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Thioacylating Agents. Use of Thiobenzimidazolone Derivatives for the Preparation of Thiotuftsin Analogs.

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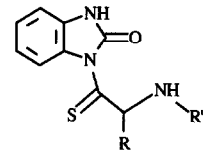
Abstract: *The properties and characteristic reactions of thioacylating reagents* ***1*** *are described These reagents are able to introduce thioamide linkages into a growing peptide at a specific site in the sequence The generality and efficiency of this methodology is demonstrated by the synthesis of the three monothioanalogues of tuftsin*

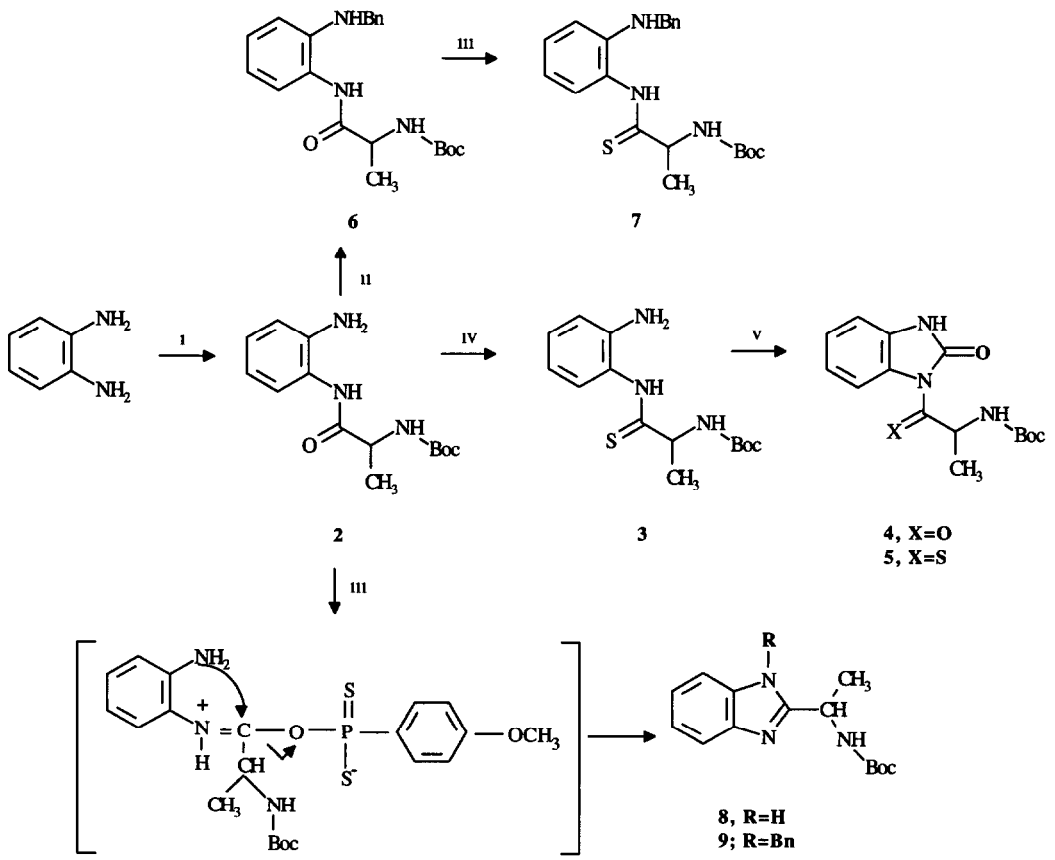
The tetrapeptide tuftsin[1](H-Thr-Lys-Pro-Arg-OH) possesses significant immunomodulatory properties It is capable of potentiating granulocyte and macrophage functions such as phagocytosis, motility, immunogenic response, as well as bactericidal and tumoricidal activity[2] Subsequently, tuftsin has a number of interesting biological properties, such as anti-infective, anti-cancer, anti-AIDS and growth factor activities[3] However, the instability of tuftsin in plasma reduces its efficacy Therefore, much effort has been undertaken to discover analogues which are more resistant to degradation[2-4] Unfortunately, such attempts have not been successful Most of the analogs either have not retained the desired activity, or are competitive inhibitors of tuftsin

