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PAPER

Thionation of di and tripeptides employing thiourea as a sulfur transfer reagent

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A simple and efficient method for the synthesis of thiopeptides by the treatment of N^{α}-protected peptide esters employing DMF/PCl_5 and thiourea as a sulphur transfer reagent is described. The conversion is carried out at room temperature within short duration of reaction time. The method is high yielding and free from racemization. Multiple thionation is demonstrated by conversion of two peptide bonds of tripeptides into thioamides. In addition, amino acid derived arylamides are also converted into aryl thioamides.

Introduction

Peptide backbone modification by replacement of an amide bond by a thioamide moiety has attracted considerable attention in recent years for several reasons. The receptor interactions of sulfur analogues of biologically active peptides may be more selective or more potent than their parent compounds. In addition, the enhanced stability against enzymatic action can be expected towards various proteolytic enzymes. It is also possible to obtain backbone-conformations compatible with the three major types of regular secondary structures (α-helix, *β*-sheet, and *β*-turn) through thioxylated peptides.[1] Thioxopeptides, popularly known as thiopeptides, can be employed for the synthesis of a wide range of peptidomimetics including triazole,[2,3] tetrazole,[4] thiazole,[5-9] and reduced amide isosteres[10] as well. Replacing the oxygen of a peptide bond with sulfur has several consequences.[11] The larger radius of sulfur and the larger charge transfer from nitrogen to sulfur make thioamide a higher barrier of the rotation about the C-N bond. The thioamide has a higher rotational barrier than does an oxoamide, owing to greater C–N double bond character. The thiocarbonyl bond is 37% longer than that of a carbonyl group, and sulfur has 32% larger van der Walls radius than oxygen, which could disturb close atomic packing within the triple helix.[12-15] Thioamides are weaker hydrogen bond acceptors as well as stronger hydrogen bond donors than is an oxoamide[16] and are known to perturb backbone n-π^* interactions.[17,18] Thioamides therefore provide a strategy for subtly modulating a variety of effects on protein stability. Recently thioamide was employed as fluorescence quenching probe in the monitoring of the unfolding of small proteins.[19,20]

Methods for the preparation of thiopeptides mainly involve the conversion of amide to thioamide using thionating agents and the use of thioacyalting agents which involve multistep protocols. Thionation of *N*- and *C*-protected peptides to thiopeptides have been reported using P_4S_{10}/triethylamine (TEA)[21] or Lawesson reagent (LR) under reflux conditions.[22,23] Belleau group employed Yakoyama's reagent which is structurally similar to that of LR. It can be used for the selective thionation of amides even in the presence of other susceptible groups such as ester, urethane, or keto carbonyls. Recently our group reported an improvement for the synthesis of thiopeptides from dipeptide esters utilizing ultrasound assisted P_2S_5.[24] This route was later demonstrated to be useful in the preparation of all thioamidated tri- and tetrapeptides including

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† Electronic Supplementary Information (ESI) available: Details of mass spectrometry, 1H and 13C NMR spectra of synthesized compounds and chiral HPLC chromatograms. See DOI: 10.1039/x0xx00000x

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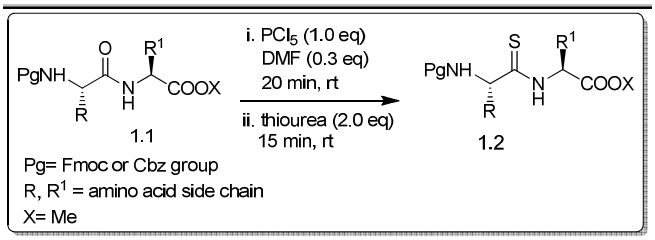
the ones containing norleucine (Nle) as well. Multiple thionation of oxygenated peptides was accomplished by treatment with P_2S_5 in anhydrous THF under ultrasound irradiation in about 3-5 hr with addition of 9-10 equiv of P_2S_5 in several portions. On the other hand, the synthesis of Ala bis and tristhioamidation involved use of excess of LR and longer reaction duration (upto nine days).[25] Aparna et al. employed thiourea as thionating agent but the reaction was carried out under solvothermal conditions in an autoclave for about 4 hours.[26] Ley et al. reported the synthesis of thioamides using aminothiophosphate resin as a polymer supported thionating agent.[27] Most of these procedures often require excess of reagents and elevated temperatures for longer durations.

