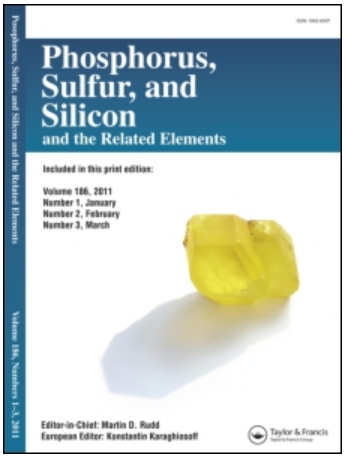

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REVIEW: ENDOTHIOPEPTIDES ALIAS PEPTIDE THIOAMIDES

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Review

REVIEW: ENDOTHIOPEPTIDES ALIAS PEPTIDE THIOAMIDES

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Key words: Peptide thioamides, O/S-exchange, thioacylation, biologically active peptides.

INTRODUCTION

Modifications of biologically active peptides are often undertaken in order to improve the properties of these peptides for more efficient use in medicine and biochemistry.[1] An important class of compounds in this field is the so-called backbone modified peptides or pseudopeptides. These are peptides or peptide-like molecules, which contain one or more non-peptide linkages between the α-amino acids or α-amino acid-like residues. Pseudopeptides are generally written with a ψ followed by the unnatural linkage in a square bracket,[1] e.g. Gly-ψ[CSNH]-Gly. Resistance towards enzymatic degradation was an early goal with pseudopeptides, but additionally their altered conformational properties can lead to powerful effects, such as enhanced activity and/or selectivity between receptors.

Among the earliest efforts in the field was the subtle thioamide substitution.[2,3] Peptides with one or more thioamides in their backbone are also known as endothiopeptides.[4] Early synthetic methods for endothiopeptide preparation required relatively harsh reaction conditions and were not universal. The real breakthrough was the application of Lawesson's reagent (**1**, Figure 1)[5,6] for O/S-exchange in protected dipeptides.[7,8] The selectivity of **1** for amide carbonyls in the presence of carbonyls of esters and urethanes (protecting groups) made this kind of chemistry possible. Selectivity *among* amides is, on the other hand, a problem with larger peptides, although some regioselectivity of **1** and its analogs have been discovered.[9] This selectivity was even found to be somewhat predictable, depending on the steric requirements of individual amino acids in a given peptide. Despite this, the O/S-exchange chemistry of **1** seems less than ideal, particularly for SPPS (solid-phase peptide synthesis), which is the standard in modern peptide synthesis.[10–12]

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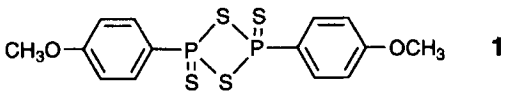

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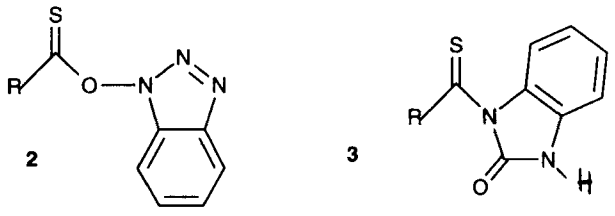
FIGURE 1 Lawesson's reagent **1**.



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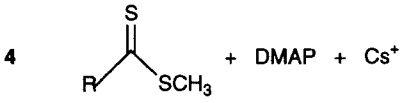

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FIGURE 2 Benzotriazolyl ester **2**, thiobenzimidazolone **3** and dithio ester **4** plus catalyst.

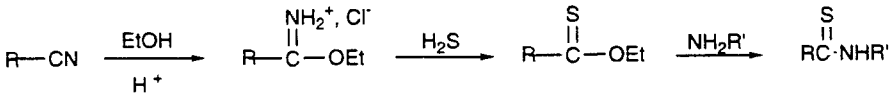


FIGURE 3 Thioacyl amino acids via ethyl thiono esters.

Recent years have therefore seen an increased focus on thioacylation methods. Thioacylation offers complete control of the regioselectivity, and its stepwise nature additionally makes it well suited for solid-phase work. Thiono analogs of the numerous active esters etc., which are well-known from peptide synthesis, were until recently unknown. This problem now seems to be solved by the arrival of *in-situ* generated amino acid benzotriazolyl thiono esters **2**,[13] amino acid thiobenzimidazolones **3**[14] and by the use of DMAP (4-dimethylaminopyridine) in combination with cesium salts for catalysis of thioacylation[15] with alkyl dithio esters **4** of amino acids (Figure 2).

These new thioacylating reagents perform reasonably well under SPPS conditions. Since the methods are quite new, they have yet to see broader acceptance. Meanwhile, **1** and its analogs retain some strengths: Initial preparation of thio derivatives of individual amino acids is unnecessary, and with e.g. cyclic peptides, the thionation can be performed as the last step, thereby limiting the potential number of side reactions.

EARLY SYNTHETIC EFFORTS

Attempts of thionating diglycyl ethyl ester by treating it with P_4S_{10} was reported as early as 1926.[2] The outcome was, however, disappointing, as only starting material

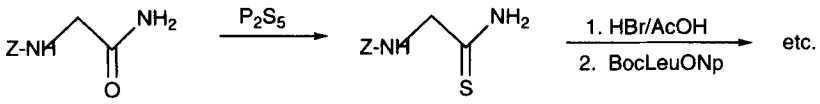


FIGURE 4 Initial steps towards an oxytocin analog (cyclic octapeptide).

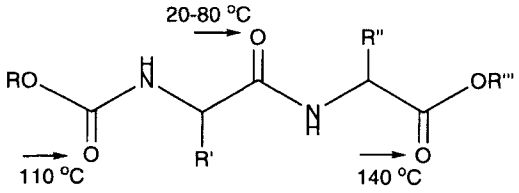


FIGURE 5 Reactivity of **1** towards the carbonyl groups of protected dipeptides, (amide > urethane > ester).

was recovered. A similar result was found with unprotected tetraglycin in 1947.[16] These observations are not surprising in the light of current knowledge: O/S-exchanges with P_4S_{10} and its derivatives are hampered by nucleophiles.[17] This means that the unprotected primary amino group of the above compounds is intolerable.

The first endothiopeptides were prepared by Ried et al. in the early sixties.[3,18,19] N-protected amino acid ethyl thiono esters, prepared from the corresponding imido esters and H_2S, were used for thioacylation of free amino acids (Figure 3). Unfortunately, the preparation of the intermediate imido esters required acidic conditions, strong enough to prevent any use of the Boc and ^{t}Bu protecting groups. Furthermore, alkyl thiono esters turned out[20] to give sufficient reactivity to only a few amino acids (Gly, Ala, Pro). Notably, Ried et al. were the first to demonstrate that protecting groups such as Z (benzyloxycarbonyl) can be removed from endothiopeptides with strong acid without affecting the thioamide function.