One approach to the stabilization of the amide linkages of tuftsin against enzymatic degradation is the replacement of the peptide bond with the thioamide linkage The larger size and lower electronegativity of sulfur, compared to oxygen, is expected to induce some conformational distortions in the modified peptide[5] While physical studies[6] have suggested that the thioamide moiety should be compatible with amides in most instances, biological studies have shown that the behavior is unpredictable[6,7] Nonetheless, the synthesis of endothiopeptides has received increasing attention Alkyl dithioester[8] derivatives of amino acids have been used as thioacylating reagents, with variable results However, apparent racemization of the final product was observed[9] An important development has been the introduction of Lawesson's 1,3-dithiadiphosphetane- 2,4-disulfide reagent[10] Thiopeptide linkages can be formed with this or similar reagents[11,12] from suitably protected peptides in a regioselective manner and in high yields This method, however, displays lack of reaction site specificity and leads to mixtures of thiopeptides which are difficult to separate[13] More recently, monothionation of peptides by use of Fmoc amino monothioacids and benzotriazolyloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBop) has been described[14] This procedure is limited to Fmoc thioacids[15] and suffers from the formation of the corresponding amides as byproducts due to the presence of oxoacids formed during the course of the reaction

Our goal, therefore, was to develop a general and simple procedure for the preparation of monothiopeptide chemoselectively In a previous communication, we reported that thioacylbenzimidazolone derivatives **1** were useful thioacylating reagents[16] It was possible to monitor the aminolysis of **1** with amino acid or peptide under neutral conditions We report here further details regarding the characteristics and the properties of these thioacylating reactions, with attention to the synthesis of thiotuftsin



1,R'=Boc, Z
R=amino acid side chains

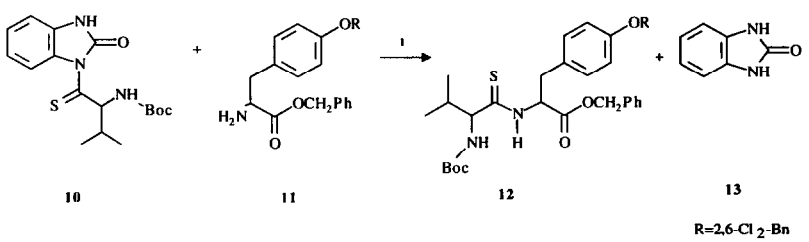


Scheme 1

Reagents and conditions (i), Boc-Ala-OH, EDC, CH_2Cl_2, (ii) Benzyl bromide, Et_3N, (iii) Lawesson's reagent, THF, (iv) P_2S_5, THF, (v) 1,2,4-Triazole, Triphosgene, Et_3N

