Contemporary Applications of Thioamides and Methods for their Synthesis

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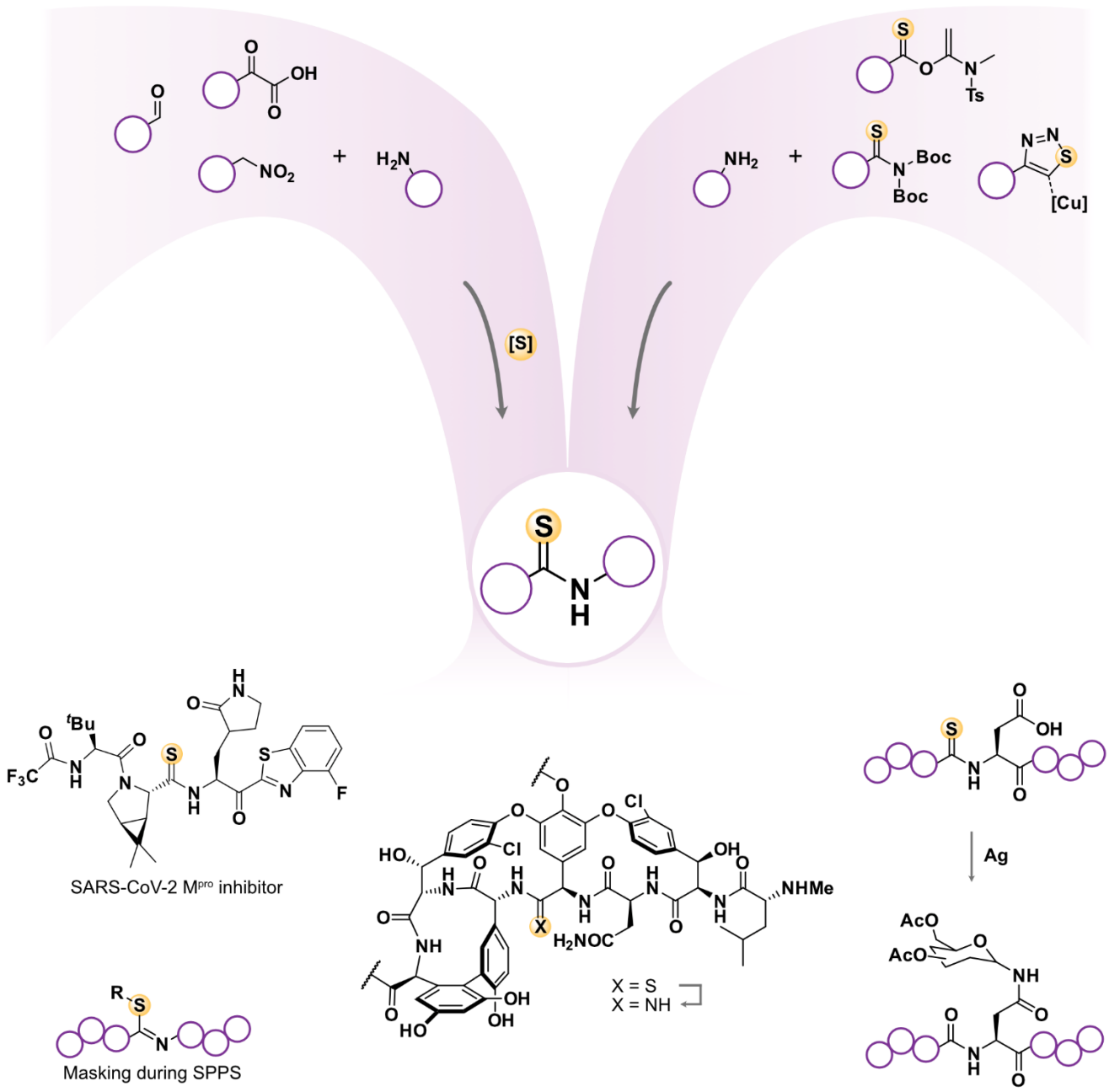

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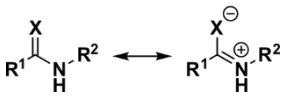
Abstract: Thioamides are naturally occurring isosteres of amide bonds in which the chalcogen atom of the carbonyl is changed from oxygen to sulfur. This substitution gives rise to altered nucleophilicity and hydrogen bonding properties with importance for both chemical reactivity and non-covalent interactions. As such, thioamides have been introduced into biologically active compounds to achieve improved target affinity and/or stability towards hydrolytic enzymes but have also been applied as probes of protein and peptide folding and dynamics. Recently, a series of new methods have been developed for the synthesis of thioamides as well as their utilization in peptide chemistry. Further, novel strategies for the incorporation of thioamides into proteins have been developed, enabling both structural and functional studies to be performed. In this Review, we highlight the recent developments in the preparation of thioamides and their applications for peptide modification and study of protein function.

1. Introduction

Thioamides are naturally occurring isosteres of oxoamides in peptide and protein backbones and their properties have been explored extensively in organic and medicinal chemistry, leading to FDA approved drugs contain thiocarbonyls such as propylthiouracil and thiamazole used for the treatment of hyperthyroidism in various diseases,[1] and the antibiotics etionamide and protionamide used for the treatment of tuberculosis.[2] At the same time, thioamides have been used as key intermediates for the synthesis of various heterocycles and for late-stage functionalization of peptides. Thioamides are also found in natural products such as the thionated nucleosides 2-thiouridine and 4-thiouridine,[3] cycasthioamide[4] the polythioamide closthioamide, isolated from the anaerobic bacterium *Clostridium cellulolyctium*,[5] and peptides such as thioviridamide[6] (Scheme 1). On the other hand, methyl-coenzyme M reductase (MCR)[7] and *E. coli* 70S ribosome[8] are the only proteins that have been shown to contain backbone thioamide bonds thus far.

The thioamide is a close isostere of the oxoamide, having both hydrogen bond acceptor and donor properties; albeit, with different strengths (Table 1). Because of the weaker bond of the carbonyl in thioamides (C=S bond length is ~1.65 Å compared to C=O with a bond length of ~1.23 Å), primarily due to the larger van der Waals radius of sulfur, there is an increased delocalization of the nitrogen lone pair into the carbonyl antibonding orbital. This, in turn, leads to a slightly shorter C–N bond (1.35 Å vs 1.37 Å)[9] (Table 1) and an increased rotational barrier around the C-N bond.[10] As a consequence, thioamides are considered stronger hydrogen bond donors but weaker hydrogen bond acceptors compared to their corresponding oxoamides (Table 1), partly due to reduced polarity and solvation by water.[11] The effect of substituting an oxoamide for a thioamide within an α-helical peptide or protein, causes the hydrogen bond distance to increase from 2.1 Å to 2.7 Å, which distorts the torsional angles of the backbone and increases the pitch per turn.[12]

Table 1. Physicochemical properties of oxoamides and thioamides.