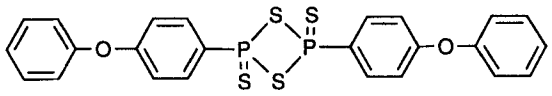
du Vigneaud *et al.* in the early seventies[21] utilized P_4S_{10}-promoted thionation of Z-Gly-NH_2 for preparation of an oxytocin thioamide analog (Figure 4). Glycinamide is placed C-terminally in oxytocin, so glycinthioamide could be used as starting point for preparing the oxytocin analog, involving a 3+6 fragment condensation. The thioamide function survived all necessary manipulations.

The chemistry used here is quite general, it has been used several times,[22-25] but recently with **1** replacing P_4S_{10}. The method has even been used for synthesis on solid-phase.[26] It is, however, limited to C-terminal thioamides, since elongation is only possible from the N-terminal.

Also during the seventies, thioasparagine was prepared by Ressler and Banerjee[27] from N-protected β-cyanoalanine, by treatment with H_2S/NH_3. This work is one of the few examples involving side-chain thioamides.[28,29] The scope of the β-cyanoalanine chemistry was later extended by Spatola and Saneii, who delayed the thioamidation until the end of the synthesis.[30]

LAWESSON'S REAGENT AND OTHER O/S-EXCHANGE REAGENTS

2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (**1**) was described by Lecher et al. already in 1956,[31] but it's potential as O/S-exchange reagent



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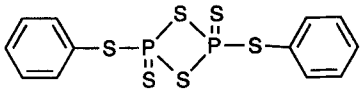

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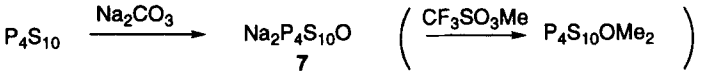

FIGURE 6 Belleau's reagent **5**, Yokoyama's reagent **6** and Brillon's reagent **7**.

was, although briefly touched in 1967,[32] not fully realized until Lawesson et al. initiated comprehensive studies of this compound in 1978.[5] The first descriptions of thionations of protected dipeptides with **1** came from Lawesson's group in 1981.[7] By boiling the substrate and **1** in benzene, followed by a chromatographic step, close to quantitative transformations could be achieved. The carbonyl functions of protecting groups such as esters and urethanes are unaffected under proper conditions (Figure 5).

Comparison of optical rotation values of desulfurized endothiopeptides (aqueous $AgNO_3$) with those of the parent peptides indicated that no epimerisation took place during the transformations.[7] This important point has later been supported by chromatographic analysis, involving LL and DL standards.[33] A single exception is the epimerisation during thionation of cyclo(Pro-Pro).[34]

Lawesson's method does not interfere with most of the common amino acid side chain functionalities,[8] but it is important to protect nucleophilic groups, such as hydroxy functions,[35,36] whereas carboxylic acids may be tolerated if the reaction conditions are carefully controlled.[29] An exception to the universality of **1** are amino acids with side chain amides, such as Asn and Gln. If thionation of the peptide backbone is attempted with Asn or Gln containing peptides, the side chain amides will likely suffer thionation too. This experiment has apparently never been done with Asn or Gln, but use of **1** with pGlu-containing peptides (pGlu, pyroglutamic acid) gives side chain thionation.[37] Compatibility of **1** with the base labile protecting group Fmoc (9-fluorenylmethoxy-carbonyl)[12] has only been little investigated,[13] but no special problem has been observed.

In 1983, Belleau *et al.* introduced **5** (Figure 6), a 4-phenoxyphenyl analog of **1**, having better solubility properties, thus allowing thionation of peptides in THF at 25°C.[9] Mild reaction conditions can be vital with sensitive peptides, such as retroinverso endothiopeptides.[38,39] The investigations by Belleau *et al.* demonstrated the first selective thionations of longer peptides, namely, **5** reacts preferably at Gly positions, or with other small amino acid residues.[9,40,41] In retrospect, the described advantages of **5** over **1** may not be as significant is originally thought. After the discovery of **5** it has been found that **1** likewise is reactive at 25°C if solvents such as THF or DME

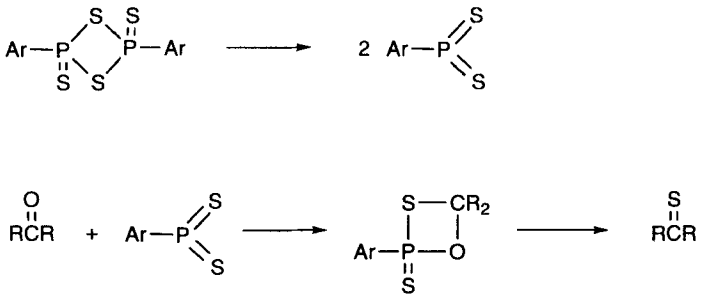

FIGURE 7 Thionation with **1** via $ArPS_2$ monomers.

(dimethoxyethane) are chosen,[42] but no actual comparative studies between **1** and **5** exist. Furthermore, as described for **5**, also **1** displays some regioselectivity towards smaller amino acid residues.[43,44] Selection of secondary amides over tertiary, such as in N-methyl peptides, has also been described.[45,46] The positions of selective thionation in polypeptides can be evaluated by the use of ^1H NMR, by exploiting the downfield shift of the CSNH proton (2 ppm), or by ^{13}C NMR due to the downfield shift of the thiocarbonyl carbon (30 ppm). See for instance Reference 46.

In 1984, Yokoyama et al. introduced **6** (Figure 6), a phenylthio analog of **1**,[47] and this reagent has become popular during recent years.[33,48-51] Comparative studies of **6** to **1** have, in terms of both yields and selectivity, disclosed **6** to be a superior reagent for peptide thionations.[47,48]

Attempts of rationalizing the relative reactivity of **1** and **6** turn out to be contradictive. The mechanism of thionation with **1** has been shown to involve dissociation to $ArPS_2$ monomers (Figure 7), followed by nucleophilic attack from the carbonyl oxygen towards the electrophilic phosphorus.[52-54]

Considering this mechanism, the decreased electrophilicity of phosphorus in **6** relative to **1** should render it actually less reactive. This is in contrast to the observations, at least with peptides. As mentioned for **5**, the explanation may lie in the better solubility properties of **6**, or alternatively, in a changed reaction mechanism. Notably, a study with lactone substrates showed agreement between the above mechanistic considerations and the reactivity of **6** relative to **1**, but the observed differences were small.[55]

Although P_4S_{10} has largely been replaced by the derivatives **1**, **5** and **6**, it has not been completely abandoned. P_4S_{10} has been used recently, but mostly in situations where reagents **1**, **5** and **6** would probably have given similar or better results.[56-58] In one case, with a cyclic peptide, P_4S_{10} was described as mandatory for achieving any thionation.[44]

Brillon's reagent **7**,[59] which is prepared *in-situ* in THF from P_4S_{10} and Na_2CO_3 (Figure 6), has the advantages of simple preparation and work-up. Water solubility of the P,S-byproducts allow aqueous work-up, whereby chromatographic steps may be avoided.[60] The reactivity of **7** seems to be somewhat limited, as sterically more crowded peptides give low yields.[59] Better results can be obtained with methylated **7**,[59] but this effort removes some of the methods simplicity, and it furthermore destroys the water solubility properties of the P,S-byproducts.

