high deprotection efficiency and minimal epimerization. This is particularly important for the synthesis of peptides containing multiple adjacent thioamides,[137] [142] motifs found in the thioviridamide family of compounds. While alternate methods such as recently developed ynamide-based couplings may lead to further improvements,[143] with these considerations, the thioacyl-benzotriazoles can be used to access many thioamide-containing peptides.

Uses of synthetic thioamide peptides

The prospects of using the nearly isosteric thioamide to intentionally modify the physical properties of the peptide backbone have long intrigued biochemists. As noted above, these properties can be roughly categorized into four categories: increased reactivity with nucleophiles, electrophiles, and soft metals; altered conformational properties; altered hydrogen-bonding propensity; and altered photophysical or electrochemical properties. All of these properties have been exploited for various purposes.

The first biochemical study of a synthetic thioamide containing peptide was published by Nobel laureate Vincent du Vigneaud in 1974.[144] This study focused on the C-terminal amide of the cyclic 9-mer peptide hormone oxytocin. It was known that the C-terminal amide was crucial for

biological activity, but none of the previously studied analogues altered the carbonyl moiety. Thioamide substitution at this site reduced the biological activity to ≤6% of oxytocin in all assays tested. This study demonstrated that although it is nearly isosteric with an amide and retains functional groups capable of donating and accepting hydrogen bonds, a thioamide modification can have significant effects on biological signaling. Since this pioneering work, thioamides have been used in biophysical studies of protein folding, as spectroscopic labels, as probes of hydrogen bonding in protein interactions, and as synthetic intermediates in the synthesis of other carbonyl modifications. For example, Boger's laboratory used thioamides both as synthetic tools and as probes of hydrogen bonding interactions with a backbone carbonyl in vancomycin.[14] Through the introduction of thioamides as useful intermediates, synthetic chemists have been able to synthesize analogues that have different hydrogen bonding patterns and test the derivatives with model target substrates to confirm the assumed mechanism of action,[14] as well as to develop more potent variants of vancomycin.[145] [146] The use of thioamides as spectroscopic labels, particularly as circular dichroism probes or fluorescence quenchers, has limited utility in complex biological environments and has been summarized elsewhere.[147] [9] Thus, we will focus here on studies applicable to cell-based or *in vivo* experiments.

One photophysical property that has a demonstrated utility in biological systems is the ability to use thioamides as photoswitches. Excitation at 270 nm or 340 nm ($\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, respectively),[21] allows for selective photoswitching of the thioamide between the *trans* and *cis* isomers. Additionally, the metastable photo-induced *cis* state has a slow thermal relaxation rate ($k < 1 \times 10^{-3}\ s^{-1}$)[148] and can populate up to 50% of the photostationary state.[149] Two studies illustrate the use of thioamide photoswitching in biological contexts. Kiefhaber and Fischer investigated the

enzymatic activity of ribonuclease S (NP_937878.1).[150] This cleaved enzyme requires the S-protein and complimentary S-peptide for activity. Incorporation of a thioamide moiety into the helical S-peptide at central positions had no significant of enzyme activity as measured by hydrolysis of cytidine 2',3'-cyclic monophosphate (cCMP). However, UV irradiation induced 30% isomerization of the peptide to the *cis* state, correlating with a 30% decrease in enzyme activity without dissociation of the protein/peptide complex. This indicated that switching the thioamide into the *cis* state lead to a conformational change that abolished enzyme activity. In another study, the conformation-activity relationship of a C-terminal pentapeptide activator of insect kinin was investigated.[149] NMR and molecular modeling studies were inconclusive as to whether a 1-4 or 2-5 β-turn was the active confirmation of the peptide.[151] In a cockroach hindgut myotropic activity assay, the photo-switched thioamide peptide in the *cis* confirmation had a 4-fold lower EC_{50} compared to the native peptide or *trans* thioamide peptide, confirming that a 1-4 turn was necessary for bioactivity (**Figure 13**).[149] These examples show that thioamide photoswitching can regulate peptide activity in cell lysates and similar systems, providing a backbone switch with much less structural perturbation than an azobenzene replacement.[152]

The distinct properties of thioamides have also been exploited in studies involving proteases. Most investigations have centered on the effects of thioamides in short peptide substrates of various proteases, with the intention of developing inhibitors and investigating the protease mechanism (e.g., favored interactions with soft metals substituted in the active sites of metalloproteases).[153] For example, Fischer and coworkers investigated thioalanine-proline-*p*-nitroaniline (Ala^S-Pro-pNA) as a substrate for dipeptidyl peptidase-4 (DPP-4, NP_001926.2), where the enzyme hydrolyses the bond between Pro and pNA.[154] They found a 1,100-fold decrease of k_{cat}/K_m

compared to the amide, which they attributed to the decrease of k_{cat}, caused by the increased rotational barrier of the thioamide. Recently, these results were extended by introducing thioamide substitutions in glucagon-like peptide 1 (GLP-1),[4] a natural substrate of DPP-4 that stimulates the release of insulin while suppressing glucagon release.[155] However, GLP-1 is rapidly degraded ($t_{1/2}$ = 2 min) *in vivo* by DPP-4, which cleaves the two N-terminal amino acids and renders GLP-1 inactive.[156] Injection of DPP-4 resistant GLP-1 variants has become a common treatment for type II diabetes.[156] The single-atom thioamide substitution at either of the two terminal positions increased the peptide half-life in an *in vitro* proteolysis assay from 2 min to up to 12 h (**Figure 14**). Competition experiments with an alternate DPP-4 substrate revealed that thioamide GLP-1 was not a competitive inhibitor, seemingly in conflict with the 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[157] reveals bifurcated hydrogen bonds with the carbonyls of the two N-terminal amino acids (**Figure 14B)**. Thioamide incorporation at either site leads to DPP-4 resistance, a finding which can be explained based on weakened hydrogen bond acceptance by the thioamide (although the rotational barrier may still play a role).

Rats injected with the thioamide containing version of GLP-1 had a significantly reduced blood glucose spike in an oral glucose tolerance test compared to those injected with GLP-1 or the vehicle control, showing that DPP-4 resistance was retained *in vivo*. In cell-based receptor activation assays, thioamide GLP-1 had comparable potency for cyclic AMP activation (which controls insulin release), but substantially lower potency for activation of β-arrestin 1 and 2 (which has

been correlated with GLP-1 effects on appetite).[158] [159] While a detailed analysis of this bias effect was not possible at the time of publication of the thioamide GLP-1 study, subsequent disclosure of a cryo-EM structure of the GLP-1 receptor (PDB code: 6B3J)could be used as a basis for modeling to explain both the signal bias as well as thioamide positional effects.[160] These studies illustrate how thioamide substitution has the potential to influence interactions of peptides with other proteins, preventing proteolytic cleavage and eliciting subtle changes in receptor activation. While thioamide substitution has been used to confer protease resistance in several other *in vivo* studies,[6] [7] we highlight the DPP-4 work because of the level of mechanistic understanding available.

A recent publication by Chatterjee and coworkers nicely illustrates how the altered conformational properties of thioamides can be exploited in medicinal chemistry applications for macrocyclic peptides.[5] Due to their high surface area per molecular weight, easy variability of functional groups, and improved bioavailability compared to linear congeners, peptide macrocycles are primed to be used as protein-protein interaction inhibitors.[161] However, in spite of their cyclic constraint, macrocycles can exhibit significant conformational flexibility, often making them low affinity or non-specific binders *in vivo*. NMR experiments, including ^{13}C spin-lattice relaxation time and temperature coefficient measurements, were used to show that introduction of a thioamide into a peptide macrocycle narrowed the breadth of the conformational ensemble, limiting the macrocycle to a single observable conformation. After initial investigation of model peptide macrocycles, the authors turned to bioactive RGD peptides. RGD peptides are known antagonists of pro-angiogenic integrins and represent attractive drug targets[161] for cancer and other indications. Cilengitide, an N-methylated cyclic peptide that reached Phase III clinical trials

against glioblastoma, was used as a reference for *in vitro* and *in cellulo* binding assays.[162] Some of the thioamide-containing RGD peptides bound more tightly to integrins than cilengitide, and computational docking of their solution NMR structures aligned well to a bound cilengitide molecule in an integrin receptor cocrystal structure (**Figure 15**).[163] These docking studies allowed them to identify the basis for increased affinity in the optimal thioamide RGD macrocycle as arising from stabilization of a certain ring conformation. Interestingly, *ex vivo* metabolic stability assays in human serum over 72 h revealed that all tested thioamide RGD macrocycles were more stable than cilengitide, independent of the position of the thioamide. Like the thioamide GLP-1 molecules, these RGD analogs show strong prospects as injectable therapeutics or vehicles for imaging probes.