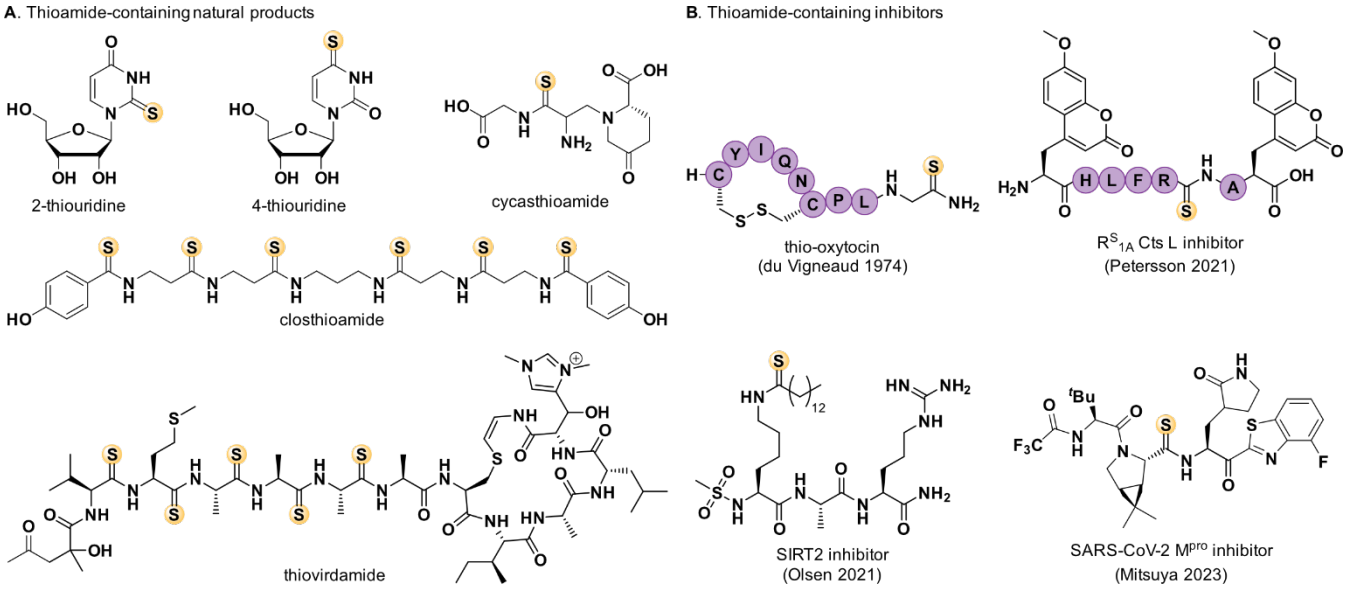
	Amide (X = O)	Thioamide (X = S)
C=X length (Å)[13]	1.23	1.65
C-N length (Å)[13]	1.37	1.35
C=X···H-N-C=O hydrogen bond (kcal/mol^{-1})[9]	6.1	4.8
C=O···H-N-C=X hydrogen bond (kcal mol^{-1})[9]	6.1	7.3

du Vineaud and co-workers were the first to synthesize a thioamide-containing peptide when they prepared oxytocin with a C-terminal thioamide (Scheme 1).[14] This single atom substitution caused a reduction of activity to only 6% of the parent peptide, highlighting the substantial effect an O→S substitution can have on biological activity. Nevertheless, thioamides have received significant attention in peptide research because they can decrease the proteolytic susceptibility of peptides,[15] and stabilize cyclic conformations of peptides.[16]

For example, Petersson and coworkers have shown that introduction of a single thioamide at a proteolysis hot spot increased the stability of the GLP1 hormone 750-fold towards dipeptidyl peptidase.[15c] More recently, Gellman and coworkers have increased the half-life of short T-cell activating peptides by incorporating up to two thioamide bonds.[15d] Furthermore Petersson and coworkers have also studied the fluorescence quenching properties of thioamides and its usage to investigate proteases.[15b, 17]

We, among others, have utilized thioamides to generate potent, tight-binding inhibitors of members of a sub class of histone deacylase (HDAC) enzymes called sirtuins (SIRTs).[18] Modification of the ε-*N*-acyllysine functionality, found in native substrates, to ε-*N*-thioacyllysine results in the formation of long lived covalent adducts in the active sites of the enzymes ($t_{½}$ >50 days),[18d] rendering these substrate analogs potent enzyme inhibitors (Scheme 1).

In addition to natural products and biologically active compounds, thioamides have also gained interest in other fields of chemistry. For example, the thiocarbonyl of thioformamide has

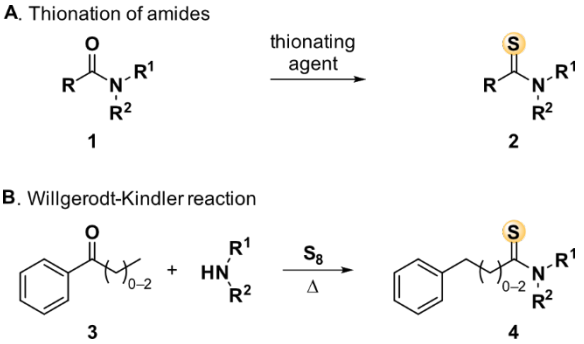


Scheme 1. Selected compounds containing the thioamide motif. A) Natural products containing thioamides, B) Selected examples of synthetic thioamide-containing compounds with biological activity.

been used in radical copolymerization to form vinyl polymers containing thioethers in their backbone. These thioethers allow for degradation of the polymer backbone and could potentially lead to novel degradable hybrid polymer materials.[19] Thioamides have also received attention in the field of prebiotic chemistry, because they can be formed by treating nitriles with either a hydrogensulfide atmosphere[20] or sodium hydrosulfide (NaSH) in formamide.[21] When the formed primary thioamides are then hydrolyzed to the monothiol carboxylate, which in turn can undergo peptide ligation with a ferricyanide catalyst.[20] Similarly, thioimidates formed by reacting nitriles with a thiol, such as in cysteine, have been shown to enable the same type of peptide ligation.[22]

Because of the unique reactivity and availability of thioamides under presumed prebiotic conditions, they have also been investigated in the field of organocatalysis.[23]

In this Review, we detail the most recent advances for the synthesis of peptides and small molecules containing thioamides and highlight recent examples of the broad applications of this functionality.



Scheme 2. Traditional syntheses of thioamides; A) using a thionating agent or B) by the Willgerodt-Kindler reaction.

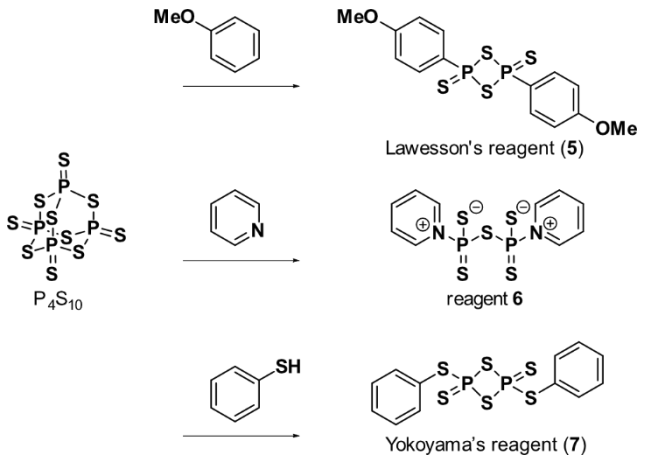
2. Synthesis of thioamides

Generally, thioamides have been incorporated into organic molecules using reagents that can thionate carbonyls to give the corresponding thiocarbonyl species, often relying on P_4S_{10} as the sulfur source, [24] or alternatively through the Willgerodt-Kindler reaction[25] (Scheme 2). The latter reaction typically involves an arylalkyl ketone, which is reacted with elemental sulfur and an amine to form an alkyl thioamide.[25b, 26] The substrate scope has been expanded substantially to include aromatic and aliphatic aldehydes, acetals, alkenes, thiols, imines, carboxylic acids, and benzyl halides.[27] However, use of the Willgerodt-Kindler reaction can be challenging due to low chemoselectivity and therefore is limited to simpler substrates. The same can be argued for thionating reagents and various methods have therefore been developed to incorporate activated thiocarbonyl-containing species into complex molecules including peptides.

2.1. Thionation of carbonyls

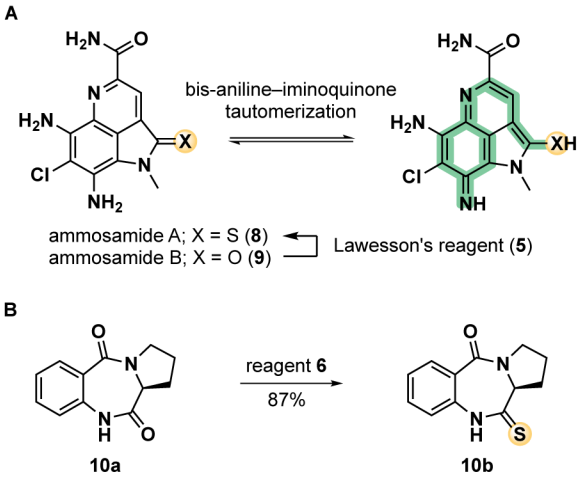
The P_4S_{10} and Lawesson's reagents have been most extensively used for the thionation of carbonyl compounds. However, often tedious separation of the desired product, low solubility of the reagent in commonly used solvents (such as THF), and decomposition at the elevated temperatures often required for thionation, have been limiting factors in the use of Lawesson's reagent.[28] Also, nitrile formation has been reported for the thionation of non-substituted amides. Some of these issues have been addressed by analogs with improved solubility[29] and alternative reagents such as the P_2S_5-pyridine complex **1**[30] and Yokoyama's reagent **7**[31] (Scheme 3). The general reactivity of oxo groups towards Lawesson's reagent was proposed by Nishio and Ori: hydroxy > amide > ketone > ester;[32] though, with steric hindrance as an additional factor to consider.[33] Thus, amides are generally converted to the corresponding thioamide at elevated temperature in THF, while ketones usually require refluxing

toluene, and esters even prolonged refluxing in toluene or xylene.[33] Therefore, alternative methods have been developed for the synthesis of thioesters, either by using P_4S_{10} and hexamethyldisiloxane[34] or by using microwave assisted heating.[35] Both carbamates and carboxylic acids are considered more inert towards Lawesson's reagent and thiocarbamates are often synthesized from isothiocyanates. Selective formation of thioamides in the presence of ester and carbamate functionalities have therefore been achieved.[24b, 33, 36]



Scheme 3. Preparation of thionating reagents: Lawesson's reagent, reagent **6**, and Yokoyama's reagent from P_4S_{10}.

