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Chemoselective thioacylation of amino acids. Preparation of the four monothiothymopentin analogs and their biological activity[1].

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Abstract: A methodology for the specific site incorporation of thioamide linkages into a growing peptide under mild conditions using thioacylating agent **1** is described. Thus, the synthesis of the four monothioanalogues of thymopentin (TP-5): [Valt^4]- **18**, [Aspt^3]- **19**, [Lyst^2]- **20** and [Argt^1]- **17** is reported. These thiopeptides retained the biological activity of the parent compound.

The potential utility of peptides as response modifiers, hormones, neuroeffectors or immunomodulators is limited by their short half-lives in-vivo. This phenomenon is primarily due to the degradation of such peptides by proteolytic enzymes. This limits their theurapeutic usefulness. It is therefore desirable to stabilize the backbone amide linkages of these peptides in order to develop new pharmaceutical agents with prolonged action or more selective properties.

Recent advances in replacement or modification of peptide linkages indicate that such linkage stabilization is feasible. Replacement of amide linkages with thioamide bonds at selected sites in the peptide backbone yields increased resistance to degradation of the peptide by proteolytic enzymes[2]. Subsequently, thiopeptide analogues of oxytocin[3], leucine-enkephalin[2b,4], cyclic enkephalin[5], demorphin[6], thyrotropin-releasing hormone[7], and growth hormone releasing hexapeptide[8] have demonstrated increased[2b,5,6], decreased[4,6,8], or nearly equipotent[7] pharmacokinetic activity relative to their parent structures.

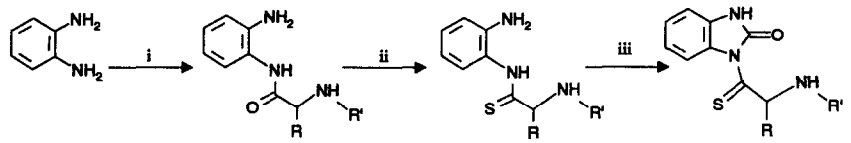
It has been shown[9,10] that the replacement of oxygen with sulfur in the amide bond does not change the geometry of the bond. However, it is expected that the presence of a thioamide bond will alter the secondary structure of the polypeptide chain due to the decreased tendency of sulfur, relative to oxygen, to participate in structural hydrogen bonds. This indicates that incorporation of thioamide bonds might be of special interest in connection with structure-function studies of biologically active peptides such as thymopentin (Arg-Lys-Asp-Val-Tyr). Thymopentin was selected because it has pleiotropic actions on neuromuscular transmission and immune functions[11], and potential theurapeutic applications in the treatment of rheumatoid arthritis[12]. However, thymopentin(TP-5) is susceptible to rapid degradation by proteolytic enzymes. This precludes oral dosage. The half-life of TP-5 is approximately 30 seconds in the plasma[12,13]. It was hypothesized that substitution of amide linkages with appropriate thioamide bonds would provide analogs of TP-5 with a longer lasting biological activity. We now report on the preparation and the activity on neuromuscular transition of the four possible monothioanalogues of thymopentin: [Tyr^5,Valt^4], [Tyr^5,Aspt^3], [Tyr^5,Lyst^2], [Tyr^5,Argt^1]-TP-5.

Thionation of peptides has until recently attracted relatively little attention[14]. This may be explained by the lack of suitable methods by which to selectively introduce the thioamide bond into peptides. Few approaches have been reported for the synthesis of endothionopeptides. Thioesters[15] and dithioesters[2c,16] have been employed in coupling reactions with α-aminocarboxylates. No information, however, has been reported regarding racemization. An important developement has been the introduction of Lawesson's

phosphetane disulphide reagent as a direct thionating agent for suitably protected dipeptides[17]. More recently, monothionation of the less hindered glycyl amide sites in tri-, tetra and pentapeptides has been described[18]. All of these methods, however, display lack of reaction site specificity. Decreased purity and overall yields have been observed because of side reactions. Furthermore, the optical integrity of the final product is often not maintained because of apparent racemization induced by the thioacylating agents[19].

Accordingly, we developed a new method for preparing thioacylating reagents of the general formula **1**. These were synthesized according to the sequence shown in Scheme I. Characterizations of these reagents as well as intermediates useful in their preparations are reported elsewhere[20].