Together, these studies show that thioamides are able to profoundly modulate the biological activity of peptides. Their unique combination of easy installation and amide mimicry with distinct physical properties allows targeted manipulation of various systems to tune protein interactions. These provide valuable tools for basic science or translational research and help to shed light on the roles of thioamides in natural peptides.

Thioprotein semisynthesis

SPPS is largely sufficient to synthesize thioamide analogs of peptide hormones or natural product derivatives. However, to investigate the role of thioamides in proteins like MCR or to use them as probes in full-sized proteins, other methods are necessary. To the best of our knowledge, the longest directly synthesized thioamide peptide/small protein was a thioleucine-modified version of the 56 amino acid containing B1 domain from protein G (GB1, UniProtKB-P06654).[136]

However, the isolated yield of this GB1 variant was very low (1%), showing the size limitations of SPPS for generating thioamide proteins. This problem has been rectified using native chemical ligation (NCL). NCL allows the ligation of two peptides or protein fragments together at low mM concentrations under neutral pH, aqueous conditions, resulting in a native amide bond.[164] The main requirement/limitation of this method is that proteins must be able to refold into their native structure after ligation in a denatured state. For this reaction to occur, the C-terminal fragment requires an N-terminal Cys or a β-, γ- or δ-thiol containing amino acid derivative, and the N-terminal fragment requires a C-terminal thioester. While the generation of C-terminal peptide fragments containing thioamides is straightforward, the generation of N-terminal fragments is complicated by the need to generate a C-terminal thioester. Thioamides have been shown to be compatible with various peptide activation methods to generate C-terminal thioesters, which have been reviewed elsewhere.[165] Many of these older methods suffer from low yields, slow reactions, or racemization. Currently, the fastest and most effective way of generating C-terminal thioesters in thioamide containing peptides makes use of acyl hydrazide methods, for which detailed procedures are available.[166] The acyl hydrazide modification is stable to most purification conditions and latent until activation using sodium nitrite at pH 4.0, which converts it to an acyl azide. Readjustment of the pH to 7.0 and addition of an aromatic thiol generates a thioester that can be immediately used in NCL reactions. Thioamide containing peptides have been shown to be compatible with this activation reaction with no observed side-reactions and have been used to generate proteins with thioamides near the N-terminus.[167]

Another concern in the field of NCL is that the requirement for a Cys residue at the ligation site forces one to ligate long fragment sequences or results in semisynthetic proteins with non-native

Cys residues included. There are two general approaches to circumventing this issue and both have been demonstrated to be compatible with thioamides (**Figure 16**). The first strategy involves masking the cysteine or analogue thereof, resulting in a mimic of another amino acid. For example, homocysteine can be used as ligation handle and treated with methyl iodide after ligation to convert it to methionine.[134] Alternatively, cysteine can be treated with alkyl halide reagents like iodoacetamide or 2-bromoethylamine which convert it to thioether mimics of (homo)glutamine or lysine**,** respectively. The second strategy, radical desulfurization, converts cysteine to alanine and converts β-, γ- or δ-thiol analogs of various amino acids to their respective canonical amino acids.[168] Desulfurization of thioamide containing peptides using classical Raney-Nickel procedures results in undesired backbone cleavage at the thioamide position.[167] The more recently developed VA-044, a water-soluble diazo compound that acts as a radical initiator, has been shown to be compatible with backbone thioamides, provided that thioacetamide is used as a scavenger to suppress an S-to-O exchange side-reaction.[167] With this combination of methods, thioamides can be incorporated in essentially any protein that is amenable to synthesis by NCL. At the mM concentrations typically used in NCL, reactions proceed in a few hours for ligations of short peptides and in 1-2 days for ligations with at least one large protein fragment (>60 residues). The limiting reagent in this process is the amount purified thioamide-containing peptide, which usually is obtained in a 20-50% yield relative to SPPS of the corresponding all-amide peptide.

Thioamide effects in full length proteins

With the possibility of incorporating thioamides as spectroscopic probes into full-length proteins[138], the question of their influence on protein stability has emerged. Due to their increased size and altered hydrogen bonding properties, they can stabilize or destabilize protein folds. While

several older studies had focused on thioamide effects on the stability of peptide models of α-helical and β-sheet secondary structure,[132 169] two recent publications have investigated their effects in full-sized proteins.[136 170] First, Raines and coworkers investigated the influence of thioamides on the thermal stability of collagen model peptides and identified thioamides as the first backbone modification that does not compromise the thermal stability of these peptides. A separate team of investigators conducted a more comprehensive investigation, examining thioamide substitutions in three different protein model systems each representing a different class of secondary structure.[136] Taken together, the two studies indicate that thioamides can have a slight stabilizing effect in α-helices and polyproline type II (PPII) helices, if the additional steric bulk of the thiocarbonyl is accommodated and the thioamide acts primarily as a hydrogen bond donor. However, in a sterically crowded environment, thioamides can be destabilizing. All tested (internal) β-sheet positions, as well as some positions in a PPII helix, had a significant negative impact on protein stability. Destabilizing effects in α-helices however, were comparatively mild, and some of the destabilizing effects in α-helices can be 'rescued' with the incorporation of a second thioamide.[142] While Raines and others have shown that thioamides can exert stabilizing effects in model peptide systems through increasing the strength of $n \rightarrow \pi^*$ interactions between i and i+1 carbonyls, these have not obviously contributed to the stabilizing effects observed to date in protein systems.[171,137,172] However, many of the observed effects cannot fully be rationalized using crystal structures of the native proteins. Determining high-resolution structures of the thioamide proteins themselves will benefit the field substantially. Combining structural information with biophysical studies should help to provide guidelines for thioamide placement beyond simple rules of avoiding steric clashes of the longer thiocarbionyl and taking advantage of stronger thioamide hydrogen bond donation.

Conclusions:

Thioamidation is an intriguing modification of the peptide unit that can have both subtle and profound effects, depending on the interactions of the thioamide site. Thioamides appear to be exceptionally rare PTMs, with only one protein example known (MCR) and a small number of peptide natural products. In these cases, the thioamide moiety has been shown to be important to biological activity ranging from copper acquisition by methanobactin to DNA gyrase inhibition by closthioamide, to imparting structural stability near the active site of MCR. Bioinformatic studies enabled by the identification of the YcaO//TfuA thioamide installation mechanisms in MCR and the thioviridamide family point to an unappreciated prevalence of thioamides in peptides and possibly in additional proteins. As the biosynthetic mechanisms of methanobactin and closthioamide are elucidated, similar genome-scanning approaches can be employed. We expect that the ability to anticipate the presence of thioamides based on bioinformatic approaches will lead to a rapid expansion of the number of identified thioamide-containing peptides and proteins, even in classes of previously-studied natural products, as illustrated by the identification of thioamide thiopeptides like saalfelduracin. Supportive evidence comes from a pre-print publication on the thiovarsolins from Truman and co-workers that appeared while this manuscript was under revision.[173] Beyond the discovery of additional thioamidated compounds, key areas for future investigation include determining the sulfur transfer mechanism during their biosynthesis and assigning biological function(s) for this rare but important PTM in peptides and proteins.

The ability to synthetically install thioamides provides opportunities to better understand natural thioamides and to apply them as tools in biophysical and medicinal chemistry. Chemical synthesis of intermediates can be used to study biosynthetic pathways, and analogs of the natural products

can be used to determine their mechanism of action. As new biological functions are uncovered for natural thioamides, these can inform the use of thioamides in a designed fashion, leading to applications beyond the current directions of improving the stability and activity of peptide drugs. Of course, the study of thioamide-containing peptides and proteins in biophysical and medicinal chemistry experiments also generates data providing insight into cryptic functions of natural products. The current genetic and synthetic tools constitute an excellent platform for further investigation of thioamide chemistry in living organisms and we hope that this review will inspire new interest in this under-studied peptide modification.

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Tables

Table 1. Physical properties of thioamides.