A modified procedure for the preparation of thioacylating agent **1**[16,17] is described in the typical example illustrated in Scheme 1 1,2-phenylenediamine was reacted with Boc-Ala-OH in the presence of EDC[18] in CH_2Cl_2 at 0°C This gave, after workup and crystallization, the desired orthoaminoanilide **2** in almost quantitative yield Surprisingly, amide formation occurs at only one of the two amino substituents on the benzene ring Compound **2** may also be synthesized with other peptide coupling reagents such as DCC, 1,1'-carbonyldiimidazole and ethyl chloroformate The preferred method employs the water soluble carbodiimide EDC because of the facile workup procedure Direct thionation of peptide **2** with Lawesson's reagent in dry THF , or dioxane at room temperature was very slow At reflux, Boc-derivative **8** was formed in high yield, presumably due to the participation of the vicinal amino group according to Scheme 1 To avoid this reaction, the amino group was protected as Bn to give compound **6** Unfortunately, reaction with Lawesson's reagent in THF under reflux gave only 10% of the expected product **7** and 75% of **9** However, facile thionation of **2** was achieved with a mixture of phosphorus pentasulfide and anhydrous sodium carbonate[16,17] in dry THF at 0°C (Scheme 1) The reaction progressed cleanly to an appreciable extent after a few hours As judged by ^1H NMR and TLC the formation of **8** was minimum Ring closure forming a cyclic urea derivative was the next step A synthesis of the urea was chosen as a model since thioamide **3** is difficult to manipulate A number of procedures were examined for cyclization of compound **2** Carbonylation using a variety of conditions including 1,1'-carbonyldiimidazole, t-butyl chloroformate, 4-nitrophenyl chloroformate, 1,1'-carbonylbis(3-methylimidazolium)-triflate[19], phosgene, and diethyl carbonate did not give satisfactory results In our hands, only 1,1'-carbonylditriazole gave the desired benzimidazolone **4** in a reasonable yield The triazole reagent was normally prepared by bubbling phosgene in a benzene solution of 1-(trimethylsilyl)-1,2,4-triazole[20] However, we replaced phosgene with triphosgene The latter was described by Eckert[21] as a safe, stable phosgene substitute and examples of its use were reported[22] In a typical experiment, a solution of 1,2,4-triazole in THF was mixed with triphosgene in the presence of triethylamine The mixture was heated for two hours at 70°C under argon The carbonylditriazole prepared in situ was then mixed with amide **2** at room temperature and the reaction was stirred overnight After workup and purification, compound **4** was obtained as a white solid In a similar manner, the pale yellow thioimidazolone **5** was prepared by reacting thioamide **3** with carbonylditriazole However, attempts to obtain **5** by direct thionation of cyclic **4** using Lawesson's reagent were not successful and starting material was recovered almost quantitatively

By following a similar pathway, as shown in Scheme 1, twenty thiobenzimidazolone derivatives **1** of natural amino acids (R= amino acid side chains, R'= Boc) were synthesized and characterized[17] These compounds are generally stable yellow solids, easy to manipulate, and can be stored for months at 0°C without decomposition However, some of them were susceptible to decomposition after standing for a few hours at room temperature For example, aspartyl or histidyl benzimidazolone derivatives of **1** where R= CH_2-$COOCH_2Ph$, R'=Boc or R= N-benzyl imidazole, R'= Boc respectively should be rapidly purified and stored below 0°C