Several groups developed thioacylating agents as monomeric building blocks for selective thionation in an oligopeptide. Rapoport and co-workers described the thioacyl benzotriazolides of amino acid derivatives as efficient thioacylating agents to obtain endothiopeptides.[28] The thioacyl benzotriazole precursors were made in three steps involving preparation of anilides, thionation and cyclization through TFA treatment resulting in the Boc protected amino acid derived benzotriazoles which were used as building blocks. Later the same group employed thioacyl nitrobenzotriazole as thioacylating agent based on requirement of good leaving group adjacent to thiocarbonyl group.[29] In addition, thioacyl benzimidazolinones, thioacylfluorobenzimidazolones, thioacyl-*N*-phthalimides[30] have been employed as thioacylating agents for the preparation of thiopeptides.[31]

Alternatively the formation of thiocarbonyl function (C=S) makes use of N^{α}-protected amino thioacids mediated by phosphorous based reagents such as benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP)[32] and bis(2-oxo-3-oxazolidinyl) phosphonic chloride (BOP-Cl).[33]

Results and discussion

In the initial part of the study, model substrate Cbz-Phg-Phe-OMe was treated with PCl_5 for 20 min at room temperature followed by the addition of thiourea (1.5 equiv) in ethanol (5 mL). After 15 min of the reaction, the expected thioxopeptide was obtained in 52% yield along with significant amount of unreacted peptide. To improve the yield, different solvents and chlorinating agents including triphosgene, oxalyl chloride, thionyl chloride, and phosphorous oxychloride were tried for the conversion of peptide into imidoyl chloride intermediate prior to thionation. None of the reagents proved beneficial than PCl_5. Further investigations revealed that PCl_5 in the presence of catalytic amount of DMF (0.3 equiv) in acetonitrile as solvent drastically increased the yield to 95%. After the initial reaction had subsided, stirring at 25 ^{o}C was continued for another 15 min. A simple work up followed by the column purification of the crude product led to the isolation of the thioxopeptide **1.2a** in good yields (IR absorption: strong peak at 1538 cm^{-1} for C=S stretching frequency; ^{13}C NMR δ 205 ppm for C=S carbon).



Scheme 1 One pot synthesis of thioxopeptides from peptides using thiourea

Table 1. Optimization of thionation of peptides.

Entry	Chlorinating reagent	Solvent	Yield
1	PCl_5	C_6H_6	52
2	PCl_5	CH_2Cl_2	48
3	triphosgene	C_6H_6	NR[a]
4	oxalyl chloride	C_6H_6	NR
5	$SOCl_2$	C_6H_6	40
6	$POCl_3$	C_6H_6	34
7	DMF/PCl_5	C_6H_6	93
8	DMF/PCl_5	CH_2Cl_2	78
9	DMF/PCl_5[b]	CH_3CN	95
10	DMF/PCl_5	Toluene	85
11	DMF/PCl_5	THF	55

[a] NR = no reaction. [b] 0.3 equiv with respect to quantity of PCl_5

The above optimized protocol was then utilized for the conversion of a series of dipeptides into thioxopeptides. Different peptide esters with Cbz- and Fmoc- as N-protectors as well as benzoyl and acetyl protected amino acid esters were converted into respective thioamides. As delineated in Table 2 it was found that all the thioxopeptides were obtained in good to excellent yields after purification by column chromatography.