Property	**Amide**	**Thioamide**	**Reference**
vdW radius (Å)	1.40	1.85	[16]
C=X BDE (kcal mol^{-1})	170	130	[12]
C=X length (Å)	1.23	1.71	[15]
C-N rotational barrier (kcal mol^{-1})	17	22	[17]
Electronegativity of heteroatom	3.44	2.58	
C=X···H-N hydrogen bond (kcal mol^{-1})	6.1	4.8	[19]
N-H pK_a	17	12	[18]
$\pi \rightarrow \pi^*$ absorption (nm)	200	270	[21]
E_{Ox} (V vs. SHE)	3.29	1.21	[23]

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

Figures and legends:

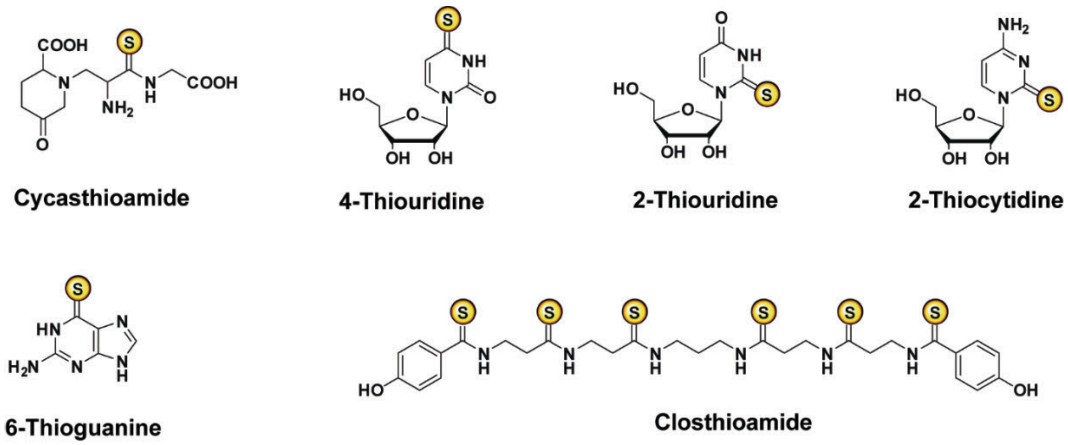


Figure 1: Structures of non-ribosomal natural products bearing a thiocarbonyl group.

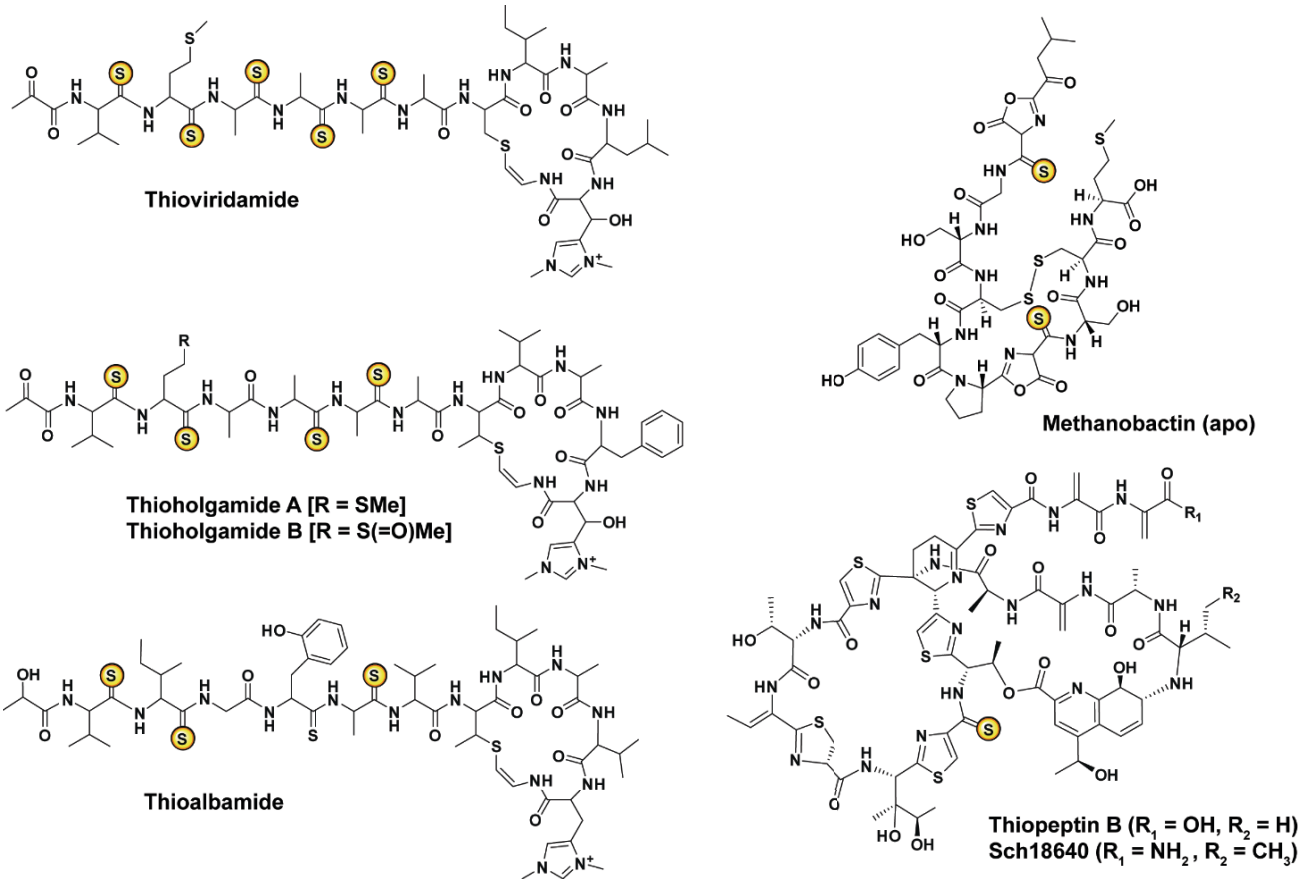


Figure 2: Structures of thioamidated ribosomal natural products.

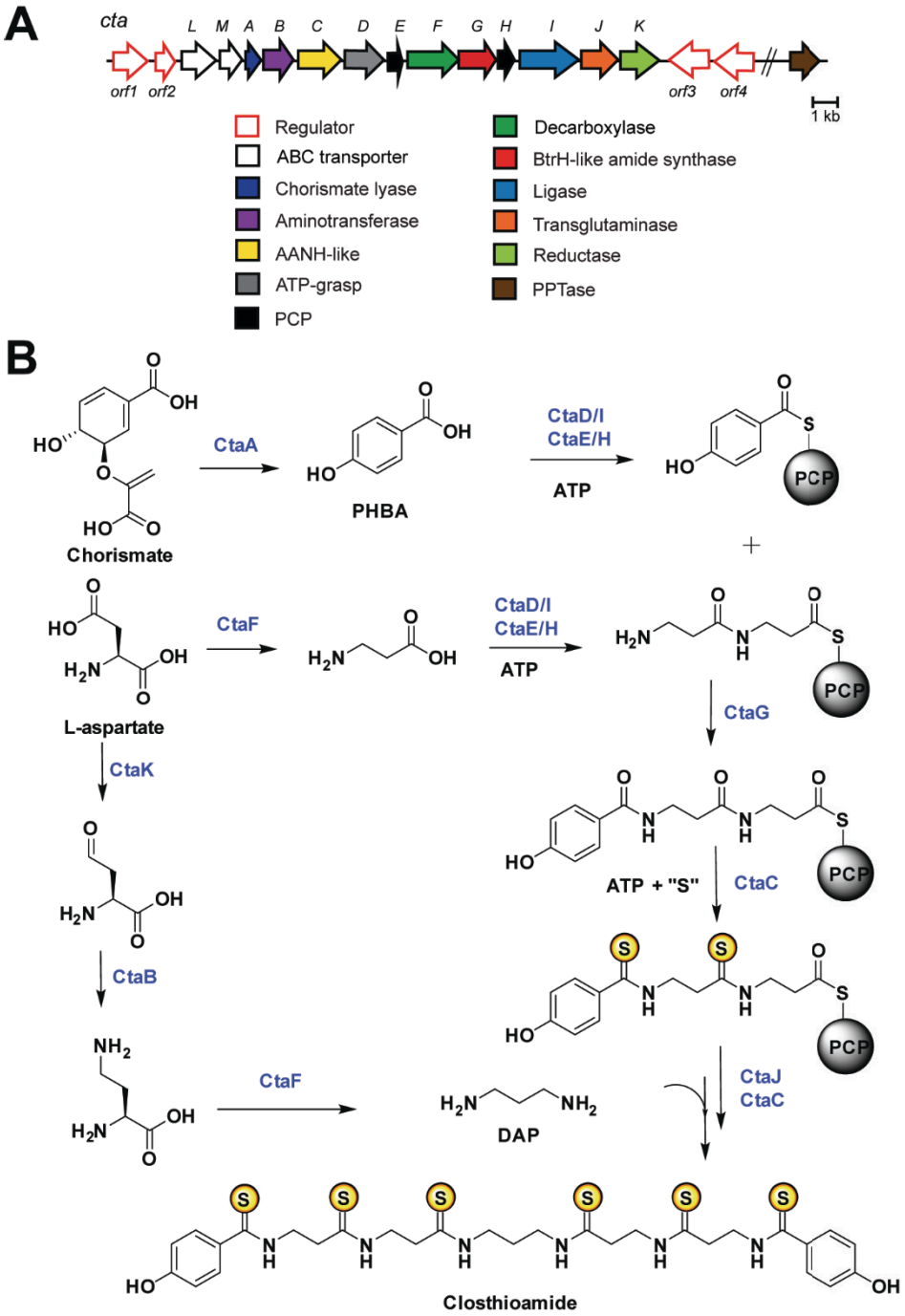


Figure 3: (A) Biosynthetic gene cluster of closthioamide from *Ruminiclostridium cellulolyticum* DSM 5812. (B) Proposed NRPS biosynthetic pathway for closthioamide that contains six thioamide groups (PCP: peptidyl carrier protein, PHBA: p-hydroxybenzoic acid, DAP: diaminopropane, AANH: alpha-adenine nucleotide hydrolase).[34]