A number of other O/S-exchange reagents are described in the literature. Most

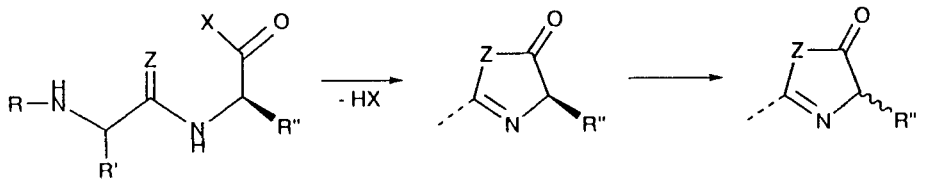

FIGURE 8 X = leaving group. R = protecting group or peptide sequence. R′ and R″ = amino acid side chains. (a) Z=O: Formation of oxazolone from activated peptide C-terminal, leading to epimerisation of the α-carbon with side chain R″. (b) Z=S: Formation of thiazolone from activated peptide C-terminal, leading to epimerisation of the α-carbon with side chain R″, or even of the α-carbon with side chain R′.[90]

elaborate further on the use of phosphorus as oxygenophile,[42,55,61,62] but others utilize similar properties in silicon[63] and boron.[64] The reagents mentioned here have, however, yet to see application to peptide chemistry.

CYCLIC ENDOTHIOPEPTIDES

Cyclic peptides are an interesting class of compounds, partly due to their tendency towards resistance against proteases, but particularly because of their restricted conformational freedom, which results in potential strengthening of receptor binding.[65,66] With regards to thionation, the conformational restrictions often seem to lead to high regioselectivity. Accordingly, cyclic hexapeptides have been selectively thionated with **1**[67,68] or with **6**.[50,69] Examples of complex mixtures (multiple products in combination with multi-thionated products) are also known, but even here some regioselectivity has been found.[44–46] The positions of thionation in cyclic peptides are often difficult to predict, at least from the primary structure alone. Even an example of totally unexpected positions of thionation exists.[50] Carbonyl groups involved in internal hydrogen bonding in cyclic peptides seem, not surprisingly, to be relatively inaccessible to the thionation reagents. Therefore, knowledge about the secondary structure of a given cyclic peptide may be helpful in predicting its reactive site(s).[50,68]

Cyclization of linear endothiopeptides has the strength of allowing control of the thioamide position, but this strategy has only few literature examples.[70,71] The danger of the thioamide function participating in the cyclisation step seems to discourage this kind of chemistry.

EXTENSIONS OF ENDOTHIOPEPTIDES

As a circumvention of the regioselectivity problems with O/S-exchange reagents, research has been focused on the possibility of extending endothio(di)peptides, stepwise or by segment condensations. Segment condensations are well-known from standard peptide synthesis, although the technique was more common before the advent of SPPS.[10–12] A problem with segment condensations of standard peptides is the much enhanced risk of epimerisation in the C-terminal part, caused by oxazolone formation (Figure 8a) in non-urethane protected residues.[72]

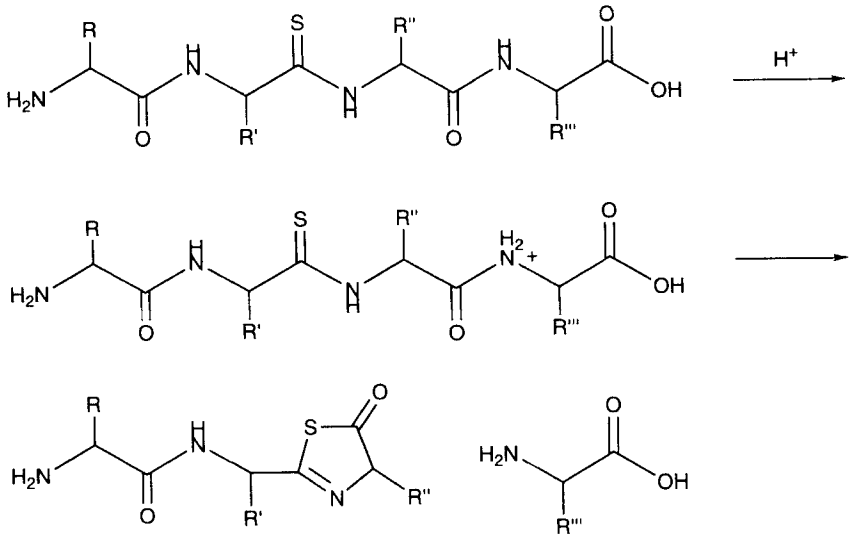

FIGURE 9 Acid catalyzed cleavage of endothiopeptides. Apparently only fatal if several R's = H.

It is important to note that this problem is usually only serious with segment condensations (non-stepwise build-up). The urethane protecting group attached to activated single amino acids limits this side reaction, because of the lower nucleophilicity of the urethane carbonyl compared to the amide carbonyl.

The first requirement for extension of endothiopeptides is, of course, cleavage of relevant protecting groups. This cleavage should take place leaving the thioamide unaffected. Usually, such deprotections do proceed smoothly. The thioamide function usually survives acidolysis of N-protecting groups such as Z and Boc,[3,73] although in some cases cleavage of the peptide chain can occur.[74,75] The cleavage takes place by a side reaction (Figure 9) similar to the Edman degradation, the standard for sequencing of peptides.[76,77] Luckily, this problem seems to be truly fatal only for peptides with several small amino acid residues. It has been observed with Leu-enkephalin analogs, Tyr-Gly-Gly-Phe-Leu.[74,75] It has not been excluded, however, whether the side reaction may lead to reduced yields with other sequences. Endothiopeptides are also compatible with the piperidine used in Fmoc-chemistry, although thioamide sensitivity towards the nucleophilic character of piperidine[78] has been suggested as explanation for low overall yields during solid-phase endothiopeptide synthesis.[14,79] C-terminal alkyl esters of protected endothiopeptides can be cleaved by mild saponification.[48] Cleavage of e.g. benzyl protecting groups by hydrogenation has apparently never been used, probably due to fear of poisoning the Pt or Pd catalysts with sulfur. The soluble Pd catalyst used in allyl deprotections ($(Ph_3P)_4Pd$) has, on the other hand, been shown to be tolerable,[80] so that allyl cleavage from endothiopeptides can take place. The work-up of such reactions is not unproblematic, however, due to complexation between Pd and thioamide.[79]

N-terminal extension of deprotected endothiopeptides result in no special problems aside for those known from standard peptide synthesis. Accordingly, many examples of step-wise or segment N-terminal extension of endothiopeptides are