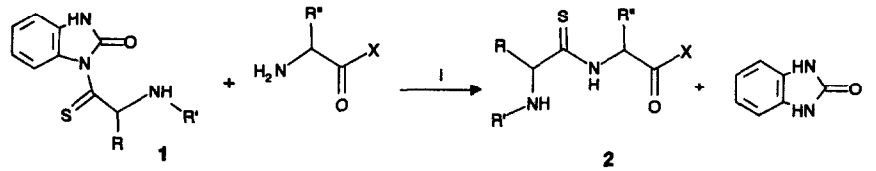
Scheme I[a]



[a]Reagents and conditions: (i),protected amino acid,DCC,CH_2Cl_2; (ii), P_2S_5 in THF; (iii), Carbonylditriazole, collidine.

A thioamide moiety may be introduced into a growing peptide chain at a specific site in the peptide sequence by reacting the thioacylating agent **1** with a protected amino acid or peptide. Scheme II describes the general pathway for the incorporation of the thioamide linkage into the peptide backbone.

Scheme II[a]

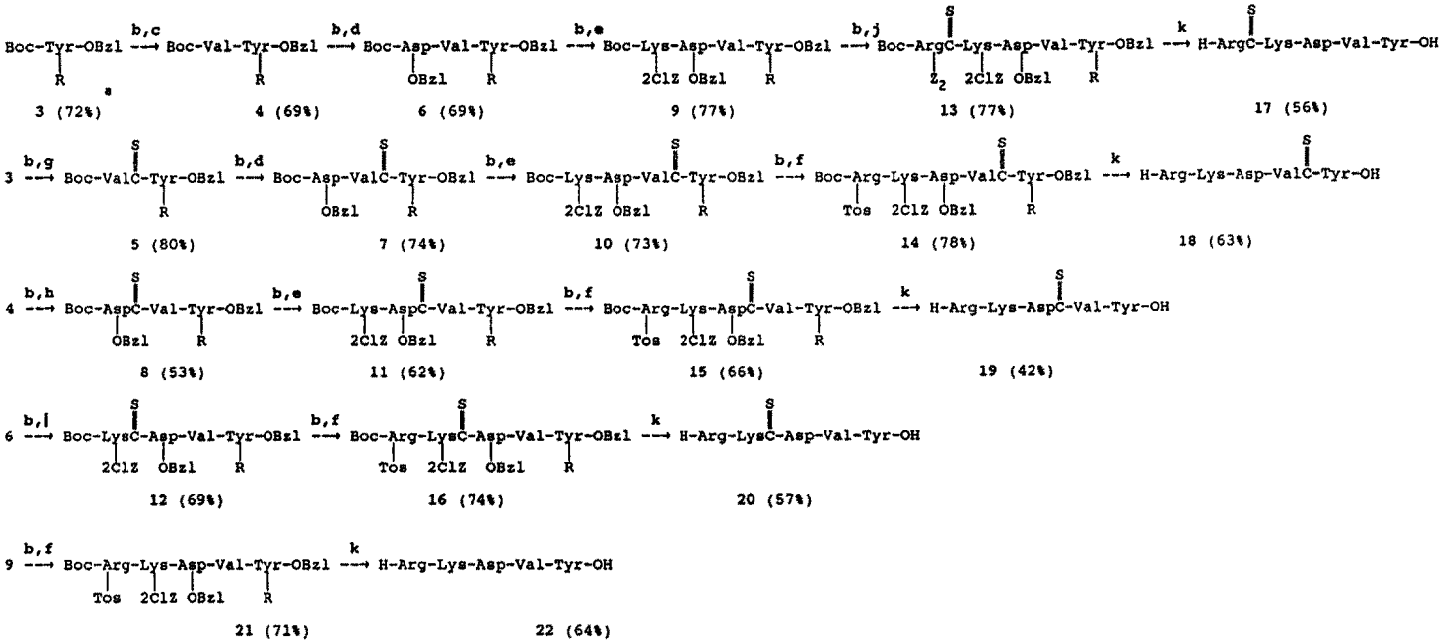


R' = Boc,Z, R and R" = amino acid side chains
X = protected carboxylate or peptide

[a]Reagents and conditions: (i), DMF or CH_2Cl_2, 25°C, 4 h to overnight.

The monitored aminolysis of **1** with an equimolar amount of amino acid or peptide in DMF or CH_2Cl_2 medium was carried out to afford Z (Boc or Fmoc)- di or poly peptide **2** in moderate to high yield. This general procedure was followed for the synthesis of the four monothioanalogues **17-20** of thymopentin[21] starting from Boc-Tyr(2,6-Cl_2-Bzl)-OBzl **3**[22] through the sequence shown in Scheme III. Until the thioamide linkage is required to be introduced, peptides **4**, **6** and **9** may be synthesized with the use of DCC-HOBt or

SCHEME III. SYNTHETIC SCHEME FOR THIOTHYMOPENTIN AND ITS FRAGMENTS [a,b]