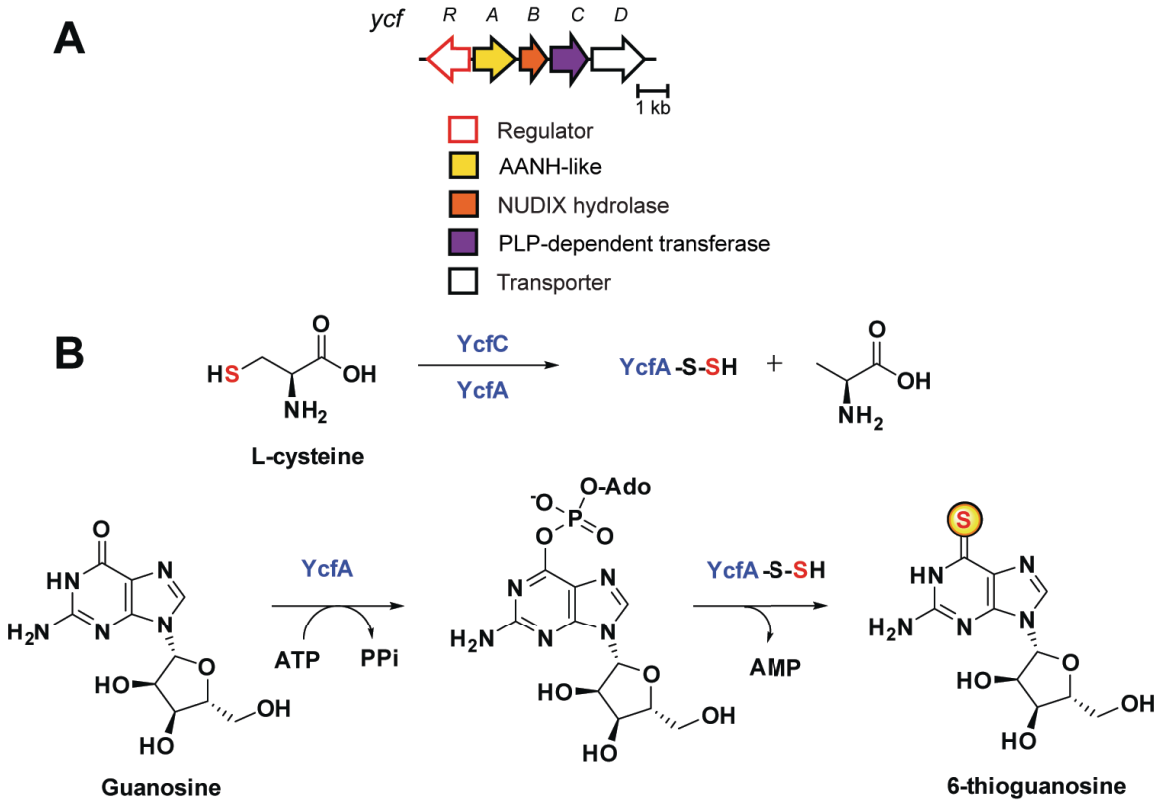


Figure 4: (A) Biosynthetic gene cluster of 6-thioguanine (*ycf*) from *Erwinia amylovora*. (B) Proposed mechanistic model for sulfur mobilization and delivery during the thioamide formation mediated by YcfA/YcfC bipartite enzyme system (AANH: alpha-adenine nucleotide hydrolase; PLP: pyridoxal phosphate).[57]

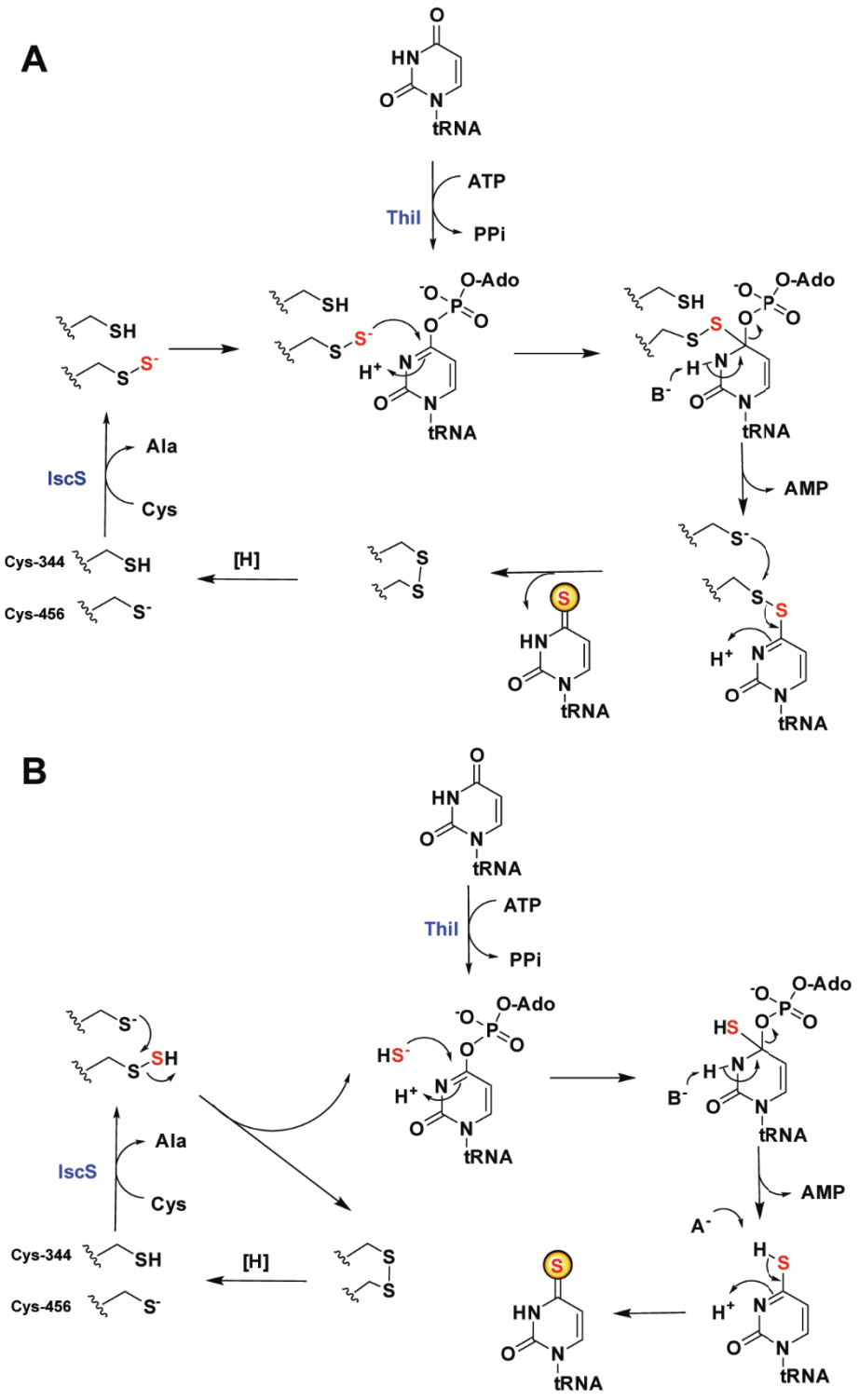


Figure 5: Proposed mechanisms for 4-thiouridine (s^4U) formation in *Escherichia coli*. (A) The persulfide group on Cys456 of ThiI acts as a nucleophile to attack the activated uridine. (B) The persulfide group on Cys456 is used as a source of bisulfide (HS^-), which nucleophilically attacks the activated uridine residue (Ado: adenosine).[30] In both the cases, the Cys456 persulfide forms at the expense of the active site persulfide group on IscS (not shown).