With the presence of multiple amide bonds, however, an early study illustrated by Jensen and Senning demonstrateted the challenges of achieved selectivity, by using model tripeptides.[37] For cyclic peptides, chemoselectivity has been reported to be higher due to amides participating in intramolecular hydrogen bonding. Using Yokoyama's reagent **7**,[31] Kessler and co-workers observed predominant thionation at a solvent exposed Phe-Phe amide in favor of a less hindered Gly-Pro β-turn motif in a cyclic heaxamer peptide.[16a] Similar findings have been reported for several naturally occurring cyclic peptides, including segetalin A and B[38], astin A, B, and C[39], cyclosporine A,[40] analogs of cyclosporine A,[41] and RA-VII.[42]

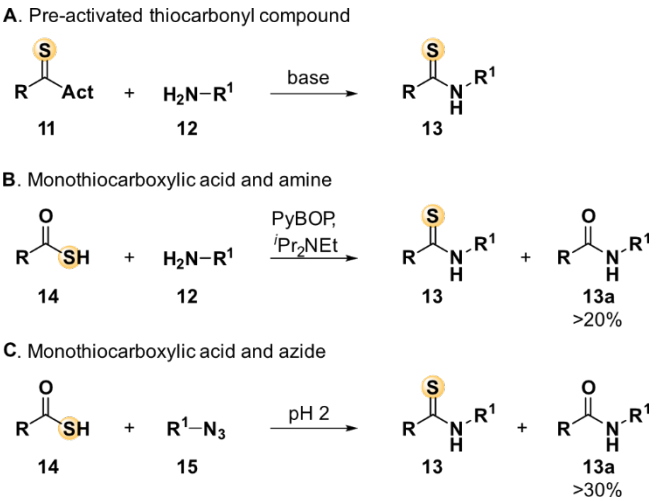


Scheme 4. Thionation of small molecules containing multiple amide bonds. A) Ammosamide B can be converted into ammosamide A, using Lawesson's reagent.[43] The iminoquinone tautomer is highlighted by green shading, B) Conversion of compound **10a** to **10b**, using reagent **6**.[30b]

For small molecules, selective thionation is rarely observed when more than one amide bond is present. The 5-membered lactam of ammosamide B have been reported to be transformed into its corresponding thiolactam ammosamide A using Lawesson's reagent; albeit, in "low yield" according to the authors (Scheme 4A).[43] The preference for this carbonyl moiety over the less hindered non-substituted amide is presumably due to its possible tautomerization to the corresponding bis-iminoquinone, revealing a free hydroxyl group which reacts more readily with the reagent than a carbonyl. Likewise, Bergman and co-workers reported selective monothionation of lactams, including the selective formation of **10b** from **10a**, presumably due to the lower nucleophilicity of the benzamide carbonyl.[30b]

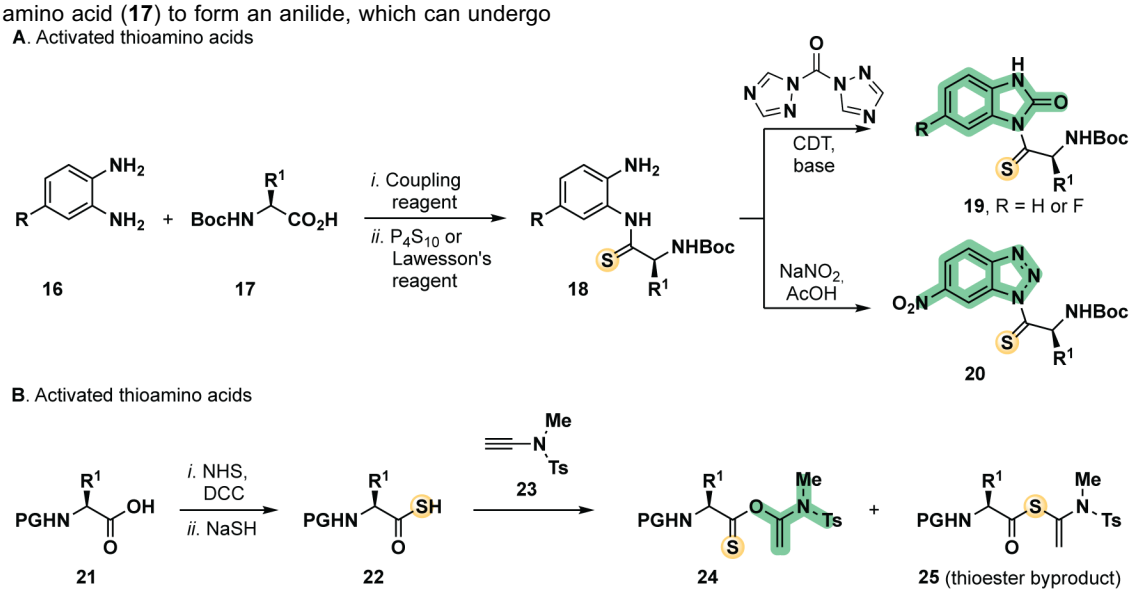
2.2. Thioacylation of amines

Although the seminal work highlighted above using thionating reagents showcase chemoselective principles, the general use of thionating reagents on complex molecules with multiple amide bonds lead to complex reaction mixtures. Therefore, strategies have been developed for introducing thioamide bonds by thioacylation of amines. This has generally been achieved by preparing an activated thiocarbonyl-containing building block, such as **11**, and reacting this with a free amine (Scheme 5A). However, thioacylation has also been attempted by direct coupling of monothiocarboxylic acids (**14**)[44] with amines using phosphorous coupling reagents such as PyBOP (Scheme 5B)[45] or with alkyl azides (**15**) at under acidic conditions (Scheme 5C).[46] Unfortunately, these latter strategies suffer from the competing formation of the oxoamide (**13a**)[45b] and from epimerization when using amino acids.[45a]

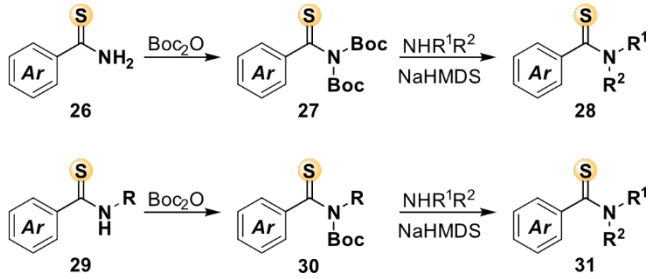


Scheme 5. Thioacylation strategies. A) General strategy for thioacylation of amines. B) Monothiocarboxylic acid activated with a coupling reagent to react with an amine. C) Monothiocarboxylic acid reacted with alkyl azides under acidic conditions. Act = activating group; PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

The first example of an activated thiocarbonyl reagent involved benzimidazolone activation of thionated, α-*N*-protected amino acids (**19**; Scheme 6A).[47] These reagents were synthesized by coupling *o*-phenylenediamine (**16**) with the corresponding *N*-Boc protected amino acid (**17**) to form an anilide, which can undergo selective thionation, using P_4S_{10}, to form a bench stable intermediate (**18**).



Scheme 6. Synthesis of activated thiocarbonyl compounds. A) Synthesis of thioacylbenzimidazolones and thioacylbenzotriazoles. B) Synthesis of α-thioacyloxyenamides. PG = protecting group. Leaving groups are highlighted with green shading. Boc = *tert*-butyloxycarbonyl.