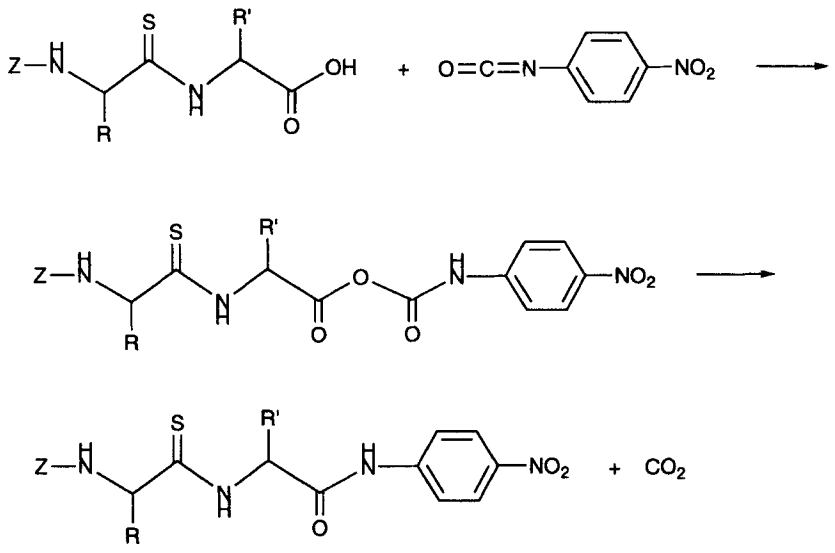

FIGURE 10 C-terminal extension by use of an isocyanate masked amine.

known.[9,21-26,33,43,48,70,74,75,81-89] When used for extension of endothiopeptides made by O/S-exchange, as in all of the just refered papers, the method is only able to provide peptides with thioamides in the C-terminal, the C+1 or maybe the C+2 or C+3 bond, due to the limited regioselectivity of O/S-exchange. This limitation can only be changed by using N-terminal extension in connection with thioacylation.

C-terminal extension of endothiodipeptides has unfortunately been found to be generally unfeasible. The formation of a thiazolone (thiazol-5(4H)-one, Figure 8b) results in inactivation of the otherwise activated C-terminal.[9,73] The thiazolone is not just relatively unreactive, it is furthermore prone to epimerisation.[90] In a few cases, C-terminal extension has actually been accomplished, but only with achiral amino acids, such as Gly and Aib[48,70,73,74,90] or with Pro,[70] which most likely does not form the thiazolone, because it would involve a strained bicyclic structure. When C-terminal extension has been accomplished afterall, yields have been low and problems with the optical purity have been discovered in the C+1 position (α-carbon at R′ in Figure 8).[90,91]

C-terminal extension of an endothiopeptide with the thioamide in a position other than that adjacent to the C-terminal (C+n, $n \geq 2$) may be less problematic, since in this case the five-ring thiazolone cannot be formed. Such chemistry is so far absent from the literature, and other problems may arise.[92] Additionally, for such chemistry to be realized, initial selective thionation of peptides longer than dimers will be required.

Enzymatic activation has been shown to be a valid alternative to the problematic chemical activation of endothiopeptides. The subtle activation by an enzyme, such as chromotrypsin, apparently does not promote thiazolone formation, thus allowing the extension to take place successfully.[80] The method seems to be quite general, but it has not yet been applied to solid-phase work. Application to solid-phase will be complicated by the required access of the enzyme to the insoluble resin particles.

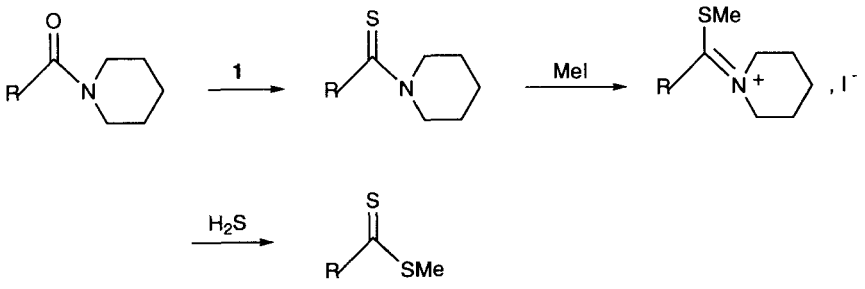

FIGURE 11 Route to dithio ester of amino acid.

An interesting alternative to the problematic activation of endothiopeptide C-terminals is the indirect activation of the *amine* component. The masked amine 4-nitrophenylisocyanate was thus reacted with an endothiodipeptide to give the anhydride (Figure 10), which decarboxylated to the corresponding amide.[51]

This new method has apparently not been tried with α-amino acids (transformed to α-isocyanato acids), and a problem may be predicted. Higher acidity of the α-proton in an α-isocyanato acid can be expected, due to the presence of two neighbouring double bonds. This may lead to enhanced risk of enantiomerisation in the amine part of the coupling.

THIOACYLATION

Considering the current, very efficient stepwise build-up of peptides on solid-phase (SPPS), thioacylation appears as the ideal method for endothiopeptide synthesis. Unfortunately, classical acylation reagents, such as acyl halides and anhydrides have no thiono equivalents among amino acids. α-Amino dithio acids are simply unknown. As mentioned earlier, the first endothiopeptides were actually made by thioacylation, by using ethyl thiono esters.[3,18,19] These esters are, however, sufficiently reactive only with small amino acids,[20] and they have only been used in solution. Alkyl dithio esters (Figure 11) are more reactive,[93,94] and these compounds were for some time thought to be quite universal.[81] Though successfully used for solution phase synthesis in a number of cases,[81,83,85,95,96] dithio esters have just as often turned out to be insufficiently reactive.[33,51,81,90] The relatively low acylating power of alkyl dithio esters in combination with the enhanced risk of enantiomerisation, which is common to all thio esters,[97] seems fatal. The risk of loosing optical purity has previously been somewhat overlooked,[83] but a recent study by Jurayj and Cushman corrected this.[33] Their study demonstrated that alkyl dithio esters are really unsuited for endothiopeptide synthesis, because serious enantiomerisation accompany the slow acylations. Jurayj and Cushman actually succeeded in preparing new dithio esters, which are more reactive than the usual methyl esters, for instance *tert*-butyl glycolate esters. Although faster acylation with these esters was indeed achieved, significant enantiomerisation (>23%) was still found. The better results with the more reactive dithio esters lead Jurayj and Cushman to suggest that development of truly potent dithio/thiono esters might solve the enantiomerisation problems. A very recent paper

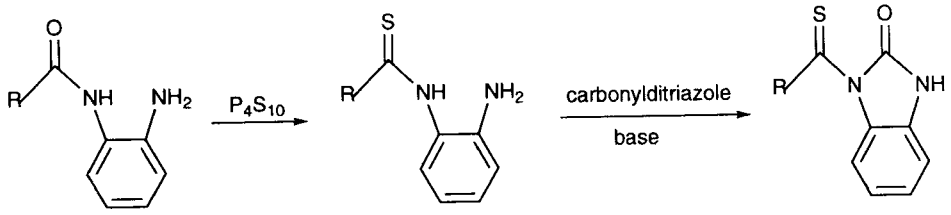

FIGURE 12 Route to amino acid thiobenzimidazolones.