Scheme 2

Reagents and conditions (i) DMF or CH_2Cl_2, 25°C, overnight

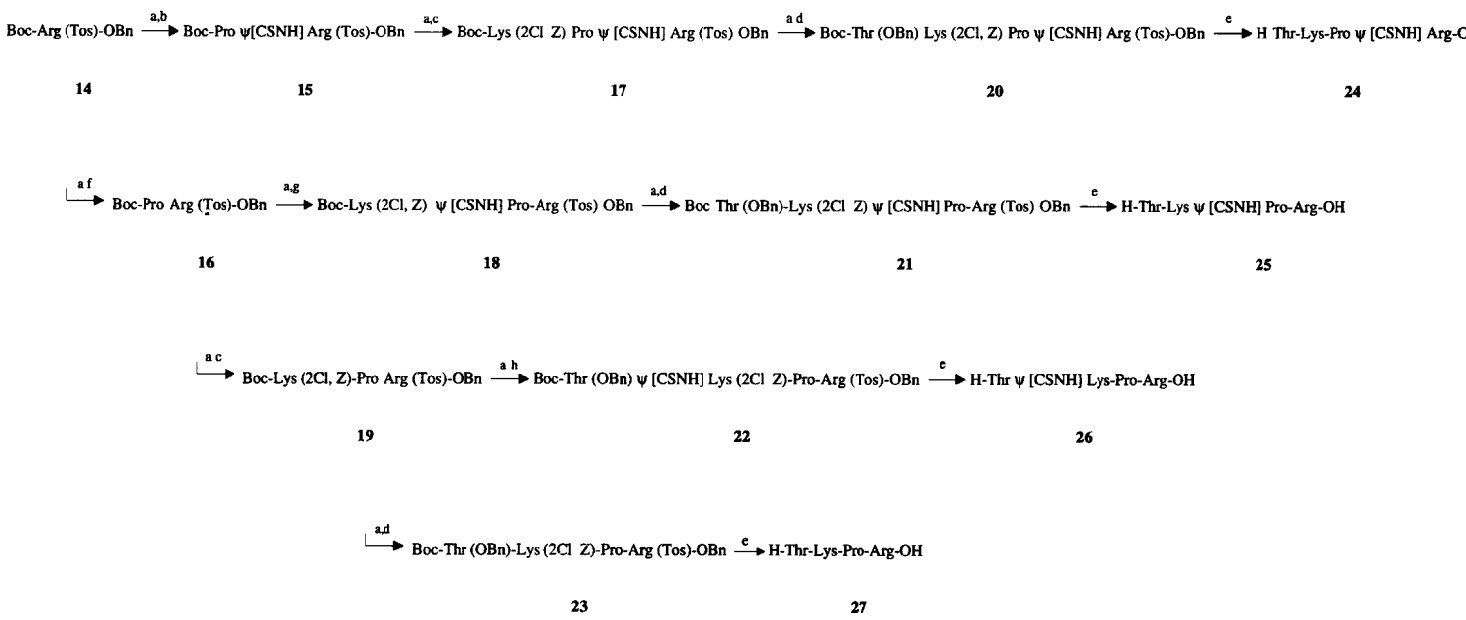
Introduction of a thioamide moiety into a growing peptide may be accomplished as represented by the example described in Scheme 2 The free amino function of tyrosine **11** was reacted with an equimolar amount of valine thiobenzimidazolone derivative **10** in DMF or CH_2Cl_2 at 25°C overnight This gave protected dipeptide **12** in high yield The byproduct benzimidazolone **13** was removed from the reaction mixture by filtration or by chromatography This procedure was repeated accordingly for the incorporation of the thioamide linkage at specific sites in the peptide sequence As a result, the three monothiotuftsin isomers were prepared starting from Boc-Arg(Tos)-OBn via the sequence shown in Scheme 3 There are two approaches to prepare thiotuftsin **24-27** The first is to prepare peptides **16** and **19** with the use of DCC-HOBt, followed by introduction of the thioamide linkage into the growing peptide The second is to introduce the thioamide bond first, as in the case of **15** and **18**, and the thiopeptide so formed may then be elongated employing, for example, DCC mediated coupling In both cases, the procedure is apparently simple and efficient For example, to prepare monothiotuftsin **20**, the Boc protected amino group of compound **17** was removed by treatment with trifluoroacetic acid (TFA) at 0°C, and the TFA salt of thiopeptide **17** was then partitioned between dichloromethane and aqueous sodium bicarbonate The free base formed was coupled with Boc-Thr-(OBn)-OH using DCC/HOBt to give the desired product **20** in a high yield

Once the incorporation of the thioamide linkage was completed, and the thiopeptides synthesized, the fully protected analogues **20-23** were treated with HF in the presence of anisole as a scavenger The crude deprotected monothiotuftsin was purified by sephadex gel filtration followed by reverse phase HPLC This gave the desired thiopeptide **24-27** No evidence of racemization was observed in all peptide and thiopeptide intermediates as demonstrated by the absence of diasteroisomers in the ^1H NMR (400 MHz) spectra

NMR data of all peptide segments and peptide derivatives in Scheme 3 were consistent with that reported for tuftsin[23] As expected[8a], the amide NH proton , H^{α} and the carbonyl carbon shifted ca 1 5 ppm , 0 5 ppm and 30 ppm respectively upon conversion of C=O to C=S The UV spectra of all thioamide-containing peptides showed the characteristic π-π^* transition in the range 267-271 nm In the IR spectra the thioamide band was observed at 1500-1525cm^{-1} The structures of the final compounds were confirmed by amino acid analysis and high resolution FABMS