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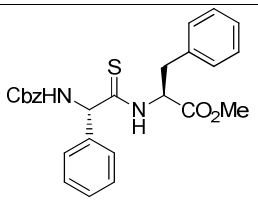
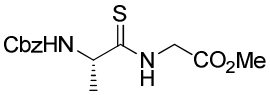
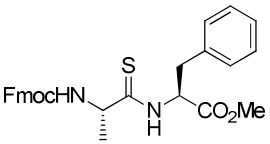
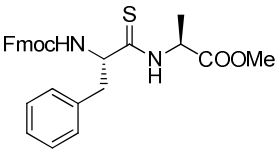
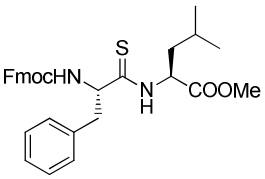
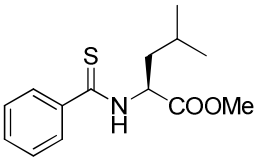
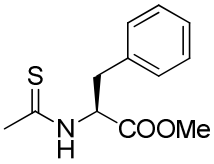
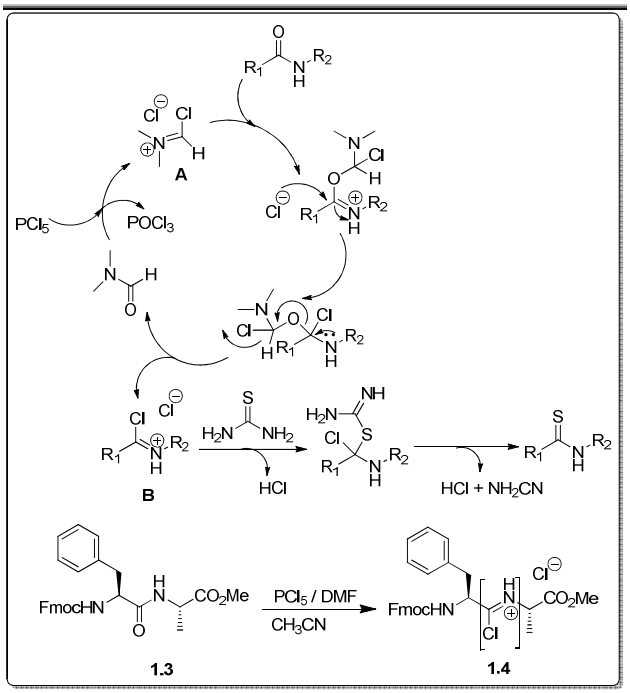
Table 2 List of thioxo dipeptide/aryl/alkyl thioamide esters synthesized

Entry	Compound	Yield[a] (%)
1.2a		95
1.2b		92
1.2c		90
1.2d		88
1.2e		86
1.2f		91
1.2g		87

[a] Isolated yields after silica gel (100-200 mesh) column chromatography.

The generation of imidoyl chloride from dipeptide employing PCl_5/DMF has been confirmed to be free from racemization.[34] The thiopeptides were analyzed by RP-HPLC for possibility of epimerization under the present conditions. The thiopeptdes Cbz-L-Phg-ψ[CSNH]-Phe-COOMe 1.2a and Cbz-D-Phg-ψ[CSNH]-Phe-COOMe 1.2a* showed retention times of 16.44 min and 12.67 min respectively. And their 1:1 mixture showed distinct retention times of 17.29 and 13.63, thereby confirming the absence of epimerization during their synthesis from respective peptides.

Based on the results of our study and previous reports, a plausible mechanism for the formation of thiopeptide has been presented in scheme 3. The first step involves the reaction of DMF with PCl_5 to give a Vilsmeier type reagent (**A**) (for ESI-MS of the intermediate **1.4** generated from Fmoc-Phe-Ala-OMe **1.3**). In the next step, the iminium chloride **A** reacts with peptide leading to the formation of imidoyl chloride tethered intermediate **B** with the release of DMF. The intermediate **B** then reacts with thiourea to produce an intermediate species, which rearranges into the desired product. The catalytic amount of DMF is crucial for the generation of imidoyl chloride intermediate as recorded in earlier reports.[35]



Scheme 2 Postulated mechanism for the DMF/PCl_5 mediated thionation of the peptide bond.

Peptide-thioxo peptide hybrids: *N*-Terminal extension of thioxo dipeptides

The chain extension from the *N*-terminus of Fmoc protected thioxopeptides leading to tripeptide bearing one thioxo peptide bond and the peptide bond/s in the backbone was undertaken. In a typical example, thioxodipeptide was treated with diethylamine in dry dichloromethane (DCM, 40%) and the free amino thioxodipeptide ester **1.6** (not isolated) was coupled to Boc-Gly-OH under standard peptide coupling conditions using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/ 1-hydroxybenzotriazole (HOBt) method. The resulting hybrid peptide **1.7** was isolated after