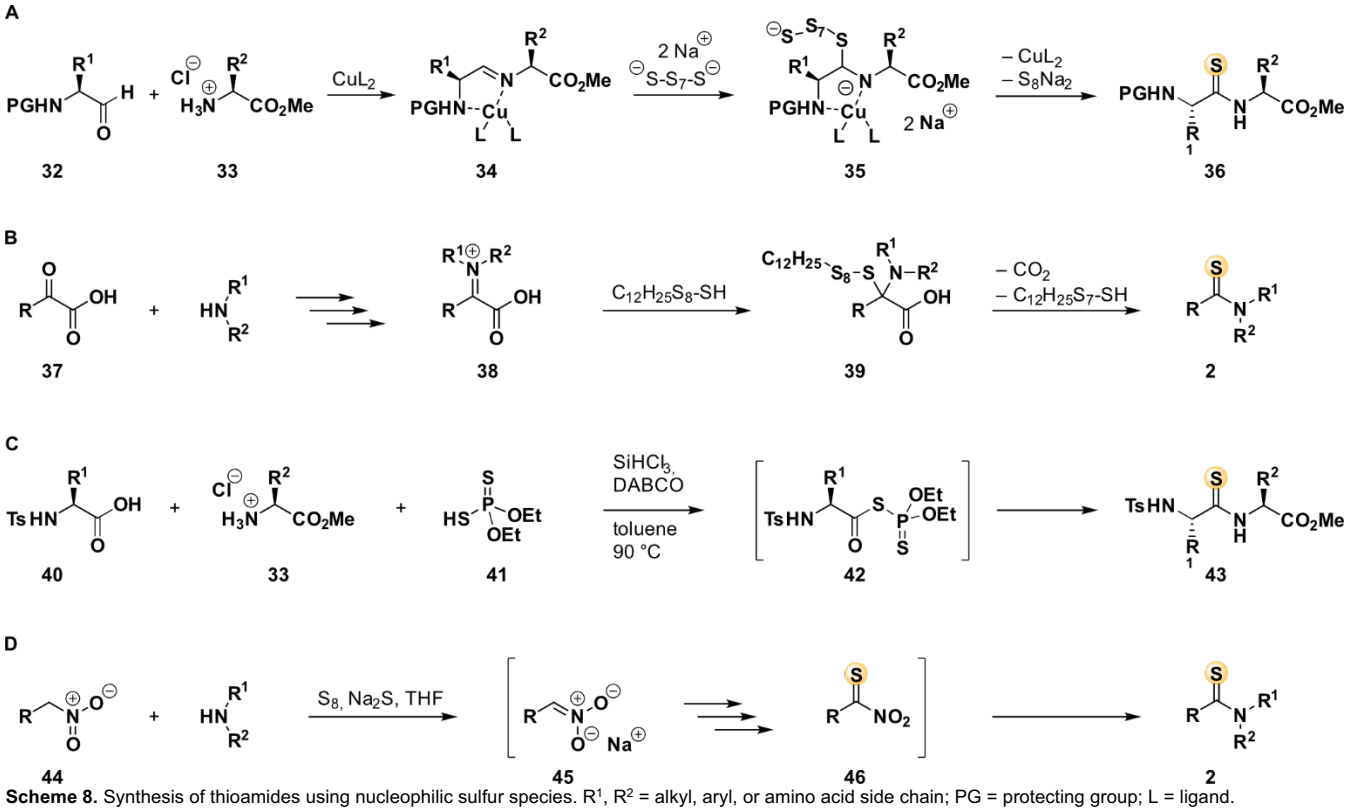


Scheme 7. Transamidation of aryl-thioamides. R^1,R^2 = H, alkyl, or aryl.

Finally, the activated thioacylbenzimidazole (**19**) can be formed by reaction with 1,1'-carbonyl-di-(1,2,4-triazole) (CDT) or alternatively with phosgene or triphosgene. The procedure has been optimized slightly by incorporation of an electron withdrawing fluorine atom to improve the leaving group properties.[48] However, this method has limitations of 1) low yielding preparation of the reagent due to the formation of a benzimidazole side product; 2) sluggish reactivity due to the non-optimal leaving group character of the benzimidazolones;[49] and 3) reported epimerization during coupling reactions.[50] Similarly, thioacyl-*N*-phthalimide has been developed, but suffers from the same drawbacks as thioacyl-*N*-benzimidazolones.[51] Another similar strategy has been reported by Shalaby *et. al.* who utilized a nitrobenzotriazole as a leaving group (**20**; Scheme 6A).[50] The synthesis of these activated species are achieved from **18** by intramolecular diazonium cyclization using $NaNO_2$ (Scheme 6A). Using H-Phe-OMe as the coupling partner, the desired thioamides could be isolated in >80% yield in enantiomerically pure form. Thus, this approach for generating thioamides has been the method of choice for thiopeptide synthesis and has been optimized for use in solid-phase peptide synthesis (SPPS); though, full conversion is not observed with Asn, Gln, Met, Thr, and His.[52]

Recently, Zhao and coworkers employed ynamides in the synthesis of thiopeptides (Scheme 6B). First, a monothiocarboxylic acid (**22**) was synthesized by formation of an activated ester, followed by treatment with NaSH. Subsequent reaction with *N*-ethynyl-*N*-methyl-toluenesulfonamide (**23**) gave a mixture of α-thioacyloxyenamide (**24**) and *S*-(1-sulfonamidovinyl) thioester (**25**) in ratios between 1:1 and 3.8:1 depending on the substrate (Scheme 6B). A higher ratio of the desired α-thioacyloxyenamide was generally observed with non-polar or protected side chains. The activated α-thioacyloxyenamide (**24**) readily reacts with amines at ambient temperatures[44b] and the utility of the method was demonstrated by a 5-step synthesis of closthioamide.[5b] The Zhao laboratory has since expanded the method for use in solid-phase peptide synthesis (SPPS) with 19 out of the 20 proteogenic amino acids.[53] The method does not tolerate histidine and some degree of epimerization (dr ≤98:2) was reported for Asp, Asn, Gln, Ser, and Arg.[53] Further, the scope of the strategy has been expanded beyond amino acids to work with aromatic α-thioacyloxyenamides and for the formation of various primary thioamides as intermediates for thiazole containing compounds that find use as peptide backbone mimics.[54] Recently, Szostak and co-workers introduced the first general method for transamidation of thioamides. Arylthioamides (**26** or **29**) were activated by reaction with Boc_2O to form **27** or **30**, which contain destabilized CS–N bonds that can undergo transamidation with primary and secondary amines in the presence of base (Scheme 7).[55] The method has been shown to work with nucleophilic amines such as morpholine and benzylamine,[55a] but works similarly well with electron deficient amines such as *p*-nitro aniline and indole in acceptable yields (>70%).[55b] At the same time, a broad range of functional groups

are tolerated, including esters, arylbromides, and phenols, and one example, using an aliphatic thioamide was also included.



Scheme 8. Synthesis of thioamides using nucleophilic sulfur species. R^1, R^2 = alkyl, aryl, or amino acid side chain; PG = protecting group; L = ligand.

2.3. New strategies to prepare thioamides

Elemental sulfur has been used to generate nucleophilic sulfur species capable of reacting with imines to form thioamides. This process has been shown by Liao and Jiang to furnish thioamide containing peptides directly from amino aldehydes (**32**) and amino acids (**33**) (Scheme 8A).[56] The methods relies on the formation of an imine, which reacts with a nucleophilic sulfur species formed from elemental sulfur and sodium sulfide. Formation of a copper complex resulted in reduced epimerization at the α-position of the thiocarbonyl moiety.

Similarly, Saito *et. al.* developed a method where α-keto acids (**37**) are reacted with primary or secondary amines to form imine/iminium ion intermediates (**38**), which can react with dodecanemercaptane-activated sulfur to give the intermediate **39** (Scheme 8B).[57] Finally, sulfur-sulfur bond reduction induces decarboxylation to give the desired thioamide (**2**). The reaction is reported to be scalable, and a wide array of amines have been used, including sterically hindered amino acids. The drawback of this method is the limited availability of α-keto acid, which then require additional synthetic steps to prepare. As an example, the α-keto acid of Cbz-Leu was synthesized and reacted with Val-OMe to give the thioamide-containing tripeptide product in 50% yield with a low degree of epimerization (dr = 19:1).