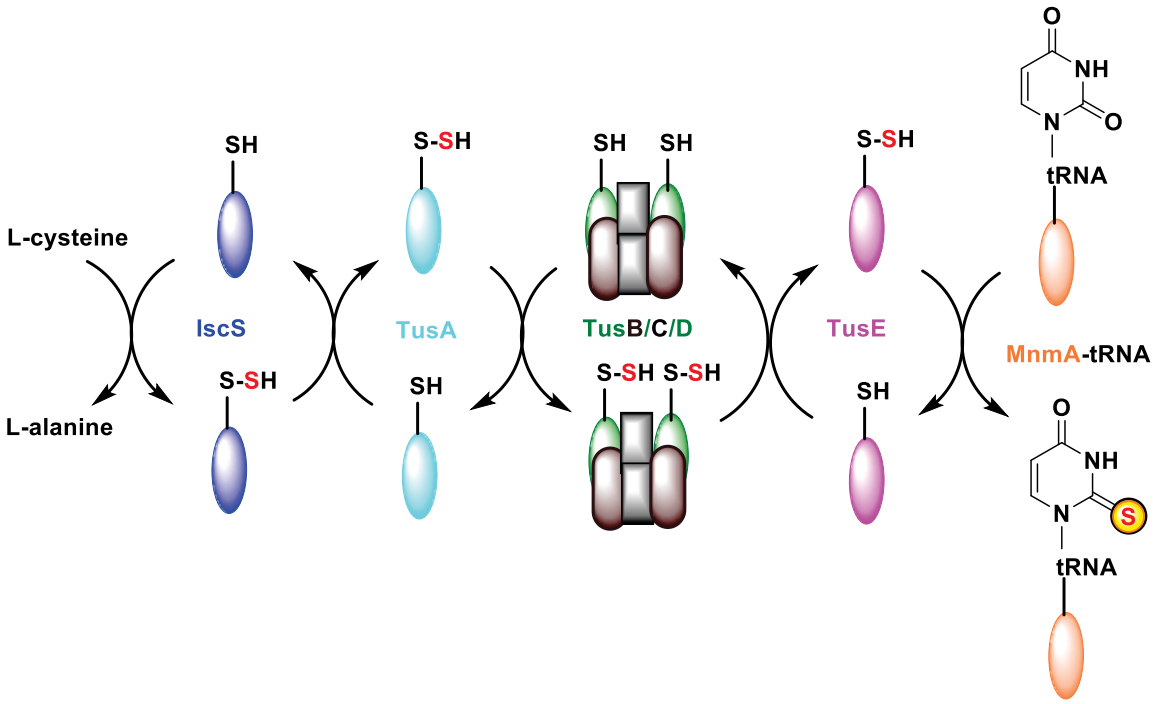


Figure 6: Sulfur relay mechanism mediated by Tus proteins during 2-thiouridine (s^2U) formation in *Escherichia coli*. Sulfur from cysteine is activated by cysteine desulfurase IscS to form the persulfide group on a conserved Cys residue on IscS. Reactive bisulfide group (HS^-) is then transferred to MnmA via TusA, TusB/C/D complex and TusE. MnmA that forms complex with tRNA finally transfers it to the activated uridine at position 2, to finally form s^2U.[30]

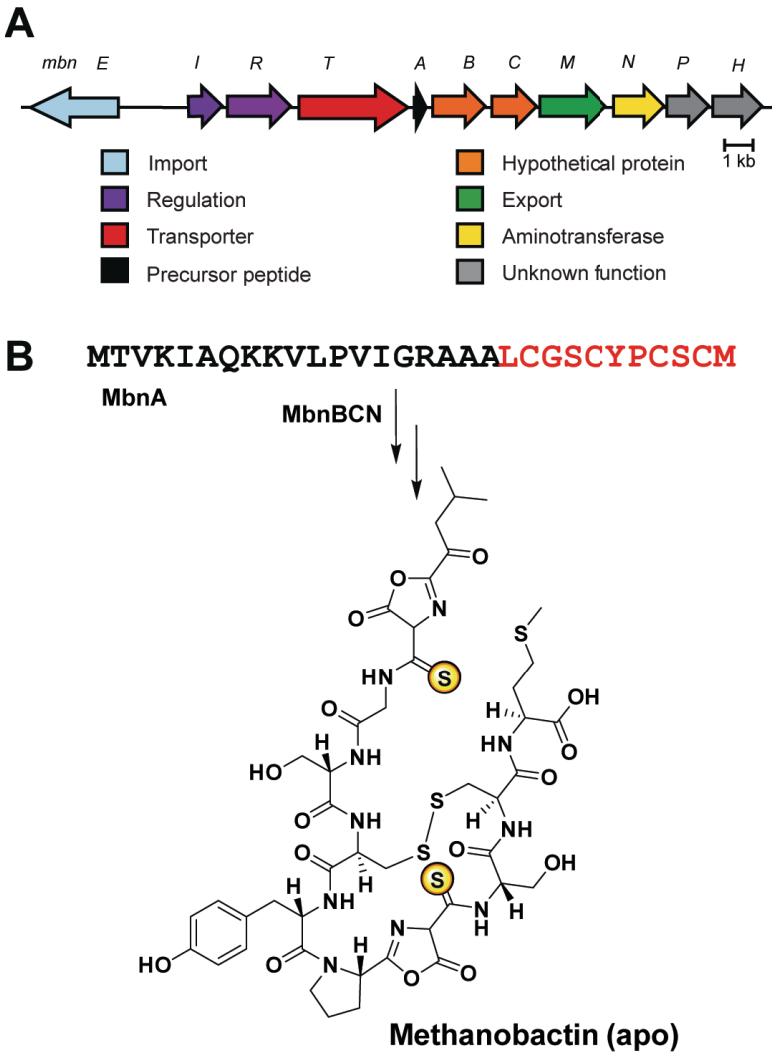


Figure 7: (A) Methanobactin biosynthetic gene cluster (*mbn*) from *Methylosinus trichosporium* OB3b. (B) Core region (shown in red) of the precursor peptide MbnA is converted to mature methanobactin that contain two oxazolone- thioamide groups by the downstream biosynthetic enzymes MbnB, MbnC, and MbnN.[35]

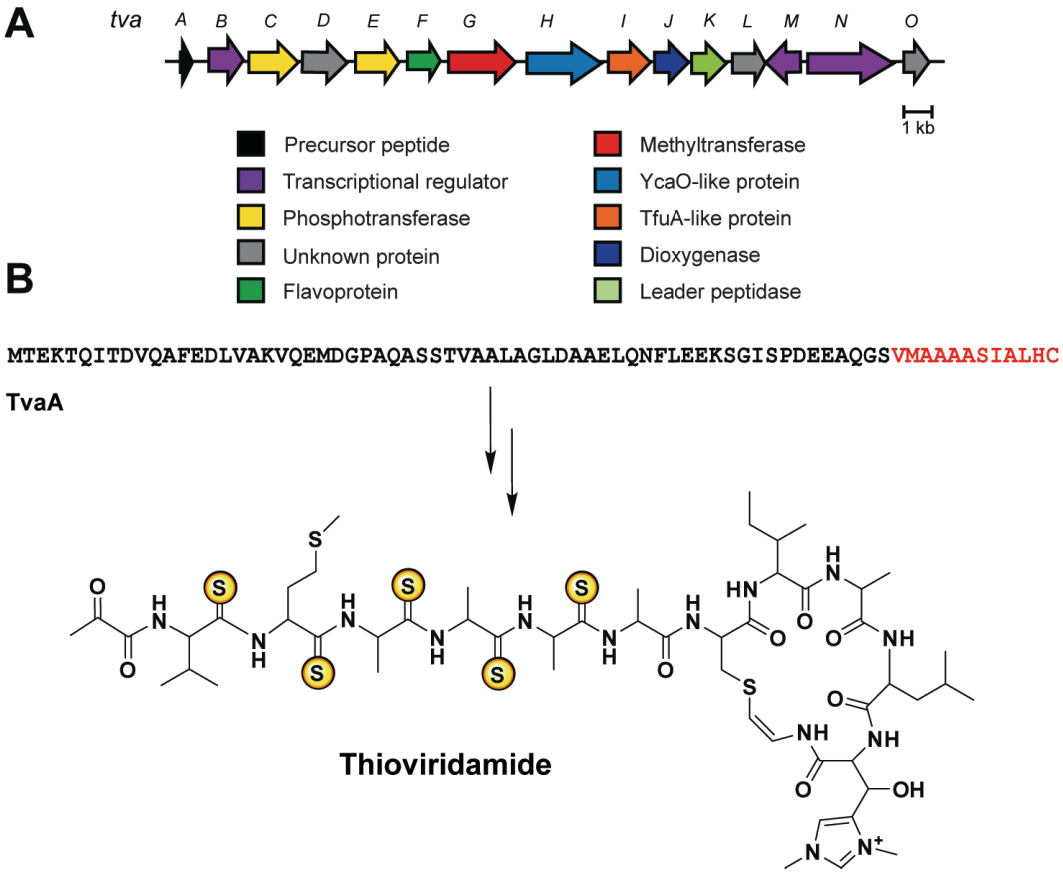


Figure 8: (A) Thioviridamide biosynthetic gene cluster (*tva*) from *Streptomyces olivoviridis* NA05001. (B) Core region (shown in red) of the precursor peptide TvaA is converted to thioviridamide that contain five thioamide groups by the downstream biosynthetic enzymes (adapted from Burkhart, B.J. et al, *Chem. Rev.* **2017**, 117, 5389).[102,103] The stereochemistry for thioviridamide has not yet been elucidated.