[a] Abbreviations: R=(2,6-dichloro-Bzl)

[b] Reagents and conditions: (a) yields; (b) TFA/CH_2Cl_2, $NaHCO_3$ (c) Boc-Val-OH, DCC, HOBt, CH_2Cl_2 (d) Boc-Asp-(OBzl)-OH, DCC, HOBt, CH_2Cl_2 (e) Boc-Lys-(2Cl,Z)-OH, DCC, HOBt, CH_2Cl_2 (f) Boc-Arg-(Tos)-OH, DCC, HOBt, CH_2Cl_2 (g) 1 (R=$CH(CH_3)_2$, R'=Boc), DMF (h) 1 (R=CH_2-COOBzl, R'=Boc), DMF (i) 1 (R=$(CH_2)_4$-NH (2Cl, Z), R'=Boc), DMF (j) 1 (R=$(CH_2)_3$-NZC(=NH)-NHZ, R'=Boc), DMF (k) HF, anisole, EtSMe, thioanisole 0°C

EEDQ coupling agents. Alternatively, the thioamide linkage may be introduced first as in the case of **5**, **8** and **12** and the thioamide so formed may then be elongated employing, for example, DCC mediated coupling. For example, to prepare monothiopeptide **7**, Boc protected amino group of compound **5** was removed by treatment with cold trifluoroacetic acid (TFA) at 0°C, the TFA salt of thiopeptide **5** was then partitioned between CH_2Cl_2 and aqueous $NaHCO_3$. The free base formed was coupled with Boc-Asp-(OBzl)-OH using DCC-HOBt to give the desired thiopeptide **7** in high yield.

Thioacylating reagents **1** are generally stable yellow compounds, easy to handle, and can be stored for a few months at 4°C without decomposition. However, on standing for several hours at room temperature some were susceptible to decomposition. Hence, Boc aspartic thioacylating reagent (**1**, R = $CH_2COOBzl$, R′ = Boc) should be prepared, purified and stored immediately at 4°C. The aminolysis of this compound is normally done at lower temperature in order to minimize side reactions. Thus, to prepare thiopeptide **8**, Boc aspartic thioacylating reagent was added to the free amine of **4** at 0°C under N_2, the reaction was brought to room temperature, and then stirred for ten hours at this temperature before workup. In general, thioacylating reagents **1** react efficently at 25°C with free amino peptides with the formation of the byproduct benzimidazolone. The latter is readly removed from the reaction mixture by filtration or by chromatography.

Once the incorporation of the thioamide linkage was completed, and the thiopeptides were prepared, the fully protected analogues **13-16**[23] were treated with HF-anisole. The deprotected monothiothymopentin was purified by sephadex gel filtration and by reverse phase HPLC. This gave pure thiopeptide **17-20**[24]. Through these sequential reactions, all peptide and thiopeptide intermediates gave no evidence of racemization as demonstrated by the absence of diastereoisomers in the ^{1}HNMR (400Mhz) spectra.

To evaluate the activity of the four monothionated analogs, their effect on neuromuscular transmission was measured as described by Audhya and Goldstein[25]. This method was used to show that thymopoietin was the essential sequence for the biological activity of bovine thymus extracts[11a,26].

The stimulation was produced by electrodes placed in the groin and an electrode inserted into the thigh muscles served to record the muscle action potentials. The stimulator was programmed to deliver 10 impulses of 0.3 msec. duration. The mean of the ratio of the height of the muscle action potential generated by the tenth impulse to the initial impulse was calculated for each experimental animal.

The control group shows no significant change of the neuromuscular response, whereas in the treated groups there is a significant decrement. This means that all four monothionated analogs produced a decrease in neuromuscular transmission indicating that the four novel compounds displayed an effect similar to the TP-5 parent in this assay. Table 1 summarizes these results.

Table 1. Representative data from electromyographic study analyzing the ratio of the tenth to the first muscle action potential. Student's test was used for the statistical analysis

Group	Compound	N	Mean	Standard deviation	P-Value
1	Control	5	0.98	0.01	
2	TP5- 10 mg	5	0.83	0.02	<0.001
3	**18** - 30 mg	5	0.84	0.03	<0.001
4	**17** - 10 mg	4	0.85	0.02	<0.001
5	**20** - 10 mg	3	0.83	0.02	<0.001
6	**19** - 10 mg	4	0.89	0.03	<0.01

In conclusion, we have developed a general and efficient method for the incorporation of thioamide linkages into a growing peptide at a specific site in the peptide sequence by reacting a thioacylating reagent with an amino terminus of a protected C-terminal of amino acid or peptide. To our knowledge, this is the first general procedure described for the incorporation of thioamide linkages into specific sites on the backbone. This is accomplished with relative ease and in substantially higher yields than observed in prior processes while retaining the optical integrity of the peptide.