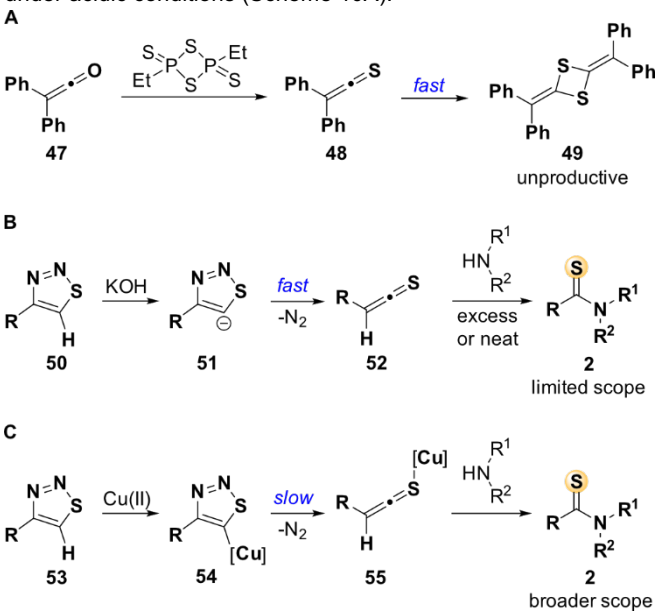
To circumvent having to prepare amino aldehydes or α-keto acids as starting materials (**40**+**33**), Jian and coworkers explored the direct coupling of amino acids. Using 1,4-diazabicyclo[2.2.2]octane (DABCO), trichlorosilane, and diethyl dithiophosphate (**41**) to activate the carboxylic acid as intermediate (**42**), allowed for the nucleophilic attack by the α-amino group of the amino acid (**33**) to form the thioamide (**43**; Scheme 8C).[58] The method was shown to have a broad substrate scope, including aliphatic amino acids, Pro, Met, Trp, Lys(Cbz), Lys(Ts), and Tyr(Ts); however, sulfonamide protecting goups (Ts and Ns) performed better than the commonly used Boc, Fmoc, and Cbz groups. Also, some degree of epimerization was observed in cases where two bulky amino acids were coupled (e.g., Phe–Phe).

Recently, sodium sulfide and elemental sulfur have been shown to activate a broad range of nitroalkane substrates (**44**) to form thioamides upon reaction with amines (Scheme 8D).[59] Mechanistic studies alluded to the reaction pursuing through a deprotonation step to give **45**, followed reaction with sulfur to give the activated thioacyl species **46**, which can then react with an amine (Scheme 8D).

Theoretically, the reaction between a thioketene (**48**) and an amine could form a thioamide. However, thioketenes are highly reactive and quickly dimerize (**49**) due to the inherit nucleophilicity of thiocarbonyl (Scheme 9A). Alternatively, 1,2,3-thiadiazoles (**50**) have been studied as precursors for *in* situ formation of thioketenes by deprotonation of the heterocycle (**51**), leading to release of nitrogen (Scheme 9B). In the presence of a large excess of amine, ranging from 10-fold to using the amine as the solvent, this method has successfully provided thioamides (**2**) in excellent yields but with a limited substrate scope.[60] Alternatively, copper-catalyzed C-H activation to give **54**, followed by slow release of nitrogen, generate a more stable copper–thioketene adduct (**55**), which can react with a wider range of amines under mild conditions (Scheme 9C). This method has been

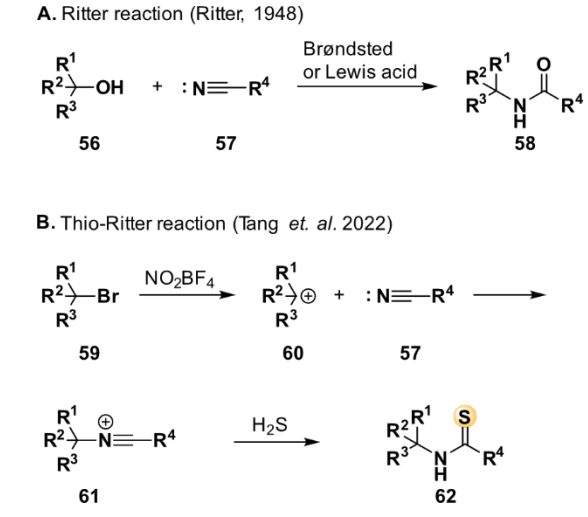
demonstrated to work with a wider range of amines, including anilines, amino acids, and sterically congested amines.[61]

The Ritter reaction provides amides from nitriles and carbocations generated from secondary or tertiary alcohols *in situ* under acidic conditions (Scheme 10A).[62]



Scheme 9. Preparation of thioamides using thioketenes. A) Thioketenes are generally short lived and will quickly dimerize. B) Thioketenes formed *in situ* from the corresponding 1,2,3-thiadiazole using strong base. C) Thioketene-copper adducts as tamed reactants for formation of thioamides with amines.

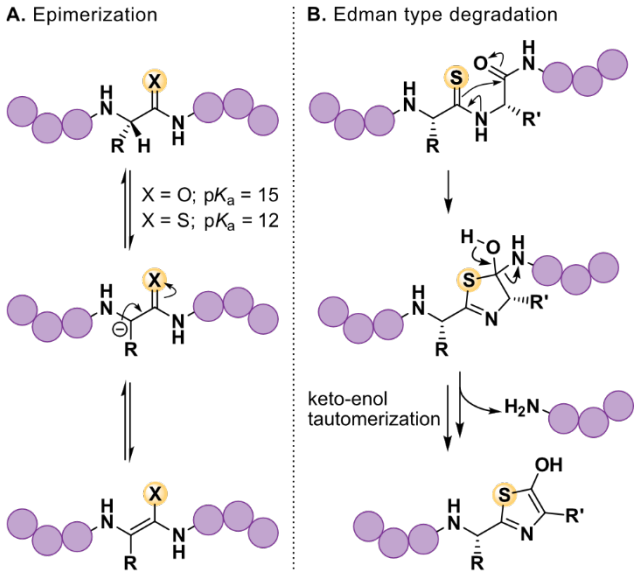
Tang *et al.* adapted this classic reaction to produce thioamides from alkyl and benzyl bromides (**59**) via their corresponding carbocations (**60**), which react with alkyl nitriles to form a nitrilium ion (**61**), similar to the intermediate in the classic Ritter reaction. Instead of reacting with water, however, treatment with hydrogen sulfide furnishes the thioamide product (**62**; Scheme 10B).[63] Due to the planarity of the carbocation intermediate, this process does give a racemic mixture of products. At the same time the use of toxic hydrogen sulfide as a sulfur donor should be carefully handled.



Scheme 10. The Ritter reaction (A) and a "thio-Ritter" reaction (B).

3. Incorporation of thioamides into peptides and proteins

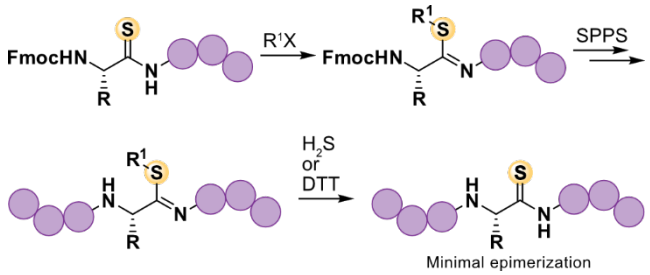
Incorporation of thioamides into peptides were initially accomplished by using solution-phase chemistry to join thioamide-containing dipeptide building blocks with other amino acids and/or peptide segments. For more efficient generation of thioamide-containing peptides, two of the above-mentioned thioacylation methods have been optimized for use in SPPS [*i.e.*, the thioacylbenzotriazoles (**20**) and the thioacyloxoenamides (**24**; *vide infra*)]. However, three fundamental challenges arise during SPPS: *i*) epimerization at the α-position adjacent to the thiocarbonyl during Fmoc removal (Scheme 11A); *ii*) potential Edman-type degradation at the n+1 position during TFA cleavage (Scheme 11B);[64] and *iii*) sulfur to oxygen exchange in the presence of water. The two latter mentioned issues can be circumvented by optimizing cleavage time and using anhydrous solvents.[64b, 65] Because of these challenges, only few examples exist where multiple thioamides have been incorporated on SPPS.[15d]



Scheme 11. Potential challenges when incorporating thioamides into peptide backbones during SPPS. A) Epimerization during Fmoc removal due to the low pKa of the α-proton. B) Edman-type degradation during TFA-mediated cleavage.