(1995) from Le *et al.*[15] promised to circumvent the described problems with the alkyl esters, by using DMAP plus cesium salts for catalysis of methyl dithio ester thioacylations. Boosted reactivity and preserved stereochemistry are described as being the results of this catalysis. DMAP is known from standard peptide synthesis as a very efficient acylation catalyst, but it is also known for enhancing the risk of racemisation.[98] Therefore the results with alkyl dithio esters are quite surprising. Nevertheless, the application of this new chemistry to SPPS seems very promising.[15] The new achievements of the amino acid alkyl dithio esters also make more interesting the recent efforts to improve the accessibility to these compounds.[99–101] The dithio esters are traditionally prepared by methods (Figure 11) that involve the use of P_4S_{10}-derivatives.[81] This requirement complicates the use of amino acids with side-chain amides (Gln, Asn, pGlu). Efforts to perform endothiopeptide synthesis via silyl ketene dithioacetal intermediates,[102] on the other hand, seem of little practical value, due to the inherent achiral character of these compounds.

In conclusion from the above, thiono or dithio analogs of the numerous active esters, which are first choice in modern peptide synthesis, are desirable for enantiomerisation-free couplings. Such active thiono compounds were until recently unavailable, but two recent efforts have attempted to solve this problem.

Zacharie *et al.* have developed thiobenzimidazolones (Figure 12)[14,103] and used them for the synthesis of two series of endothiopeptides, thymopentin (Arg-Lys-Asp-Val-Tyr) and tuftsin (Thr-Lys-Pro-Arg) analogs. The new amino acid derivatives are apparently sufficiently reactive for SPPS, although long reaction times are often required, e.g. 80 h. The yield of an endothiopentapeptide by SPPS was low, 15% overall,[14] but this is not necessarily due to insufficient thioacylation. Side reactions during the deprotection steps etc. may be the cause as well (piperidine and acid sensitivity, as described above). According to NMR, the thiobenzimidazolones seem to couple under retention of chirality, but a direct chromatographic proof involving diastereomeric standards has yet to be reported. Since the synthetic steps toward preparation of amino acid thiobenzimidazolones involve the use of P_4S_{10}, peptides with backbone thioamides at Asn, Gln and pGlu positions seem to be unavailable by this method. Use of the thiobenzimidazolene of Val for thioacylation of side chain unprotected Ser has recently been demonstrated.[104]

HOBt (1-hydroxybenzotriazole) esterification[105] is the perhaps most popular activation method in contemporary peptide synthesis.[11,12] Many popular peptide coupling reagents contain "build-in" HOBt, and among these are the phosphorus-containing BOP (benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate)[106] and PyBOP (benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate).[107] Exploitation of the oxygenophilicity of phosphorus has opened a

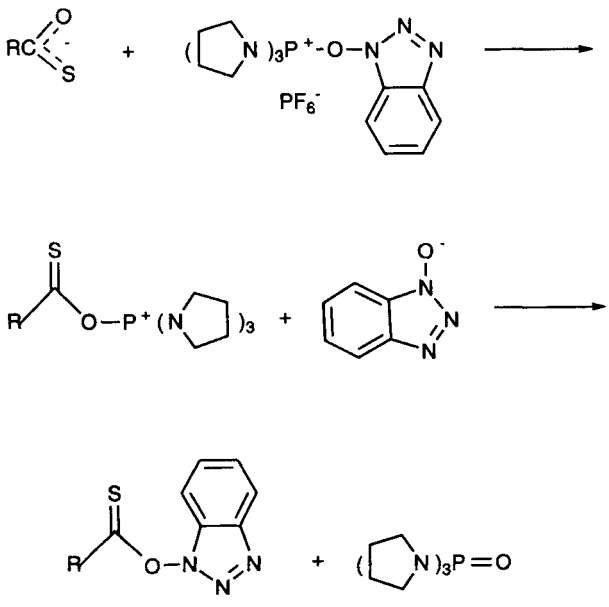

FIGURE 13 Bt thiono ester from monothiocarboxylate and PyBOP.

route to *in-situ* generation of amino acid benzotriazolyl thiono esters (Bt thiono esters), namely by activating N-protected amino monothio acids with phosphorus-based coupling reagents (Figure 13), an elimination of water can be achieved, thereby forcing the sulfur to occupy a thiono function.[13] This chemistry leads to the formation of active thiono esters, well-suited for use in SPPS.[79] Best results have been achieved with faster activating reagents, such as PyNOP,[60] a 6-nitro substituted analog (better leaving group). Importantly, this new thioacylation method preserves amino acid stereochemistry well, as documented by chromatographic analysis.[108] Unfortunately, normal amides as side products can not be avoided. Yields are therefore limited, and chromatographic purification of the final products is necessary.[79] Chromatographic purification of normal peptides is often done anyway, so this obstacle seems acceptable. The required starting materials, the monothio acids, are prepared from active esters, such as N-hydroxysuccinimide esters, by reaction with a H_2S-salt.[109,110] Since no P_4S_{10}-derivatives are involved, side-chain amides are allowed with this particular method.

ENDOTHIOPEPTIDES AS SYNTHETIC INTERMEDIATES

Endothiopeptides can be used as intermediates for the synthesis of other pseudopeptides. The "reduced amide," or methylene amino bond, is perhaps the most widespread peptide backbone modification. This modification is most often introduced by reductive amination of amino aldehydes,[111-113] but it is also accessible by reduction of thioamide linkages (Figure 14). Some problems with stereochemical lability of the amino aldehydes and related intermediates exist.[114] Besides, if the thioamide modified peptide is already at hand, reduction can be convenient route. Endothio-

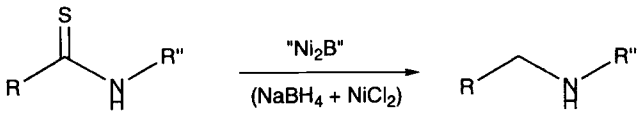

FIGURE 14 Reduction of thioamide to methylen amine.

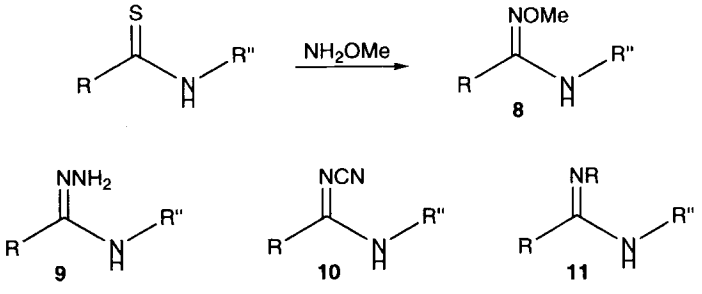

FIGURE 15 Amidoximes, amidrazones, cyano amidines and alkyl amidines.