An alternative approach for introduction of thioamide linkages is via solid phase (Merrifield) synthesis As a first application of this approach, (L)Leuψ[CSNH]Phe-OH **28** and (D)Leuψ[CSNH]Phe-OH **29** were prepared in 95% and 90% yields respectively (Scheme 4 Path A) Thus , Boc-Phe copolymer Merrifield resin was prepared by well-known solid phase methods To achieve the coupling of phenylalanine to leucine, the Boc group of phenylalanine was removed by treatment with 55% TFA in dichloromethane After washing the resin with dichloromethane, it was neutralized with 10% triethylamine in methanol The coupling of leucine was carried out with 2x1 5 equivalents of Boc-Leu-thioacylating reagent in DMF with an interval of sixteen hours The suspension was stirred for a total of 80 hours at room temperature The peptide was cleaved from the resin, deprotected with HF and purified by HPLC to give pure thiodipeptides In a related manner, this procedure was applied to the synthesis of another monothiopeptide, monothiothymopentin Arg-Lysψ[CSNH] Asp-Val-Tyr **30** (Scheme 4 Path B). Thymopentin is an immunomodulatory pentapeptide[24] The Merrifield resin linked tripeptide Fmoc-Asp(OtBu)-Val-Tyr(2,6-Cl_2-OBn) was synthesized by conventional solid phase procedures, and was reacted after deprotection with 20% piperidine with thioacylating reagent **1**(R =$(CH_2)_4$-NH-Boc, R' = Fmoc) (ca nine equivalents) in anhydrous DMF at room temperature The resulting resin was deblocked with 20% piperidine in DMF and coupled to Boc-Arg(Tos)-OH to afford the desired product The thiopeptide may be liberated from the resin by using established methods i e HF containing dialkyl sulfide with anisole and thioanisole at 0°C However, the yield of the desired thiopentapeptides did not exceed 15% Several unsuccessful attempts were made to improve the yields, for example changing the solvent from DMF to N-methyl pyrrolidone or to N,N-dimethylacetamide or the concentration of the reagents It is possible steric hinderance becomes important in the reaction between the thioacylating reagent and the growing tetra- or pentapeptide Alternatively, thioamides are sensitive to nucleophiles, and the use of piperidine for Fmoc

Synthetic Scheme for Thiotuftsin and its Fragments



Scheme 3

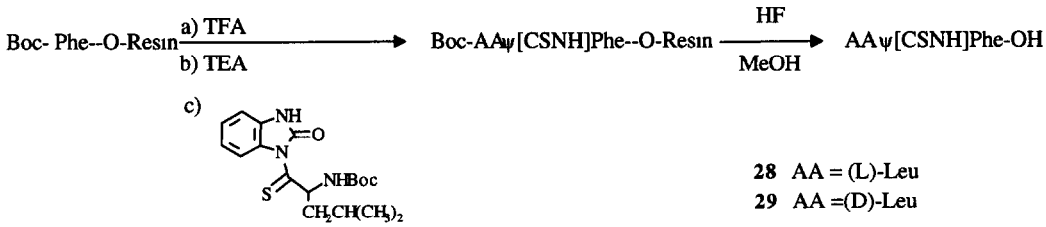
Reagents and conditions (a) TFA/CH_2Cl_2, $NaHCO_3$ (b) Boc Proline thiobenzimidazolone, DMF (c) Boc-Lys (2Cl, Z)-OH, DCC HOBt, CH_2Cl_2 (d) Boc Thr-(OBn)-OH DCC HOBt, CH_2Cl_2 (e) HF, anisole, EtSMe, thioanisole 0°C (f) Boc-Pro-OH DCC HOBt, CH_2Cl_2 (g) **1** [R= $(CH_2)_4$-NH (2Cl Z), R =Boc] (h) **1** [R=-CH (OBn) CH_3 R'=Boc]

deprotection could have resulted in many side reactions This appears to be a limitation for this type of thioacylating agent and further studies for the preparation of less hindered reagents or the use of a non nucleophilic base for Fmoc cleavage such as DBU are in progress