The increased epimerization observed for thioamides is caused by the acidity of the α-proton, which is around three orders of magnitude lover for a thioamide compared to an oxoamide.[65-66] Standard SPPS Fmoc removal by piperidine–DMF (1:4) has been shown to cause ~20% epimerization of model peptide Cbz-Phe^{S}-Ala-OMe in 1 hour.[66] Lowering of the piperidine concentration has been shown to reduce the degree of epimerization,[67] but the longer deprotection time then needed for sterically hindered amino acids has led to truncation of the peptide.[52c, 68] Substituting piperidine for DBU and/or piperazine generally appears to lower the extent of epimerization. However, when multiple deprotection steps are needed after introduction of the thioamide, epimerization still occurs.[52c, 65] Thus, utilizing these optimized Fmoc removal strategies must be performed carefully and preferably using an automated SPPS system to ensure short reaction time.

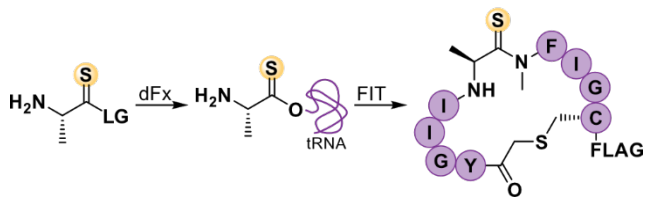
To reduce the pKa of the α-proton in the thioamide-containing residue, VanVeller and coworkers has introduced the masking of the thioamide group as a thioimidate.[66, 69] The thioimidates are easily prepared by treating the thioamide with an alkylating agent such as methyl iodide or p-azido benzyl bromide (*p*-N_3-BnBr), and upon completion of the SPPS, the thioimidate can be cleaved with hydrogen sulfide or dithiothreitol (DTT) prior to TFA cleavage from the solid support (Scheme 12). Due to the hydrogen sulfide being a flammable, toxic, and corrosive gas, the azido-benzyl group that can be removed with DTT appears to be preferred. Both protection and deprotection of the thioamide is reportedly quantitative, rendering the reaction suitable for SPPS. The thioimidates were reported to be stable to treatment with 20% piperidine in DMF, but epimerized rapidly when treated with 2% DBU.[66] Importantly, the thioimidate harbors very low stability towards dilute TFA (2%), which was recently addressed by applying milder cleavage conditions [i.e., mixtures of hexafluoro isopropyl alcohol (HFIP)–CH_2Cl_2].[64b, 69] Alternatively, the application of a more labile linker (e.g., 2-CTC) allows for resin cleavage before thioamide deprotection using HFIP–CH_2Cl_2 (1:4).[66, 69]



Scheme 12. Protection of the thioamide as a thioimidate protects against epimerization during SPPS. R^1 = Me or *p*-N_3-Bn; X = Br or I; DTT = dithiothreitol.

Because of the limited length of thioamide-containing peptides that can be achieved using SPPS, semisynthetic methods[70] have been pursued to incorporate small thiopeptide segments into larger proteins by native chemical ligation (NCL).[71] The subject has been entertained previously[72] and, more recently, semi-synthesis has been expanded to non-cysteine residues, by utilizing radical desulfurization to remove the mercapto group after the ligation step.[73]

Due to the inherent challenges associated with the introduction of thioamides using SPPS, Maini *et al.* have also investigated the use of ribosomal incorporation of thioamide bonds into peptides.[74] First, the authors utilized the flexizyme dFx[75] to attach Ala^S to tRNA. They then utilized various Ala^S-tRNAs in their flexible *in vitro* translation (FIT) system[76] to translate the desired peptide sequence (Scheme 13).



Scheme 13. Incorporation of thioamides into cyclic peptides utilizing genetic code expansion. LG = (3,5-dinitrobenzyl)oxy; FIT = flexible *in vitro* translation; FLAG = DYKDDDDK.

Utilizing this method, the authors managed to synthesize both linear and cyclic peptides, where the cyclization was achieved by reacting an N-terminal chloroacetamide with an internal cysteine residue, showing that the thioamide is inert in reaction with the chloroacetamide under the translation conditions. Further, the the system accepted an N-methylated phenylalanine residue to give N-methylated thioamide-containing peptide macrocycles (Scheme 13). Though, generally oxoamides were observed as side products.

The ε-*N*-thioacyllysine residues comprise attractive isosteres of acylated lysine, endowed with increased stability toward hydrolytic enzymes.[18b, 77] Söll and coworkers have pushed the *in vitro* translation technology further to use flexizyme followed by FIT to incorporate ε-*N*-thioacetyllysine into multiple sites in the histone 3 (H3) protein, mimicking the native histone acetylation marks.[78]

More recently, Petersson and co-workers attempted to incorporate the ε-*N*-thioacetyllysine residue into proteins in *E. coli*, using amber codon suppression; albeit, with limited success so far.[79] Cole and coworkers, on the other hand, succeeded in the sortase-mediated semi-synthesis of thioacetylated H3 at Lys9 as well as an even more stable methylthiourea analog that could be incorporated into reconstituted nucleosome particles to study chromatin–sirtuin enzyme interactions.[80]

Pless and coworkers have utilized tandem protein trans-splicing to insert peptide segments containing ε-*N*-thioacetyllysine into membrane-bound proteins in live cells.[81] This approach was confirmed by investigation of the protein's function post-insertion.

Alternatively, post-translational conversion of oxoamides to thioamides could be envisioned, inspired by natural product biosynthetic pathways, as previously reviewed.[82] In support of this idea, Koehnke and coworkers have recently achieved the reconstitution of the biosynthesis of thioholgamide (analog of thioviridamide) to enzymatically introduce thioamides *in vitro*.[83]

4. Applications of thioamides

4.1. Fluorescence quenching by thioamides

As briefly mentioned in the introduction, thioamides can act as fluorescent quenchers of both Förster resonance energy transfer (FRET) and photoinduced energy transfer (PET). Thioamides have been found to quench the fluorescence of the canonical amino acids, Trp and Tyr, as well as *p*-cyanophenylalanine,[17c, 84] 7-azatryptophane,[17b] 7-methoxycoumarin-4-yl alanine,[85] and acridon-2-yl alanine.[17b] This quenching mechanism has been utilized to study protein dynamics in relation to both folding and aggregation. By expanding the scope of thioamide quenching to various fluorescent dyes (e.g., fluorescein, BODIPY FL, Alexa Fluor 448, and rhodamine 6G), Petersson and coworkers have expanded the scope of this strategy to be suitable for microscopy.[17a]

The thioamide-based quenching mechanism has also been utilized to study protease activity in continuous high-throughput format by installing a thioamide on one side of a protease cleavage site and a PET donor on the other.[15b] This method has

also been used to identify stabilizing O→S substitutions in cancer imaging peptides towards serine proteases,[86] and to identify how thioamides can improve the stability of peptides towards cysteine proteases.[17d] Furthermore, this approach has been used to identify selective protease inhibitors for the cathepsin protein family,[87] and Schutkowski and coworkers have incorporated thioamides into ε-*N*-acyllysine-containing substrates to develop an assays for continuous monitoring of the activity of histone deacetylase and sirtuin enzymes.[88]

4.2. The stereo electronics of thioamides in molecular interactions

The n→π* interaction, along the classical Bürgi-Dunitz trajectory of nucleophilic acyl substitutions,[89] has been investigated by Raines and coworkers in the stabilization of protein secondary structure.[90] In their detailed investigations of the *cis–trans* isomerization of amide bonds at proline, Raines and coworkers took advantage of the altered donor and acceptor properties of the thiocarbonyl, using thioamides as amide bond surrogates.[91] Likewise, thioamides have been applied to investigate the *cis–trans* isomerization of the amide bonds in peptoids[92] and β-peptoids.[93] In the latter study, X-ray crystallography and natural bond order (NBO) calculations indicated the presence of a thiocarbonyl–H-C_{sp2} interaction.