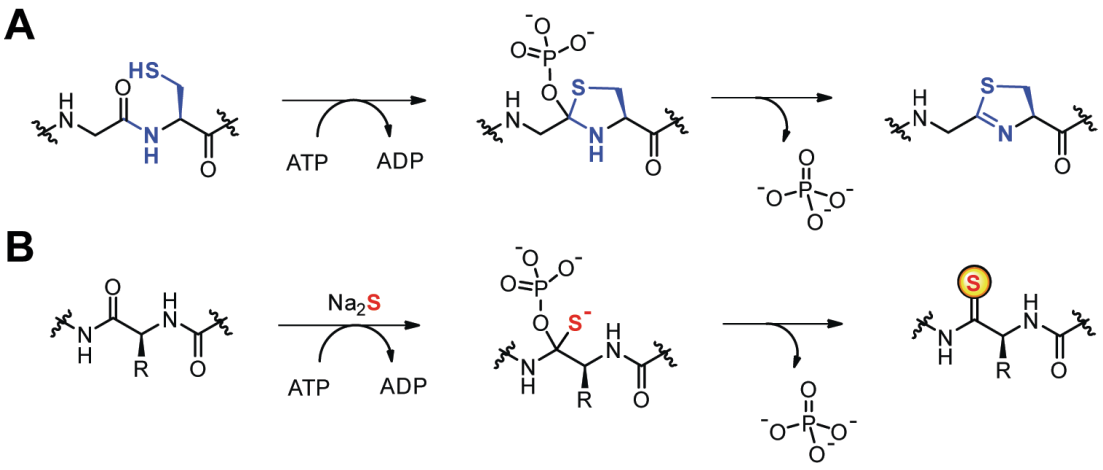


Figure 9: Comparison of reactions catalyzed by YcaO enzymes. (A) Biochemically characterized YcaO proteins involved in the biosynthesis of azol(in)e-containing ribosomal natural products catalyze the ATP-dependent cyclodehydration of cysteine, serine, and threonine.[103] Shown is the transformation of peptidic cysteine to thiazoline which proceeds via an *O*-phosphorylated hemiorthoamide intermediate. (B) An analogous reaction is proposed for the biosynthesis of thioamides on peptidic backbones (in thioviridamide and MCR), with an exogenous source of sulfide (Na_2S) acting as the nucleophile in place of the adjacent cysteine.

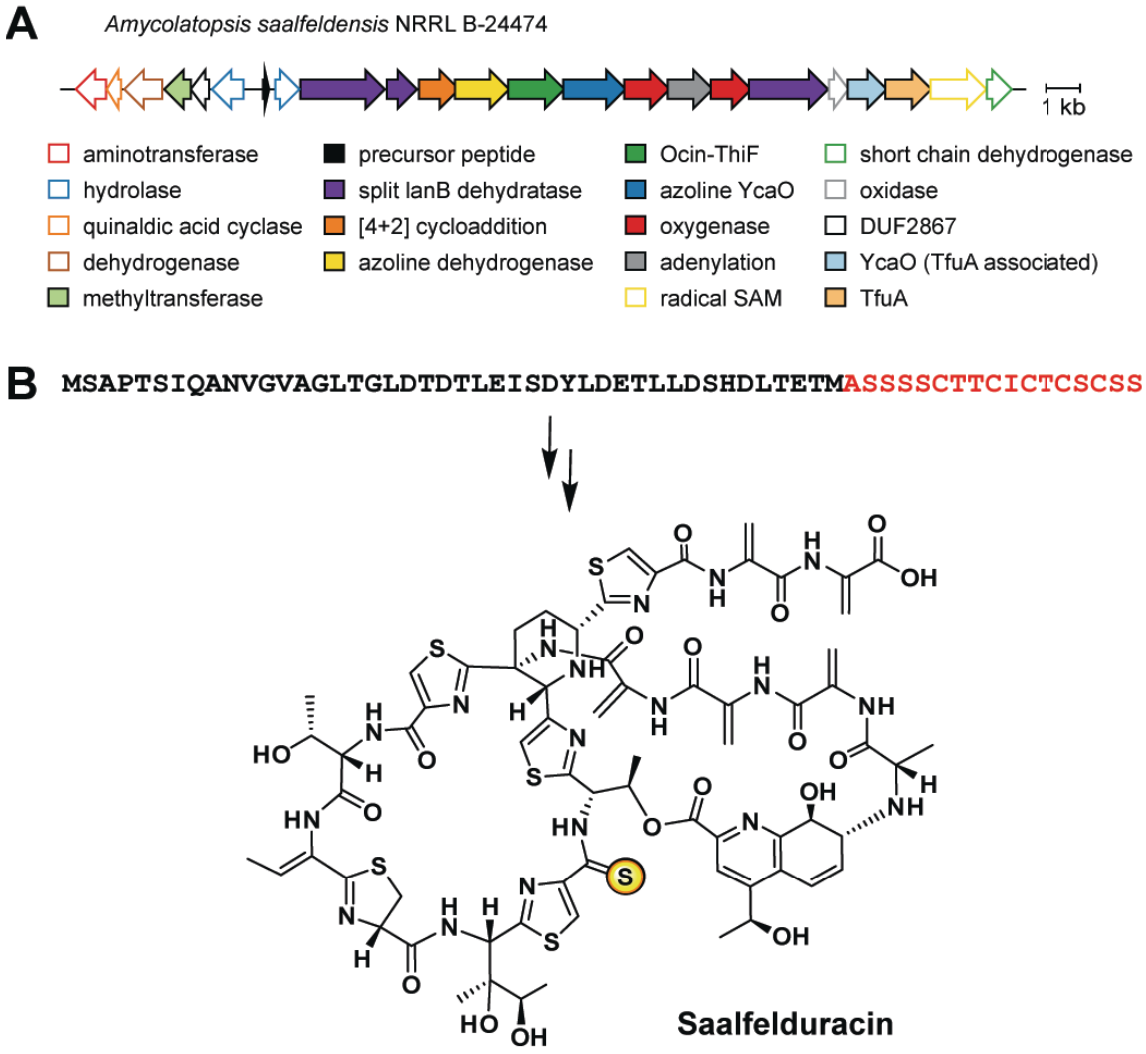


Figure 10: Thioamidated thiopeptide Saalfelduracin (A) Saalfelduracin biosynthetic gene cluster from *Amycolatopsis saalfeldensis* NRRL B-24474. (B) Predicted precursor peptide of saalfelduracin (with the predicted core peptide in red) and the proposed structure (adapted from Schwalen, C.J. et al, *J. Am. Chem. Soc.*, **2018**, 140, 9494).[29]