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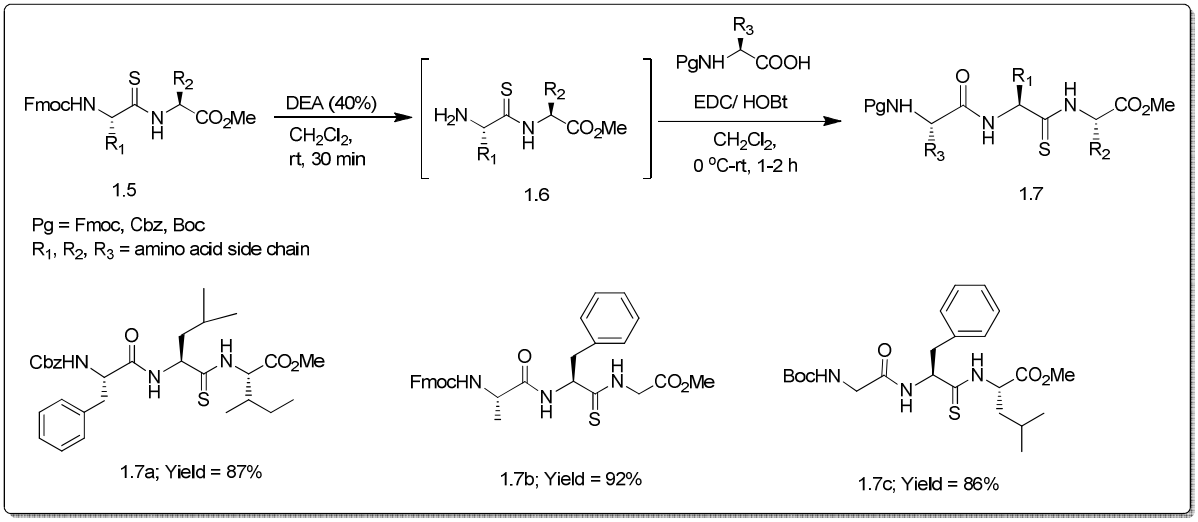
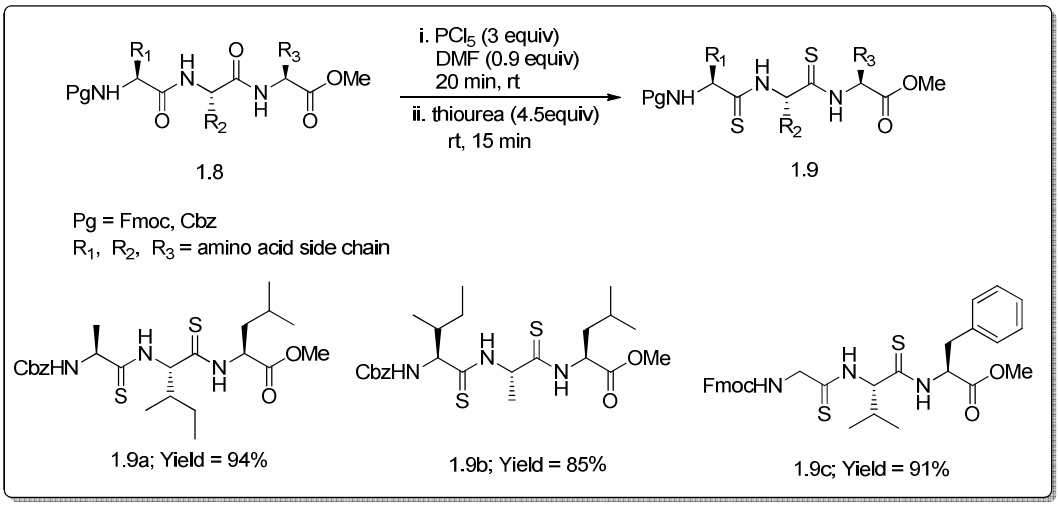
column purification in good yield (**Scheme 3**).



Scheme 3 Synthesis of thioxo peptide-peptide hybrids

Dithioxo Tripeptides: Tripeptides possessing two thioxo bonds

The scope of the present protocol is then extended to thionation of two peptide bonds of tripeptide esters. For this, initial experiments were carried out using three equiv of each reagent; however, it led to the isolation of the product **1.9** in about 45−60% yield. Later, on careful optimization, it was found that three equiv of PCl_5, 0.9 equiv of DMF, and 4.5 equiv of thiourea was necessary to obtain the acceptable yield of products **1.9** (Scheme **4**).



Scheme 4 Synthesis dithioxo tripeptide esters.

Thionation of amino acid arylamides

Amino acid arylamides are widely used in preparation of polymers, peptidomimetics, and pharmaceuticals. The present study also encompassed the conversion of amino acid arylamides to thioamides. The arylamides were then treated with PCl_5 mediated by DMF and thiourea by using the optimized conditions shown in scheme 5. The products were obtained in moderate to good yields (Table **3**).