The biological results of three Thiotuftsin analogues will be reported elsewhere

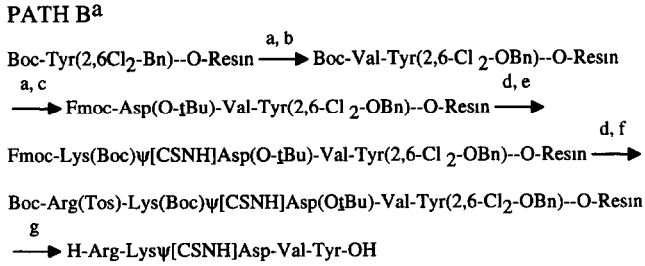
In conclusion, we describe herein the synthesis, properties and the behavior of new thioacylating reagents These compounds are able to incorporate thioamide linkages into a growing peptide at a specific site in the peptide sequence by reacting with an amino terminus of a protected C-terminal of amino acid or peptide The potential utility of this method has been illustrated by the facile synthesis of the three monothionated analogues of tuftsin, and it is expected that the procedure will find widespread use

PATH A



28 AA = (L)-Leu
29 AA =(D)-Leu

PATH B[a]



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Scheme 4

[a] Key (a) TFA,CH_2Cl_2, (b) Boc-Val-OH, DCC, HOBt, DMF, (c) Fmoc-Asp(O-tBu)-OH, DCC, HOBt, DMF, (d) 20% Piperidine, DMF, (e) **1** (R = $(CH_2)_4$-NH(Boc),R' = Fmoc), DMF, (f) Boc-Arg(Tos)-OH, (g) HF, anisole, 0°C

EXPERIMENTAL SECTION

Progress of reactions and purity of products were determined by analytical TLC on silica gel plates, visualized by UV, iodine vapor, ninhydrin or Ehrlich's reagent, and by analytical HPLC (Millipore-Waters apparatus) on a 300x3 9 mm BONDAPACK C18 (5μ) reverse phase column with a gradient of 100% H_2O to 50% A/B over a period of 50 min , flow rate, 1 ml/min (solution A, 0 04% TFA in CH3CN, solution B 0 04% TFA in H_2O) using a UV detector at an absorption of 214 nm for detection Products were purified by preparative thin-layer chromatography (PTLC) on 20x20 cm silica gel GF uniplates (Analtech 0 25-1 0 mm thickness), by silica gel column (E Merck 230-400 mesh), and/ or by reverse-phase high-performance liquid chromatography (HPLC) using Whatman Partisil 10 ODS (20x250 mm) using the same gradient described above Final deprotected products were lyophilized from H_2O and often retained as a partial solvate Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected IR spectra were obtained on a Perkin-Elmer model 21 spectrophotometer, while UV spectra were recorded on Perkin-Elmer 402 spectrophotometer Proton NMR spectra were obtained on a Bruker WH-90 or WH-400 spectrometers

Mass spectra were recorded on a Kratos Ms-50 TA instrument The FAB (fast atom bombardment) ionization was obtained with a FAB saddle field source operated with xenon atoms at 7 kV. Elemental analyses were performed by Guelph Chemical Laboratories Ltd , Ontario (Canada) and are reported in percent Amino acid analyses were carried out on a Varian 5500 analyzer by Biochem Immunosystems after hydrolysis with 6 M HCl containing 0 1 % phenol, at 110°C for 20 h

Boc-Alanyl-2-Aminoanilide (2). To a mixture of Boc-Ala-OH (2 08 g, 11 mmol), 1,2-phenylenediamine (1g, 9 2 mmol) and TEA (1 5 ml, 11 mmol) in CH_2Cl_2 (50 ml) was added EDC HCl at 0° C After stirring at this temperature for two hours and then at room temperature overnight, 5% HCl (25 ml) was added and the mixture was washed with brine, 5% sodium bicarbonate, and brine, and dried over Na_2SO_4 Evaporation of the solvent and crystallization of the residue from CH_2Cl_2/hexanes afforded pure **2** (2 4 g, 94%) as a colorless solid m p 122-24°C, R_f = 0 64 (EtOAc/hexanes 1 1), ^1H NMR ($CDCl_3$) δ 1 46 (d, 3H, J =6 Hz), 1 47 (s, 9H, 3xCH_3 Boc), 3 00 (bs, 2H, NH_2), 4 29 (m, 1H, H^{α}), 5 04 (d, 1H, NH, J = 4 55 Hz), 6 78 (m, 2H, aromatic), 7 05 (m, 1H, aromatic), 7 26 (m, 1H, aromatic), 7 96 (bs, 1H, NH)