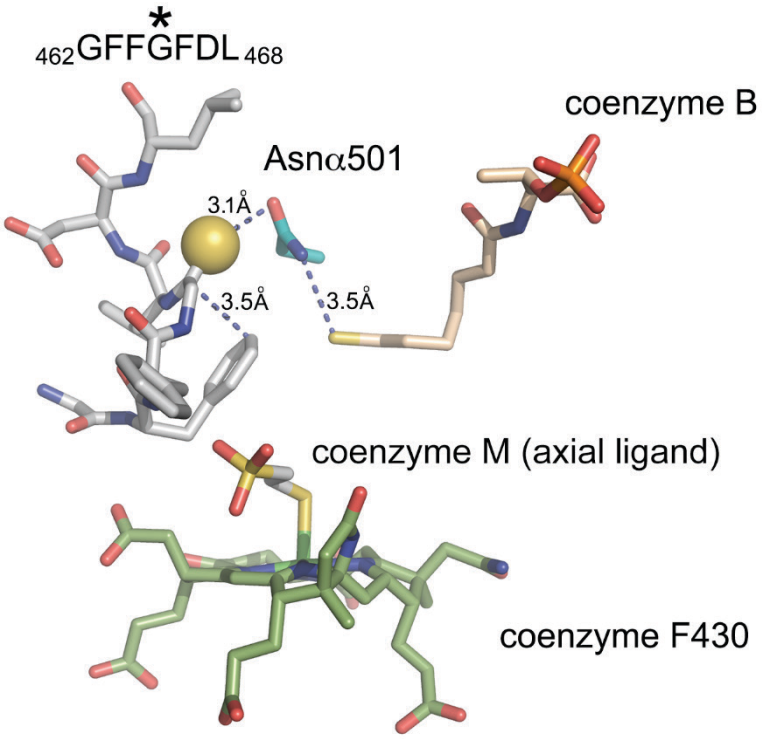


Figure 11: A view of the MCR active site with the thioglycine involved in several stabilizing interactions using the crystal structure of *Methanosarcina barkeri* (PDB code: 1E6Y),[174] (adapted from Nayak, D.D. et al, *eLife*, **2017**, 6, e29218).[32]

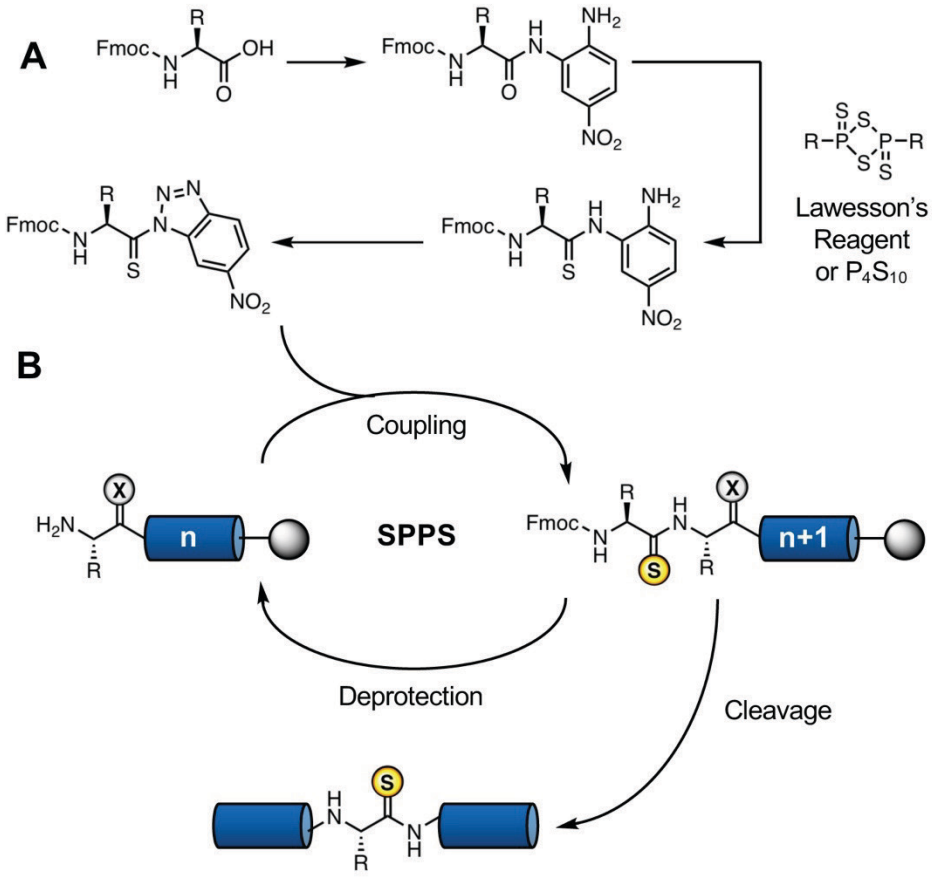


Figure 12. Site-specific incorporation of thioamides using solid phase peptide synthesis (SPPS). (A) Thioacylbenzotriazole monomers can be synthesized in three steps from Fmoc-protected amino acids using Lawesson's Reagent or P_4S_{10} to thionate. (B) The thioacylbenzotriazoles can be used to introduce the thioamide during SPPS with minor modifications of standard coupling, deprotection, and cleavage protocols.

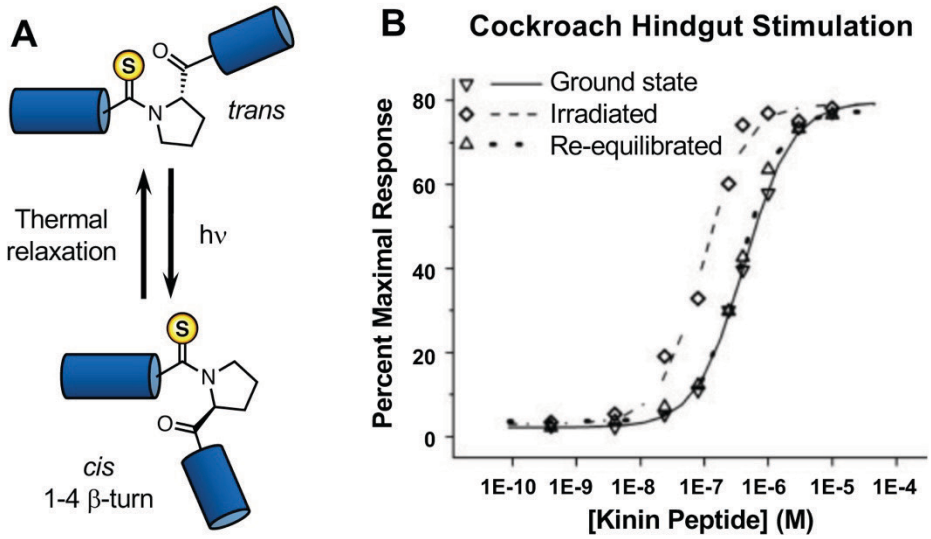


Figure 13. Controlling bioactivity though thioamide photoisomerization. (A) Insect kinin-derived thioamide pentapeptide can undergo UV-induced photoisomerization. When exposed to cockroach hindgut extract in an *ex vivo* myotropic contraction assay, the irradiated *cis* peptide (adopting a 1-4 β-turn) shows higher activity than the *trans* peptide. (B) Titration of isolated *cis* or *trans* peptide to determine EC_{50} values. The *cis* peptide shows a 4-fold lower EC_{50} (adapted from Huang, Y. et al, *J. Peptide Sci*., **2008**, 14, 262).[149]