More recently, the favorable desolvation of the relatively apolar thioamides in water has been shown by Chatterjee and coworkers to stabilize the microenvironment in β-sheets. Thus, introduction of these amide bond surrogates in proximity to lipophilic side chains lead to a significant increase in the thermal stability of the WW domain of Pin1.[94] Further, the same laboratory has found O→S substitution to increase membrane penetration of peptides, as an alternative to more traditional methods such as N-methylation of backbone amide bonds.[95]

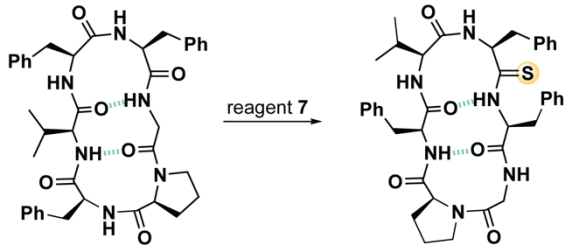
Also, thioamides, as well as selenoamides, have been shown to stabilize the supramolecular assembly of benzene-1,3,5-triamide polymers.[96]

4.3. Conformational impact of thioamides in peptides

In seminal work by la Cour, describing the restrictions of dihedral angles around the α-carbon of thioamides in peptides, it was found that dihedral angles in both α-helices and β-sheets allow the introduction of thioamides.[97] It was also speculated that the thioamide would destabilize secondary structures due to the altered hydrogen bonding properties and size of the sulfur atom,[97] which was later investigated in more detail.[12] On the other hand, it was found that introduction of thioamide bonds can stabilize β-turns,[97] which has been confirmed experimentally.[33, 98]

Peptide cyclization has long been used to restrict the conformational ensemble of biologically active peptides.[99] Initially observed by Kessler and coworkers, further conformational restraints can be introduced by backbone thionation; exemplified by the selective introduction of a thioamide bond into cyclic hexapeptide **63** to form **64**.[16a] This thionated homologue forms a stabilized β-turn structure that mimics the active conformer of the natural hexapeptide, resulting in increased potency against its target, trypanosomal triosephosphate isomerase (TIM). More recently, Chatterjee and coworkers investigated the effect of the introduction of thioamide bonds on conformational space in cyclic peptides more systematically, and generally observed an increase in conformational homogeneity.[16b]

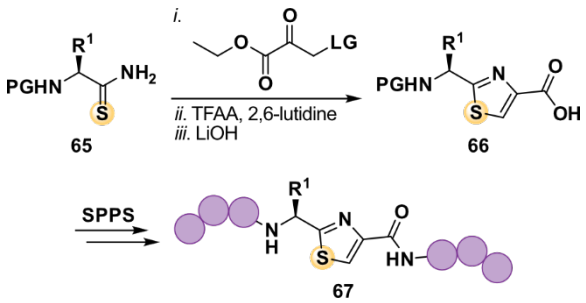
In another example of achieving an increase in peptide potency by thioamide substitution, Spatola and coworkers achieved this for cyclic enkephalin analogs; though, no structural evaluation was included in this study.[100]



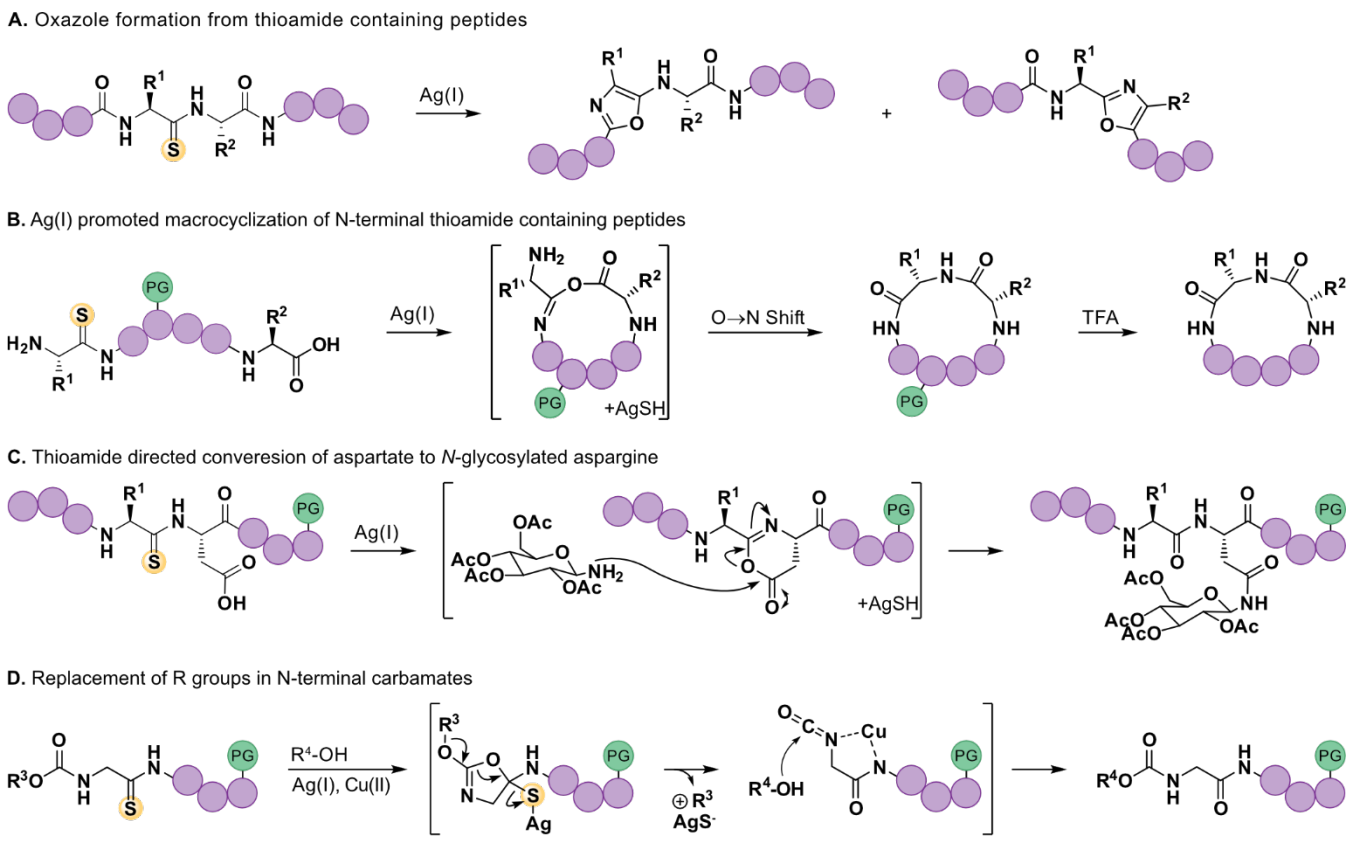
Scheme 14. Active conformer of **63** locked by selective thionation between the two Phe residues, using Yokohama's reagent (**7**) to give **64** that adopts a stable β-turn.

4.4. Synthetic applications of thioamides

Besides the potential advantages of thioamides in peptide and protein backbones, including enhanced proteolytic stability and desolvation properties, thioamide-containing peptides have also been used as intermediates in the syntheses of complex molecules. For example, non-substituted C-terminal thioamides (**65**) have been condensated with ethyl bromopuryvate under Hantzsch conditions (Holzapfel-Meyers-Nicolaou modification) to form 5-carboxy-2-methylaminothiazole building blocks (**66**), which can be incorporated into peptide backbones (**67**; Scheme 15).
To avoid epimerization, the cyclization with ethyl bromopuryvate (or similar) is performed at reduced temperatures (–40 °C to –20 °C), followed by dehydration with trifluoroacetic anhydride (TFAA) and lutidine.[101] This approach has been used to synthesize the macrocyclic natural product sanguinamide A[102] and the central heterocyclic domain of micrococcin P1, which contains four thiazole moieties.[101b]



Scheme 15. Synthesis of thiazole-containing dipeptide mimics, which in turns can be incorporated into peptide backbones. R^1 = (protected) amino acid side chain; PG = protecting group; LG = leaving group such as Br, Cl, I, OTf, OMs, and OTs.



Scheme 16. Ag(I) mediated functionalization of thioamides. A) Potential oxazole formation when a thioamide is activated by Ag(I). B) Head-to-tail macrocyclization of N-terminal thioamide-containing peptides. C) Late-stage N-glycosylation of aspartate residues in peptides containing an adjacent thioamide. D) Carbamate replacement of N-terminal thioamide-containing peptides. R^1, R^2 = (protected) amino acid side chains; R^3 = *t*Bu, Allyl, Bn, $Me_3SiCH_2CH_2$-; R^4 = *t*Bu, Allyl, Bn, Et, CF_3CH_2-, $(CF_3)_2CH$-, CCl_3-CH_2-, $Me_3SiCH_2CH_2$-, 9-fluorenylmethyl. TFA = trifluoroacetic acid.