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21. All the peptide segments and peptide derivatives in scheme III displayed the expected signals in their ^{1}HNMR spectra. Chemical shifts of H, H^{α}, H^{β} and signals belonging to the Boc, Z, Bzl are comparable to those found for TP-5[27]. Upon conversion of C=O to C=S, the amide NH proton shifted ca. 1.5 ppm downfield. The assignments are in accordance with those reported for protected thiopeptides[14]. IR spectroscopy showed the ester band at 1730 cm^{-1}, urethane at 1692-1705cm^{-1}, amide I at ca. 1680- 1690 cm^{-1} and thioamide II at 1500-1525cm^{-1}. The U.V. spectra of all thioamide-containing peptides showed the characteristic π-π^* absorption in the range 268-272 nm. In the mass spectra the m/e (+ FAB) was observed in some cases i.e. protected **3, 5, 8, 14, 15** and unprotected **17, 18, 20**. For the Boc protected di- tri- and tetrapeptide a general trend was the loss of m/e $C_6H_5CH_2$ (91) and $C_6H_3Cl_2CH_2$ (159).
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23. Compounds **13-16** gave satisfactory elemental analyses. **13** : m.p. 122-24°C; R_f = 0.75 EtOAc (Alumina); IR υ_{max} ($CHCl_3$) 1490 (thioamide), and 1740 cm^{-1} (ester); UV λ_{max} ($CHCl_3$) 271 nm (log ε = 4.00); $[\alpha]_D^{20}$ -22.5° (c= 0.2, $CHCl_3$). **14** : m.p. 105-107°C; R_f = 0.73 6% MeOH/$CHCl_3$); IR υ_{max} ($CHCl_3$) 1710 (urethane), 1695 (amide), 1510 (thioamide), and 1720 cm^{-1} (ester); UV λ_{max} ($CHCl_3$) 271 nm (logε = 4.20); MS(FAB) 1368(M^+-$C_6H_5CH_2$); $[\alpha]_D^{20}$ +9.1° (c = 0.55, $CHCl_3$). **15**:m.p. 88-90°C(d); R_f = 0.73 6% MeOH/$CHCl_3$ (Alumina); UV λ_{max} ($CHCl_3$) 274 nm (logε = 4.08); MS(FAB) 1458 (M^+); $[\alpha]_D^{20}$ = 58° (c = 0.8, $CHCl_3$). **16**: m.p. 64-66°C(d); R_f = 0.75 6% MeOH/$CHCl_3$ (Alumina); UV λ_{max} ($CHCl_3$) 272 nm (logε = 4.10).
24. ^{1}HNMR of compounds **17-20** were consistent with the expected structure. **17**: m.p. 148-50°C; R_f =0.38 n-butanol:AcOH:Pyridine:H_2O (4:1:1:2) (silica gel); UV λ_{max} (MeOH) 269 nm; HRMS m/e cald for $C_{30}H_{50}N_9O_8S$ 696.3505, found 696.3531; MS m/e 696 (M^+); α_D^{26} =-30.47 (c = 0.105, MeOH). **18**: m.p. 146-48°C; R_f =0.45 n-butanol:AcOH:Pyridine:H_2O (4:1:1:2) (silica gel); UV λ_{max} (MeOH) 269 nm; IR ν_{max} (KBr) 1670 cm^{-1}(amide) and 1525 cm^{-1} (thioamide); Amino acid analysis Arg (1.00), Asp (0.80), Lys (1.00), Tyr (0.85), Val (1.00); HRMS m/e cald for $C_{30}H_{50}N_9O_8S$ 696.3505, found 696.3465; MS m/e 696 (M^+); α_D^{26} =-17.33 (c = 0.52, MeOH). **19**: m.p. 140-42°C; R_f =0.41 n-butanol:AcOH:Pyridine:H_2O (4:1:1:2) (silica gel); UV λ_{max} (MeOH) 272 nm (logε =3.99); Amino acid analysis Arg (1.27), Asp(0.88), Lys (1.22), Tyr (0.75), Val(1.00); MS m/e 678 (M^+- H_2O). **20**: m.p. 133-35°C; R_f =0.40 n-butanol:AcOH:Pyridine: H_2O (4:1:1:2) (silica gel); UV λ_{max} (MeOH) 268 nm; HRMS m/e cald for $C_{30}H_{50}N_9O_8S$ 696.3505, found 696.3576; MS m/e 696 (M^+); α_D^{26} =-4.00 (c = 0.25, MeOH).
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