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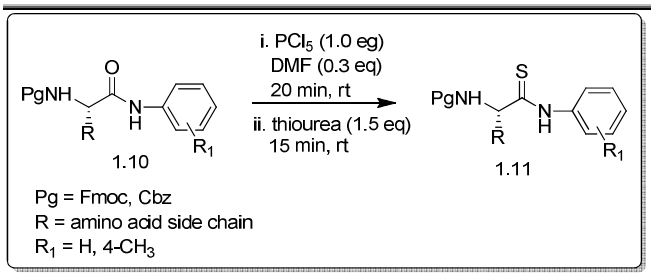

Scheme 5 Synthesis of amino acid derived arylthioamides.

Table 3. List of amino acid arylthioamides

Entry	Pg	R	R_1	Yield (%)
1.11a	Fmoc	$CH_2CH(CH_3)_2$	4-CH_3	87
1.11b	Fmoc	CH_3	H	86
1.11c	Fmoc	CH_2COOBn	4-CH_3	84
1.11d	Cbz	$CH_2C_6H_5$	H	83
1.11e	Cbz	H	H	79

Conclusions

In summary thioxopeptides and oligothioxopeptides can be prepared in good yields from corresponding peptides employing thiourea as a thionating reagent. The method offers a high-yielding, racemization-free, and cost effective way to access the title compounds. The versatility of the method is demonstrated by its application to peptides containing differential N^{α}- and carboxyl protection. Further, peptide-thioxopeptide hybrids can be prepared by coupling to N^{α}-free amino thioxopeptides in good yields.

Experimental Section

General

All chemicals were used as obtained from Sigma Aldrich Company, USA. All the solvents were dried and purified using recommended procedures in the literature whenever necessary. High resolution mass spectra were recorded on a Micromass Q-TOF micromass spectrometer and ESMS on a LCQ Deca XP MAX using an electron spray ionization mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker NMR AV 400 MHz and 100 MHz spectrometers, respectively, at the Indian Institute of Science, Bangalore. RP-HPLC analysis of epimers was carried out by using a LCQ Deca XP MAX VWD at λ = 254 nm; flow rate: 1.0 mL min−1; column: Thermoscientific C18 syncronis, pore size-5 µm, diameter × length = 4.6 × 250 nm; method: gradient 0.1% TFA water–acetonitrile; acetonitrile 30–100% in 30 min. Melting points were determined in an open capillary and are uncorrected. TLC experiments were performed using MERCK TLC aluminum sheets (silica gel 60 F254) and chromatograms were visualized by exposing in an iodine chamber or to a UV-lamp. Column chromatography was performed on silica gel (100–200 mesh) using ethyl acetate and hexane mixtures as the eluent.

General procedure for the synthesis of thioxodipeptide/aryl/alkyl thioamide esters 1.2a-1.2g, 1.11a-1.11e.

To a stirred suspension of protected dipeptide ester (1.0 mmol) in acetonitrile (5 mL) was added PCl_5 (205 mg, 1.0 mmol) and DMF (0.025−0.03 mL) at room temperature under nitrogen atmosphere. A clear yellow solution was formed after 10 min. The stirring was continued for another 10 min. Then, a prepared ethanolic solution of thiourea (360 mg, 1.5 mmol, 0.947 M) was added. The reaction was found to be complete in 15 min (TLC analysis). The solvent was evaporated under vacuum and diluted with EtOAc (10 mL). Organic phase was washed with 1 N $NaHCO_3$ (3 × 10 mL), 1 N citric acid (2 × 10 mL), H_2O (2 × 10 mL), and saturated NaCl (10 mL) solution and dried over Na_2SO_4. The solvent was filtered and evaporated under reduced pressure. The crude reaction mixture was purified under column chromatography using hexane/ethyl acetate (8:2) as the eluent to afford the desired product.

General procedure for the preparation of thioxo peptide-peptide hybrids 1.7a-1.7c

Step 1. To a solution of Fmoc protected thioxo dipeptide ester **1.5** (1.0 mmol) in CH_2Cl_2 (10.0 mL), 18 mL of 40% DEA in CH_2Cl_2 was added, and the solution was stirred for 20 min at rt under nitrogen atmosphere. After complete deprotection of the Fmoc group (observed by TLC analysis), the solvent and excess DEA were removed completely under reduced pressure with repeated co-evaporation with CH_2Cl_2. The resulting amino free thioxo dipeptide ester was dissolved in anhydrous THF (5.0 mL) and maintained at 0 °C.