Silver(I) carboxylates, as well as with Hg(II) and Cu(I) salts, have been characterized as thiophilic metals and have been studied in desulfurization reactions of thioamides to generate imides, amides, amidines, and nitriles.[103] Such desulfurization methods have been utilized for late-stage functionalization of peptides as well. When the thioamide is coordinated to Ag(I), making the C=S bond more electrophilic and hereby prone to cyclization with the neighboring amides (Scheme 16A).[104] By having other functional groups in the molecule, it is possible to diminish both desulfurization and oxazole formation.

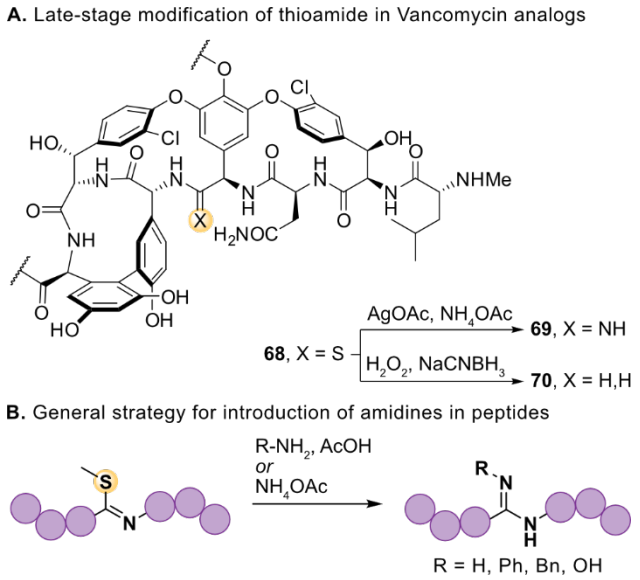
Hutton and coworkers have investigated this chemistry to synthesize amide bonds from N-thioacylated amines and carboxylic acids, making it possible to synthesize peptides from the non-standard N→C terminal.[105] Further, the method has been optimized to include head-to-tail peptide macrocyclization at faster rates than for usual coupling reagents, arguing that Ag(I) is capable of coordinating both the carboxylate and sulfur atom to bring the two ends of the peptide in close proximity (Scheme 16B).[106] Furthermore, the method has been utilized for lactam stapling[107] and macrolactonization.[108] Most recently, the group has further developed the method to achieve late stage glycosylation, by converting aspartate residues to *N*-glycosylated asparagine residues (Scheme 16C).[109]

Ibara *et al.* have similarly used Ag(I) to selectively replace carbamates at the N-terminus of thiopeptides (Scheme 16D).[110] Thus, achieving the substitution of the *tert*-butyl alcohol in the Boc group with benzyl alcohol to convert a Boc group to a Cbz group. Similar substitutions also worked to introduce for example Fmoc and Teoc groups (Scheme 16D).

The Boger laboratory has studied the total synthesis of the antibiotic vancomycin and developed derivatives with efficacy against bacterial strains that have developed resistance towards the natural product.[111] To achieve potent binding to the mutated peptidoglycan terminus (D-Ala-D-Lac motif instead of the native D-Ala-D-Ala motif) in the vancomycin-resistant bacterial strain *E. faecalis*, the highlighted amide bond was replaced by an amidine functionality (**68**; Scheme 17A).[111a] This substitution was installed by late-stage functionalization of a thioamide (**69**), which was selectively installed early in the synthetic sequence and carried through multiple steps.[111c] Preparation of an amino methylene analogue (**70**) was achieved by oxidation of the thiocarbonyl to the corresponding S-oxide, which is desulfurized by treatment with sodium cyanoborohydride (Scheme 17A). An earlier version of the synthesis of vancomycin analogs also provided N'-hydroxy amidine analogs by reaction of thioimidates with hydroxylamine.[111a]

Neither the work by Boger and coworkers, nor the recent work cyclic peptides by Yudin and coworkers,[112] reported the formation of oxazoles during thiocarbonyl activation with Ag(I), likely due to the structural constrains imposed by the cyclic structures of their substrates. VanVeller and coworkers have recently applied thioimidates, as initially introduced for protection against epimerization (*vide infra*), to convert the thioimidate into amidines (Scheme 17B).[113] This approach allowed for the installation of amidines both in solution and in SPPS, where both

ammonium, benzyl amine, and aniline were introduced in good yields. At the same time, the method avoids the use of Ag(I), minimizing the extent of side reactions. Moreover, epimerization was not observed. Introduction of secondary amines such as piperidine was only successful in solution and with unhindered thioimidates. In SPPS, standard protecting were allowed and the main limitation was that thioimidates could not be formed at Aa-Pro junctions.



Scheme 17. Amidine formation from A) thioamide in the synthesis of aglycon Vancomycin analogs or B) thioimidates in peptide backbones.

5. Summary and Outlook

Thioamides are naturally occurring isosteres of amide bonds and have found use synthetic surrogates of amides in a variety of contexts, including medicinal chemistry and peptide and protein science. For example, the thioamide functionality has recently been applied to improve the proteolytic stability of potential drug candidates, including GLP-1 analogs and inhibitors of the SARS-CoV-2 M^{pro} protease. Further, thioamide-containing analogs of cyclic peptides have been shown to harbor improved cell-penetrating properties over oxoamide counterparts, which is a major challenge in peptide medicinal chemistry. Further, recent advances in protein science have allowed for the introduction of ε-*N*-thioacetylated lysine residues in nucleosomes and membrane proteins, by using sortase-mediated semi-synthesis and tandem protein trans-splicing in cells, respectively.

In addition to these recent advances in applications of thioamides, a wide range of new synthetic methods, involving this functionality, has surfaced within the last few years. Examples of which include creative strategies based on novel starting points for the preparation of thioamides, such as ketenes, keto acids, amino aldehydes, nitroalkanes, and copper-activated 1,2,3-thiadiazoles, as well as development of a thio-Ritter reaction.

Also, novel thioacylating reagents based on α-thioacyloxyenamides have been developed for solution-phase and solid-phase peptide synthesis and methods to mask the thioamide bonds during peptide synthesis as thioimidates masking to circumvent epimerization in SPPS.

Finally, utilization of thioamides in chemical modification of peptides and natural products have been explored by the laboratories of Hutton, VanVeller, and Boger, respectively. Typically, the thioamides are activated by a Lewis acid [e.g., Ag(I) salts] in these works to achieve peptide cyclization, peptide glycosylation, and amidine formation in both peptides and complex natural products.

Although, the area has experienced substantial advances, most syntheses of polypeptides still involve the generation of the thioamide bonds by using an activated thiocarbonyl species. However, based on the works discussed herein, we foresee a bright future for further development and optimization of methods to address still remaining challenges such as epimerization, conversion to oxoamide, and robust incorporation into protein backbones.

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Christian A. Olsen received his M.Sc. from the Technical University of Denmark in 2000 and his Ph.D. from the Danish University of Pharmaceutical Sciences in 2004. After independently working on the development of novel peptidomimetics at the same institution, he did his postdoctoral fellowship with Prof. Ghadiri at Scripps Research. In 2010 he returned to a faculty position at the Technical University of Denmark and in 2014 he accepted his current position as professor at the University of Copenhagen. He is recipient of the Lundbeck Foundation Fellowship, the EFMC award for a young medicinal chemist in academia, and an ERC Consolidator grant.

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Keywords: Amide isosteres • Peptides • Peptide functionalization • Protein modification • Natural products

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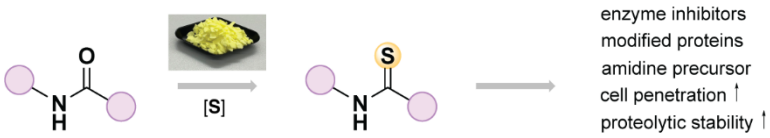

Substitution of amide bonds with thioamides can have a beneficial influence on the stability of peptides towards proteolysis and affect folding among other effects. In this Review, we highlight the recent developments in the preparation of thioamides and their applications for modification of peptides and natural products as well as new frontiers in the introduction of thioamide functionalities into proteins.

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