Boc-Alanyl-2-Aminothioanilide (3). Under a flow of argon, phosphorus pentasulfide (0 75 g, 1 68 mmol) was mixed with sodium carbonate (0 18 g, 1 7 mmol) in dry THF (100 ml) The mixture was stirred for 1 hour at 25 °C and then cooled to 0°C To this clear solution was added anilide **2** (0 5 g, 1 79 mmol) and the reaction was kept at this temperature overnight To workup, an aqueous solution of sodium tribasic phosphate was added (12%, 7 ml) followed by EtOAc (20 ml) and heptane (20 ml) The organic layer was separated and washed with brine (2x30 ml) Solvent was then evaporated and the residue was purified on silica gel using EtOAc hexanes (1 2) as eluant This gave pure **3** (0 28 g, 53%) as a pale yellow solid m p 124-26°C, R_f = 0 75 (EtOAc/hexanes 2 3), ^1H NMR ($CDCl_3$) δ 1 44 (s, 9H, 3xCH_3 Boc), 1 59 (d, 3H, CH_3, J = 6 9 Hz), 3 94 (bs, 2H, NH_2), 4 62 (m, 1H, H^{α}), 5 35 (d, 1H, NH, J = 6 87 Hz), 6 8 (m, 2H, aromatic), 7 15 (m, 2H, aromatic), 9 60 (bs, 1H, NH)

N-(Boc-Alanyl)-2-Benzimidazolone (4) A solution of 1,2,4-triazole (0 25 g, 3 62 mmol) in dry THF (40 ml) was mixed with triphosgene (0 18 g, 0 61 mmol) and triethylamine (0 52 ml, 3 65 mmol) under a flow of argon The mixture was refluxed for 2 hours then cooled to room temperature To this solution was added anilide **2** (0 50 g, 1 81 mmol) and the reaction was stirred overnight at 25 °C Insolubles were removed by filtration and the solvent was evaporated under reduced pressure The residue was chromatographed on silica gel using EtOAc hexanes (1 1) as eluant This gave pure benzimidazolone **4** (0 34 g, 62%) as a white solid R_f = 0 52 (EtOAc/hexanes 2 3), ^1H NMR ($CDCl_3$) δ 1 47 (s, 9H, 3xCH_3 Boc), 1 51 (d, 3H, CH_3, J = 7 02 Hz), 5 34 (d, 1H, NH, J = 7 02 Hz), 5 70 (m, 1H, H^{α}), 6 9-7 1 (m, 3H, aromatic), 7 99 (d, 1H, aromatic), 9 31 (bs, 1H, NH) This compound was characterized as the hydrochloride salt Thus, Boc derivative **4** (0 12 g, 0 39 mmol) was dissolved in dry ether (30 ml) at room temperature under argon HCl gas was bubbled through this solution for ten minutes and the reaction mixture was stirred for one hour at this temperature The solution was then evaporated to half of its volume and kept at 4°C for four hours The precipitate was collected and washed with cold dry ether (10 ml) This gave the hydrochloride salt of **4** (0 081 g, 86%) as a white solid mp 52°C (d), R_f = 0 28 (20% MeOH/EtOAc), ^1H NMR (DMSO) δ 1 52 (d, 3H, CH_3, J = 6 26 Hz), 5 09 (m, 1H, H^{α}), 7 09-7 27 (m, 3H, aromatic), 8 01 (d, 1H, aromatic, J = 7 69 Hz), 8 48 (b, 3H, NH_3), 11 76 (s, 1H, NH), MS (FAB, thyioglycerol, m/e), 206 (M+1), HRMS m/e Calcd for $C_{10}H_{12}N_3O_2$ 206 0931, Found 206 0949