Step 2. Activation of Fmoc/Z-amino/dipeptide acid (1.1 mmol) was carried out separately by dissolving with dry THF (5 mL) and cooled to 0 °C. EDC (1.0 mmol) and HOBt (1.2 mmol) were added to the above solution and stirred for 10 min. While maintaining the temperature at 0 °C, the above amino free thioxo peptide ester (1.1

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mmol) was added, and the resulting mixture was stirred for 1−2 h, during which the coupling was complete. The solvent was evaporated under a vacuum and diluted with EtOAc (10 mL). Organic phase was washed with 1 N $NaHCO_3$ (3 × 10 mL), 1 N citric acid (2 × 10 mL), H_2O (2 × 10 mL), and brine (10 mL) solution and dried over Na_2SO_4. The solvent was filtered and evaporated under reduced pressure. The crude reaction mixture was purified under column chromatography using hexane/ethyl acetate (8:2) as the eluent.

General procedure for the synthesis of dithioxo tripeptide esters 1.9a-1.9c

To a stirred suspension of protected tripeptide ester (1.0 mmol) in acetonitrile (10 mL) was added crystalline PCl_5 (615 mg, 3.0 mmol) and DMF (0.062 mL). A clear solution was formed within 20 min at rt under nitrogen atmosphere. Then, a prepared ethanolic solution of thiourea (1.082 g, 4.5 mmol, 2.847 M) was added. The reaction was completed in 15 min (TLC analysis), solvent was evaporated under vacuum and diluted with EtOAc (10 mL). Organic phase was washed with 1 N $NaHCO_3$ (3 × 10 mL), 1 N citric acid (2 × 10 mL), H_2O (2 × 10 mL), and brine (10 mL) solution followed by drying over Na_2SO_4. The solvent was filtered and evaporated under reduced pressure. The crude reaction mixture was purified under column chromatography using hexane/ethyl acetate (8:2) as the eluent.

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Thionation of di and tripeptides employing thiourea as a sulphur transfer reagent

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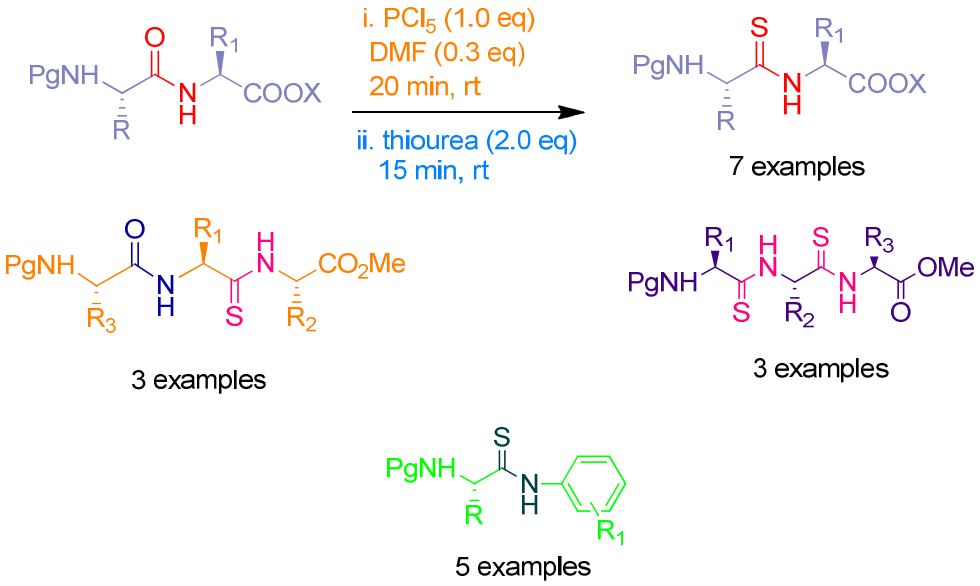

Keywords: Peptides • peptidomimetics • thiourea • thionation • thiopeptides

Thiopeptides has been introduced into peptide backbones by thionation employing DMF/PCl_5 and thiourea as a sulphur transfer reagent. The protocol was also successfully used for the thionation of two peptide bonds, tripeptide bearing thioxopeptide-peptide hybrids, amino acid derived arylamides to aryl thioamides in good yields.