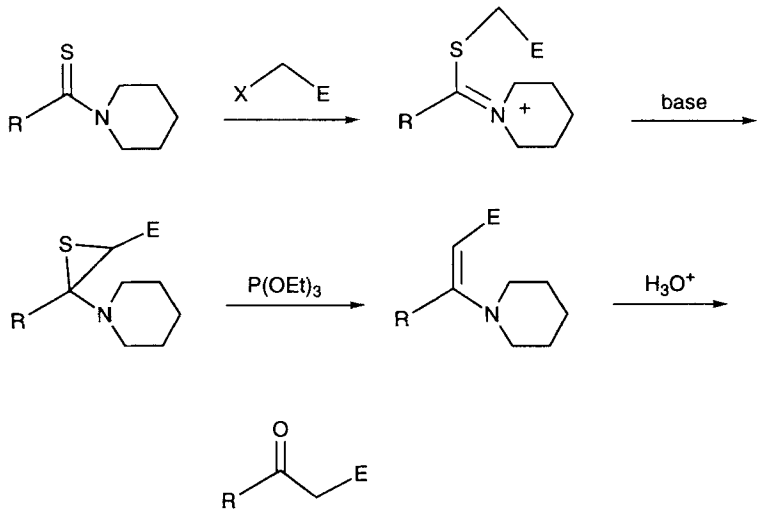

FIGURE 16 Amino acid enamines or ketones from thioamides, X = halide, E = electrophile (CO_2Me, COPh, CN, NO_2 and others).

peptide reduction has been performed by use of Raney nickel,[67] but comparative studies have shown nickel boride (from $NiCl_2$ and $NaBH_4$) to be more efficient.[115] Accordingly, this possibility seems to be the method of choice today.[116-118]

Hydroxylamine can react by nucleophilic displacement with peptide thioamides to form peptide amidoximes **8** (Figure 15). Other nucleophiles such as hydrazine, cyanamide and amines need the catalysis by mercury ions to give reasonable yields of amidrazones **9**, cyano amidines **10** and alkyl amidines **11**, respectively.[78] At least this is the case with the sterically relatively buried backbone thioamides, whereas side-chain thioamides can be more reactive.[119]

Cyclic endothiopeptides have been S-alkylated by treatment with benzyl bromide

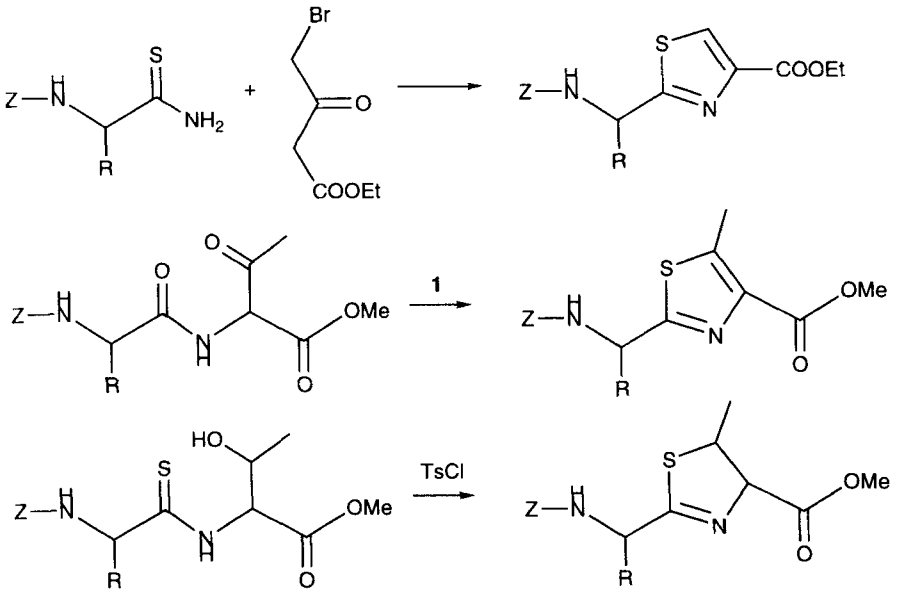

FIGURE 17 Hantzsch reaction to give peptide thiazoles (top). Peptides with β-functionalization transformed to thiazoles (middle) or thiazolines (bottom).

under strongly basic conditions (30% NaOH) to give the corresponding peptide benzyl thioamidates. These could be hydrolysed selectively at the thioamidate position with 6 M aqueous HCl in acetonitril.[36]

Amino thiopiperidides react with methylen halides bearing electron-withdrawing groups, such as α-bromo methyl acetate, to give α-thioiminium salts, which rearrange to episulfides (Figure 16). These can subsequently be desulfurized with triethyl phosphite to yield enamines and, upon hydrolysis, methyl ketone derivatives.[120] Difunctionalised enamines are likewise available from amino acid thioamides by intermediacy of thioiminium salts.[41]

The Hantzsch reaction (Figure 17) can be used for preparing peptide thiazoles by reaction of amino acid thioamides with α-halo carbonyl compounds,[121,122] but there are problems with the stereochemical purity.[123] An alternative is the thionation of dipeptides containing a C-terminal β-oxo amino acid, inducing spontaneous cyclisation.[124,125] A similar chemistry gives peptide thioazolines from peptide β-hydroxy thioamides.[126,127] Finally, peptide imidazolines are available from α-amino thioamides by reaction with α,β-diamino acids.[128,129]

Parenthetically, endothiopeptides may be desulfurized back to the parent amides by aqueous hydrolysis aided by silver ions.[7]

CONFORMATIONAL ASPECTS

The conformation of endothiopeptides and the structure of the thiopeptide bond have been thoroughly investigated by the use of NMR,[41,46,50,68,70,73,130-135] X-ray

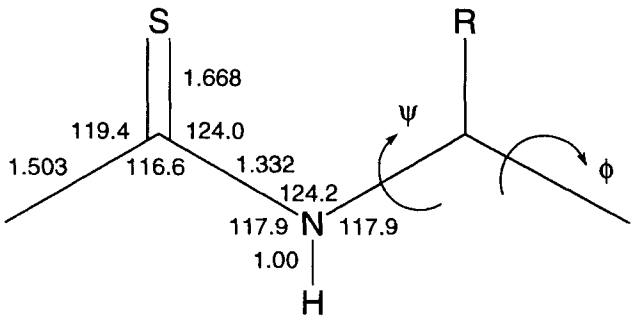

FIGURE 18 Mean geometry of the thiopeptide bond.[138] Bond distances (Å) and angels (°).

crystallography,[34,43,46,67,87,134,136-142] molecular modelling,[46,50,70,134,135,138,141,143] IR,[132-134] and CD.[134,144,145]

UV and MS have been used as well, but these techniques give little structural information.[7,8,146] Fluorescence spectroscopy has been used for measuring intramolecular distances in endothiopeptides, by exploiting the fluorescence acceptor properties of the thioamide.[23]

The thioamide pseudopeptide bond has been found to resemble the normal peptide bond quite well. This is the inevitable conclusion from all of the work refered above. Just like the normal peptide bond, the CSNH bond is usually planar and in a trans configuration.[147] ‡ A higher energy barrier for rotation around the CSNH bond is present, relative to the CONH bond.[132] This is due to more C═N character. The C═S bond is approximately 0.5 Å longer than the C═O bond (Figure 18), and the larger covalent[148] as well as van der Waal radius of sulfur[149] limits the available ϕ, ψ space (Figure 18).[136] This is due to interaction of the amino acid side chains with sulfur. Nevertheless, thioamide linkages can be accepted in usual peptide structures, such as β-turns,[43,139,150,151] γ-turns,[70] α-helixes[138] and β-sheets,[87] with only minor distortions. The refered experimental results are complemented by theoretical investigations using hard-sphere calculations.[143]

Concerning hydrogen bonds, which are essential for the formation of ordered peptide structures, the thioamide function can take part in three different patterns, NHC═O—HNCS, NHC═S—HNCO and NHC═S—HNCS. These patterns have been named MIH (mixed intramolecular hydrogen bonds), IMIH (inverse mixed intramolecular hydrogen bonds) and TTIH (thioamide-thioamide intramolecular hydrogen bonds) respectively.[132] In accordance with the higher acidity of the thioamide function relative to normal amides,[152] the MIH, which is the strongest of the three, is also stronger than the usual amide hydrogen bond. The lower basicity of CSNH relative to CONH means that the IMIH is normally expected to be weaker than usual amide hydrogen bonds,[139] but this is not always the case, due to the role of polarizability.[133] Interestingly, the strong MIH opens the possibility of constructing analogs of biologically active peptides with strengthened bioactive conformations.[68,153] It

‡Peptide chemists generally use cis/trans nomenclature to designate peptide configuration. In Figure 18 the two α-carbons are in a trans configuration around the C—N bond (a partial double bond, due to amide tautomerism). This "trans" form is the normal configuration in peptides. By E/Z-nomenclature the "trans" form should rightfully be called the Z configuration because oxygen/sulfur has higher priority than carbon.