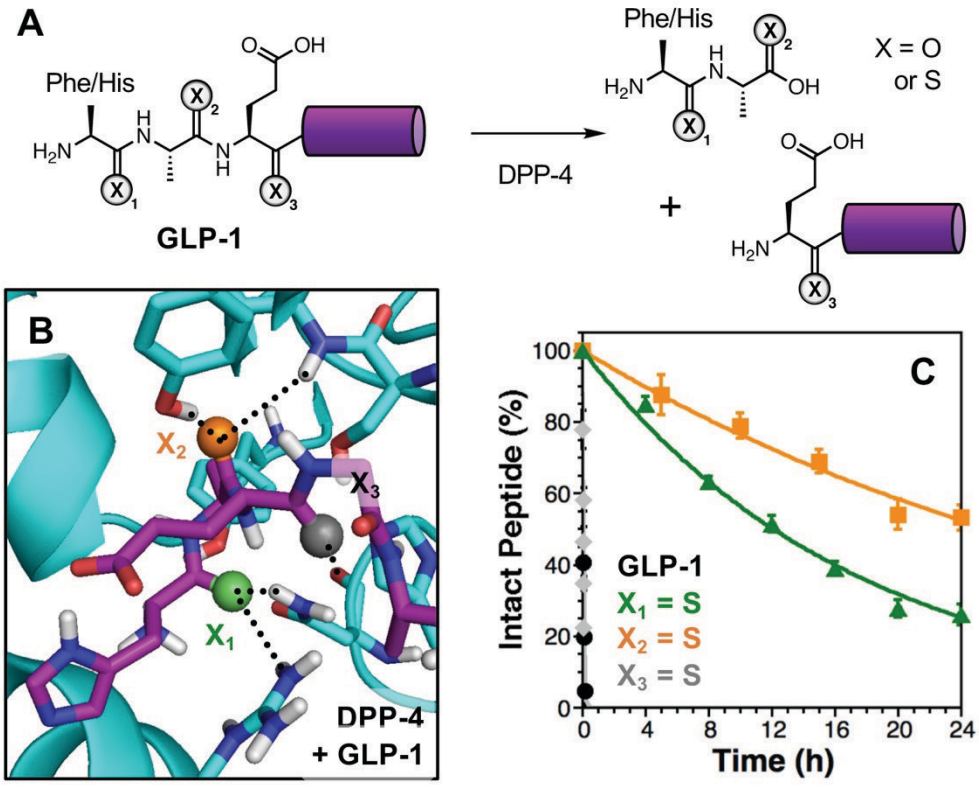
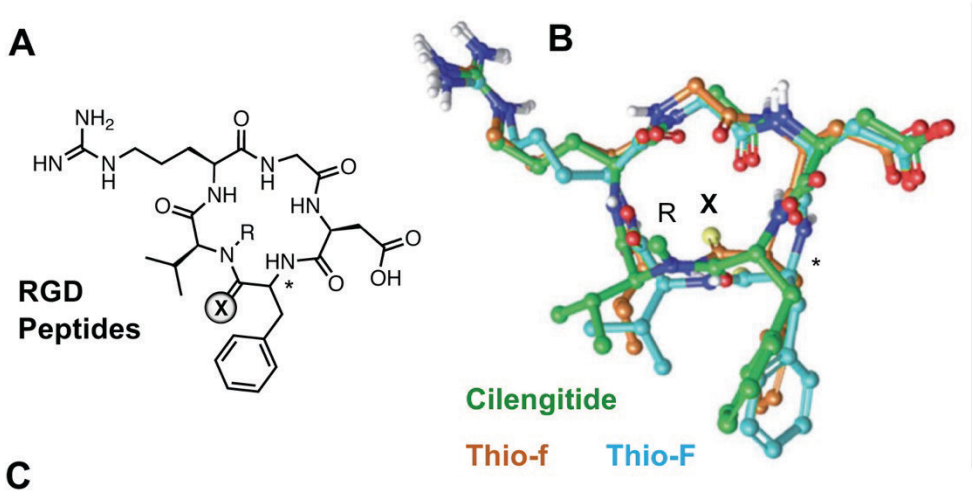


Figure 14. Glucagon-like peptide 1 (GLP-1) is stabilized by thioamide incorporation. (A) Incorporation of a thioamide into GLP-1 at the X_1 or X_2 positions prevents dipeptidyl peptidase 4 (DPP-4) degradation of GLP-1. (B) An image of the DPP-4 (cyan) active site with a GLP-1 N-terminal fragment (purple) bound, modeled based on the neuropeptide Y bound DPP-4 structure (PDB code: 1R9N).[157] The X_1, X_2, and X_3 carbonyl oxygens are highlighted as green, orange, and grey spheres, respectively. Key interactions with DPP-4 are shown as dashed lines. (C) *In vitro* degradation assay shows that incorporation of a thioamide at X_1 and X_2, but not X_3, can effectively prevent proteolysis by DPP-4 (adapted from Chen, X. S. et al, *J. Am. Chem. Soc.*, **2017**, 139, 16688).[4]



C

RGD Peptide	$t_{1/2}$	Breast Cancer	Glioblastoma
Cilengitide X = O, R = Me, *D-Phe	12 h	1.2 nM	3.0 nM
Oxo-f X = O, R = H, *D-Phe	8 h	$>10^5$ nM	$>10^5$ nM
Thio-f X = S, R = H, *D-Phe	9 h	4 .0 nM	326 nM
Oxo-F X = O, R = H, *L-Phe	7 h	$>10^5$ nM	$>10^5$ nM
Thio-F X = S, R = H, *L-Phe	36 h	1.0 nM	1.6 nM

Figure 15. Thioamide incorporation increases activity of integrin agonist RGD peptides. (A) Chemical structures of RGD peptide macrocycles, including therapeutic candidate cilengitide and two matched amide/thioamide pairs. (B) NMR structures of the Thio-f (orange) and Thio-F (cyan) peptides are shown overlaid on the structure of cilengitide bound to αvβ3 integrin (PDB code: 1L5G).[163] (C) Summary of peptide serum stability ($t_{1/2}$) and activity (IC_{50} for binding to breast cancer cells and for glioblastoma cells). Macrocycles Thio-f and Thio-F show higher stability and activity than the corresponding amide peptides. Thio-F is superior to cilengitide in all assays. Figures reproduced with permission from Verma *et al*., *Chem. Sci*. 9, 2443 (2018).[5]

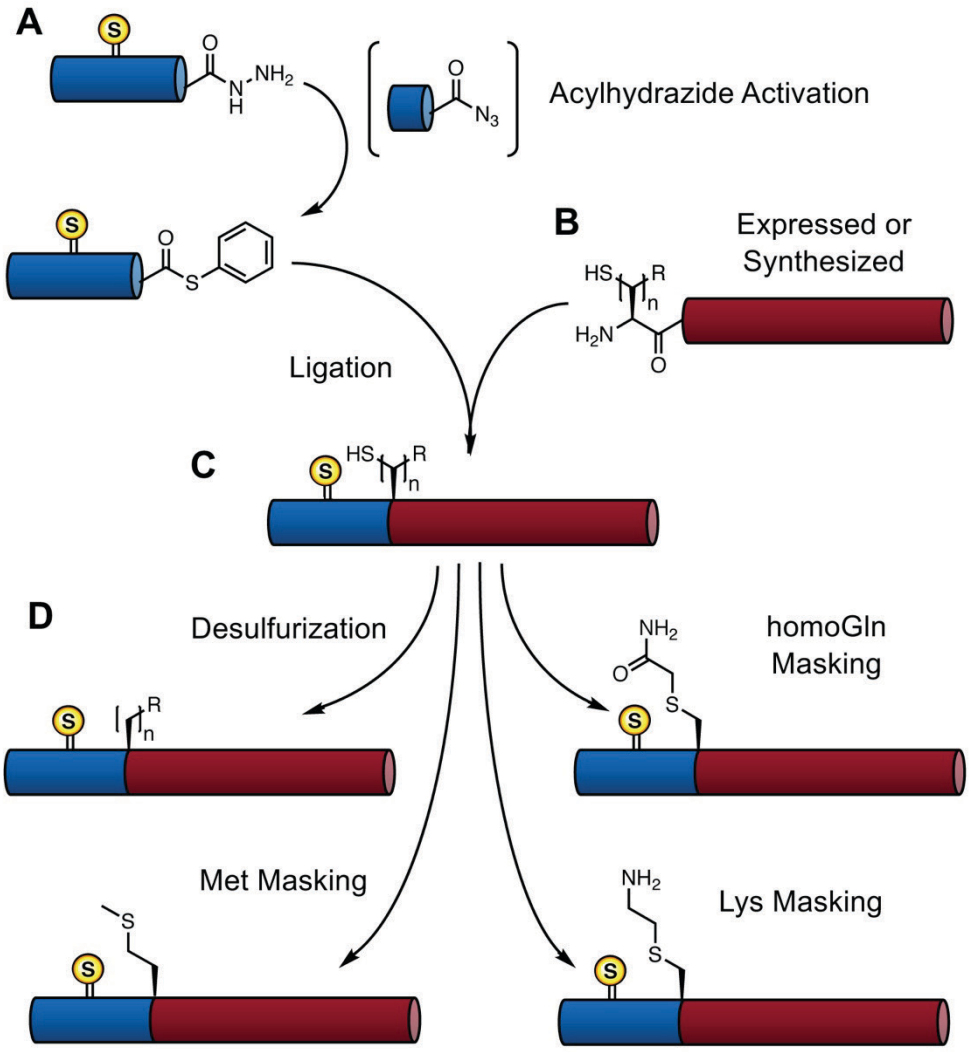


Figure 16. Traceless ligation to synthesize thioamide-containing proteins. (A) Thioamide-containing thioester peptides (blue) are synthesized from acylhydrazide precursors by oxidation to form an acylazide which is displaced with thiophenol. (B) N-terminal cysteine fragments (red) are synthesized or recombinantly expressed. Synthetic peptides can contain β-, γ- or δ-thiol cysteine analogs (n = 1, 2, or 3) which can be converted to native amino acids after ligation. (C) Ligation can be performed without special precautions for the thioamide. (D) Masking of the ligation site can occur through: Desulfurization – thiol-selective radical desulfurization using sacrificial thioacetamide, Met Masking – methylation of homocysteine, homoGln Masking and Lys masking – alkylation of cysteine with iodoacetamide or bromoethylamine, respectively.