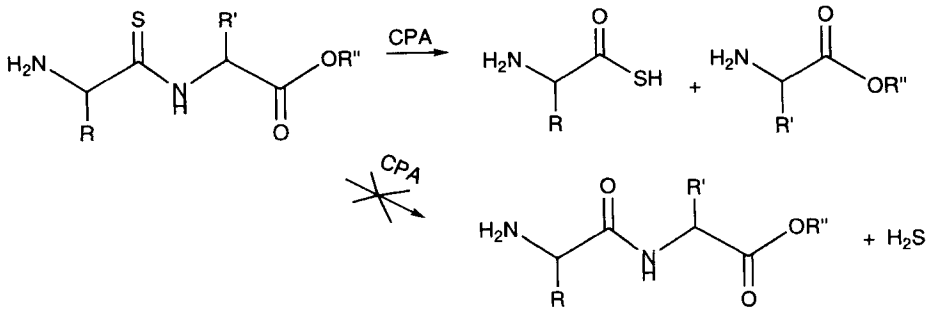

FIGURE 19 Proteolysis to monothioacid and amine, not to amide and H_2S.

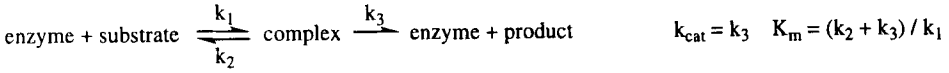

FIGURE 20

should be noted, however, that despite the only subtle differences between the thiopeptide and the normal peptide bond, dramatic shifts in conformation of e.g. cyclic peptides have been observed upon thioamide incorporation.[50,142]

PROTEOLYTIC BEHAVIOR

Not surprisingly, thioamides are hydrolyzed by acid and base at rates comparable to or larger than those of their parent amides. It has been shown that thioacetamide is hydrolyzed by hydroxide about 80 times faster than acetamide,[154] while the rates of acidic hydrolysis are approximately equal.[155] On the contrary, enzymatic hydrolyses of endothiopeptides have been found to be often significantly slower than with the natural peptides. The differences have, however, been found to be highly dependent on individual substrates. Accordingly, the mostly studied enzyme, carboxypeptidase A (CPA), hydrolyses endothiopeptide substrates at rates (k_{cat}/K_m)§ between 10 and 10,000 times lower than usual.[56,57,95,96] The altered values are almost entirely due to changes in k_{cat}. K_m-values for the normal peptides and the thiopeptides are of similar size, indicating that the enzyme binds these substrates at similar strengths. The endothiopeptides are therefore only weak inhibitors of CPA, and as a general property, this can be important for potential medical purposes, since the enzyme is thus not prevented from its normal function. Notably, the investigated degradations of endothiopeptides are regular proteolyses, resulting in the formation of amine and monothio acid (Figure 19), not just hydrolysis to H_2S and amide.[56]

Native carboxypeptidase A contains Zn(II) at the active site, but substitutions with other metal ions have lead to interesting results.[57,95] Cd(II) yields an enzyme that is more active towards endothiopeptides, but relatively inactive towards natural peptides, while the effects of Co(II) and Ni(II) are less pronounced. Mn(II)-CPA is

§K_m, the Michaelis constant, expresses the substrate-enzyme affinity, whereas k_{cat}, the turnover number, expresses the rate of substrate processing (Figure 20).

almost inert towards endothiopeptides, but the activity towards oxo substrates is only slightly reduced. The Hg(II)- and Cu(II)-CPA's are practically inactive, also towards oxo substrates.[57]

Investigations with aminopeptidase P have shown proteolysis of an endothiopeptide substrate to be 1100 times slower than for the corresponding oxo peptide, again only due to changes in k_{cat}.[51] With dipeptidyl peptidase IV the effect of a thioamide substitution "transferred" to the neighboring amide bond, thus leaving this bond approximately 100 times more resistant to hydrolysis. This effect was partly due to a significantly altered K_m-value.[51] Leucine aminopeptidase was found to be totally unable to affect three endothiopeptides, but again the K_m-values were comparable to those of the corresponding oxo substrates, thus only moderate inhibition was observed.[91] Proline oligopeptidase hydrolyzed two endothio substrates more than four orders of magnitude slower than it affected the oxo peptides,[88] while five cysteine proteases of the papain family were slowed down by an endothiopeptide 2–3 orders of magnitude as compared to rates of the usual substrate. Contrary to the above results, angiotensin-converting enzyme hydrolyzed an endothiopeptide substrate at almost unchanged rate, while a second endothiopeptide, which was only slightly different from the first (Ala for Gly substitution), was completely resistant towards proteolysis.[130] This was explained by considering steric repulsion (sulfur-methyl), which disfavored amide bond rotation. Another atypical example is found with a thiopeptide hormone analog (TRH, thyrotropin releasing hormone, pGlu-His-Pro-NH_2), which was degraded by human plasma 3 times faster than the natural hormone.[156]

Work with enzymatic hydrolysis of peptide ethyl thiono esters showed cysteine proteases to attack these at rates similar to the parent esters,[157] whereas serine proteases did not hydrolyze such substrates at a measurable rate.[158]

Curiously, bacterial ribosomes have been shown to be able to catalyze endothiopeptide synthesis by the coupling of N-acetyl leucine 5′-adenylic thiono ester to Phe-tRNA.[159]

BIOLOGICAL ACTIVITY

While the effect of thioamide substitution on peptide proteolysis is most often as expected (some proteolytic resistance), the effect on biological activity is much more unpredictable, if not even unintelligible. This situation was naturally not what was hoped for, when the subtle thioamide peptide modification was originally introduced. Nevertheless, it has become gradually apparent that even the subtlest modification can have a large effect on e.g. peptide conformation, resulting in drastically altered bioactivity. Examples of all possible outcomes are known: Endothiopeptides with considerably higher potency, approximate equipotency, reduced or even no potency.

The first study of peptide thioamide bioactivity involved thioamide substitution in the C-terminal amide of deamino-oxytocin. This yielded an analog with only 6% oxytocin activity and 1.5% vasodepressor activity,[21] relative to the parent peptide. On the other hand, thioamide substitution in a cyclic oxytocin peptide ligand has resulted in a compound having higher affinity (6–7 times) for both oxytocin and vasodepressor receptors.[67]

Substitutions in Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) were investigated simultaneously by two groups, using almost identical synthetic routes and biological assays, guinea pig ileum and mouse vat deferens. The results published by the two groups were similar, although some contradictions did exist. Spatola *et al.*[153] found one enkephalin analog to be practically inactive in both assays, while two other analogs had enhanced potency in both assays (3–13 times). Belleau *et al.*[83] likewise found decreased potency with the first analog. The two other analogs were found to be more active (3–9 times) and almost equipotent, respectively. Belleau *et al.* additionally studied a fourth analog, which Spatola *et al.* did not succeed in preparing,[75] despite the fact that very similar synthetic routes were followed. The previously mentioned acid catalyzed Edman-like degradation of thiopeptides seems to the reason for Spatola *et al.* not obtaining Tyr-Gly-ψ[CSNH}-Gly-Phe-Leu. Differences in the choice of protecting group strategies lead to differences in choice of deprotecting acids (HF or formic acid). Apparantly, formic acid was acceptable, whereas HF cleaved the thiopeptide. Bellaeu's fourth analog was equipotent or about 6 times less active, respectively, in each of the two assays (guinea pig ileum and mouse vat deferens).

Binding studies by both groups revealed behavior generally in accordance with the bioactivity studies. Spatola *et al.* furthermore described the 5 times higher receptor selectivity of one analog relative to native Leu-enkephalin, for the δ-receptor over the μ-receptor. Enhanced receptor selectivities were later also found with three cyclic enkephalin mimics bearing thioamide substitutions.[44] The potencies of these compounds were, on the other hand, univocally lower than that of the parent cyclic mimic, and the potency decreased with increasing number of thioamide modifications (one, two or three). Four Leu- and Met-enkephalin N-methylthioamides (C-terminal thioamide) showed analgetic activity in mice up to 100 times stronger than Met-enkephalin, and the effect was also found to be longer lasting. However, in rats the same compounds were found to have no activity at all.[82]

Early studies of a C-terminally thioamidated TRH analog (pGlu-His-Pro-ψ[$CSNH_2$])[84,156,160] have later been complemented by a reexamination of this and of two other thioamidated TRH analogs.[37] The results from the two studies were similar. All TRH analogs showed thyroid stimulating hormone releasing activities close to the values of the natural hormone, although the potency for a dithioamidated analog was down towards 50%.[37] Significant selectivity between pituitary and cortical receptors was found for two of the analogs.[37] Furthermore, binding studies showed that one analog, despite its almost unaltered potency, gave significantly lower binding (4 times) than TRH.[37] This observation was ascribed to increased receptor response or alternatively, to proteolytic resistance. Sleeping times and motoric activity of rats were similarly affected by a thioamidated TRH analog and the natural hormone.[160]

The root of rubia akane is a source of a couple of potent cyclic peptide antitumor agents. Thioamide substitution in these peptides have lead to four analogs, which possessed either enhanced (up to 8 times) or preserved antitumor activity, as measured by *in vitro* studies on murine lymphotic leukaemia and epidermoid nasopharynx carcinoma cells.[68]

A tripeptide with side-chain thioamidated Asn was, like its parent peptide, a substrate for enzymatic glycosylation, and bindings to the enzyme had similar strengths.[28] Four endothio analogs of thymopentin all retained biological activity

close to the parent compound, which has pleiotropic effects on neuromuscular transmission.[103] Immunosuppressive cyclosporin A, a cyclic decapeptide, was modified to generate nine mono-, di-, tri- and tetrathio analogs, not all structurally resolved. All nine analogs had lower activity than cyclosporin A, but two monothioamides were close to equipotent. The activities of the other analogs decreased with increasing number of sulfur atoms.[46]

Five thioamide analogs of a chemotactic tripeptide showed from 25% activity (release of lysozyme from neurophils) down to complete inactivity.[161] Another disappointing result was obtained with an analog of growth hormone releasing hexapeptide including a C-terminal thioamide. This compound was found to have lost all activity.[26] Further along these lines, a C-terminally thioamidated Substance P hexapeptide analog had a relative potency of only 1.3% (contraction activity on isolated guinea pig ileum).[24]

Turning to endothiopeptides investigated solely for inhibitory effects, a cyclic endothiohexapeptide inhibited trypanosomal triosephosphate isomerease 25 times stronger than the corresponding oxo peptide.[49] A thioamide analog of a tetrapeptide inhibitor of farnesyl transferase had about twice the potency of the parent peptide.[162] A dipeptoid cholecystokinin antagonist thioamide analog was binding to both rat pancreas and mouse cerebral cortex receptors at roughly half strength, while a slightly different dipeptoid structure gave a thioamide analog with once again approximately half the usual affinity for one receptor, but only 4% for the other.[163] Attempts of finding an antagonist for gastrin by preparing thionated analogs of its C-terminal tetrapeptide gave no inhibitory effect.[58] A study of thioamidated betaine, a peptide-like aminopeptidase inhibitor, showed its inhibitory effect to be decreased 5 to 40 times towards different aminopeptidase variants.[35]

MISCELLANEOUS

Amino acid and peptide thioamides are potent ligands for complexes with Cu(II) and Ni(II). The stability constants for the thioamide complexes are orders of magnitude higher than for the corresponding amides.[164] Similar results are found for complexes with Cd(II),[165] and with Pt(II) and Pd(II).[166]

Semi-automated amino acid analysis of endothiopeptides can be performed in the usual fashion, typically involving hydrolysis in boiling 6 M HCl for 12–24 h, followed by phenylisothiocyanate derivatisation and HPLC analysis. Not surprisingly, the thioamidated amino acids undergo considerable stereochemical isomerization during the hydrolytic process, as shown by analysis upon derivatisation with Marfey's reagent (N-(2,4-dinitro-5-fluorophenyl)-L-alaninamide).[167] This is, however, no problem for standard amino acid analysis, since here no chiral environment is present. Additionally, the sulfur atom is lost in the hydrolytic process, so the usual amino acid standards are valid.

ABBREVIATIONS

Boc	*tert*-butyloxycarbonyl
BOP	benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate

Bt	benzotriazolyl
'Bu	*tert*-butyl
CPA	carboxypeptidase A
DMAP	4-dimethylaminopyridine
DME	dimethoxyethane
Fmoc	9-fluorenylmethoxycarbonyl
HOBt	1-hydroxybenzotriazole
HONSu	N-hydroxysuccinimide
IMIH	inverse mixed intramolecular hydrogen bonds
MIH	mixed intramolecular hydrogen bonds
Np	4-nitrophenyl
pGlu	pyroglutamic acid (5-oxoproline)
PyBOP	benzotriazol-l-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
PyNOP	6-nitrobenzotriazol-l-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
SPPS	solid-phase peptide synthesis
THF	tetrahydrofurane
TRH	thyrotropin releasing hormone
TsCl	tosyl chloride
TTIH	thioamide-thioamide intramolecular hydrogen bonds
Z	benzyloxycarbonyl

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