N-(Boc-ThioAlanyl)-2-Benzimidazolone (5)[17]. Prepared according to the procedure described above for benzimidazolone **4.** (0 30 g of **3,** 0 99 mmol) yield (0 16 g, 51%), m p 101-03°C, R_f =0 65 (EtOAc/Hexanes 2 3), ^1H NMR ($CDCl_3$) δ 1 46 (s, 9H, 3xCH_3 Boc), 1 49 (d, 3H, CH_3, J = 6 8 Hz), 5 55 (d,

1H, NH, J = 6 87 Hz), 6 29 (m, 1H, H^{α}), 6 73-7 08 (m, 3H, aromatic), 8 65 (d, 1H, aromatic), 9 35 (bs, 1H, NH)

Boc-Alanyl-2-N-Benzyl-Aminoanilide (6). To a solution of anilide **2** (0 15 g, 0 53 mmol) in dry THF (30 ml) at 0°C was added triethylamine (0 08 ml, 0 63 mmol) followed by benzylchloroformate (0 1 g, 0 58 mmol) under an atmosphere of argon The solution was stirred at this temperature overnight Solvent was evaporated and CH_2Cl_2 (30 ml) was added The organic layer were washed with 5% $NaHCO_3$, brine and dried over anhydrous Na_2SO_4 The crude product was purified on silica gel using EtOAc hexanes (1 3) as eluant to afford pure **6** (0 11 g, 50%) as a white solid m p 135-37°C , R_f = 0 23 (30% EtOAc/hexanes), ^1H NMR ($CDCl_3$) δ 1 39 (s, 9H,3xCH_3 Boc), 1 46 (d, 3H, CH_3, J =5 98 Hz), 4 26 (m, 1H, H^{α}), 4 36 (s, 2H, CH_2-Ph), 5 11 (d, 1H, NH, J = 6 04 Hz), 6 7 (m, 2H, aromatic), 7 06 (t, 1H, aromatic), 7 26 (m, 7H, aromatic + 2 NH), 7 89 (s, 1H, aromatic) MS (FAB, thioglycerol, m/e) 370 (M+1)

Boc-ThioAlanyl-2-N-Benzyl-Aminoanilide (7). Anilide **6** (0 10 g, 0 24 mmol) was dissolved in dry THF (25 ml) under a flow of argon To this solution was added Lawesson's reagent (0 19 g, 0 47 mmol) and the reaction mixture was refluxed for 5 hrs Solvent was then removed and the residue was chromatographed on silica gel using EtOAc hexanes (1 2) as eluant This afforded two compounds thioamide **7** (0 011 g, 11%) as a white solid m p 52°(d), R_f = 0 6 (30%EtOAc/hexanes), ^1H NMR ($CDCl_3$) δ 1 38 (s, 9H, 3xCH_3 Boc), 1 59 (d, 3H, CH_3 J = 6 9 Hz), 4 39 (s, 2H, CH_2), 4 58 (m, 1H, H^{α}), 5 20 (d, 1H, NH, J = 5 32 Hz), 6 69-6 76 (m, 2H, aromatic), 7 17-7 37 (m, 8H, aromatic), 9 25 (bs, 1H, NH), MS (FAB thio glycerol, m/e) 386 (M+1) The second compound was identified as benzimidazole **9** (0 071 g, 84%), m p 180-82°C, R_f = 0 4 (EtOAc/hexanes 1 1), ^1H NMR ($CDCl_3$) δ 1 41 (s, 9H, 3xCH_3 Boc), 1 55 (d, 3H, CH_3, J = 6 8 Hz), 5 19 (m, 1H, H^{α}), 5 41 (d, 1H, NH), 5 50 (s,2H, CH_2), 7 12 (m, 2H, aromatic), 7 3 (m, 6H, aromatic), 7 79(d, 1H, aromatic) MS (FAB, thio glycerol, m/e) 352 (M+1)

2-(α -N-Boc)-Ethyl Benzimidazole (8). **2** (0 20 g, 0 71 mmol) was dissolved in dry THF (35 ml) and treated with Lawesson's reagent (0 52 g, 1 28 mmol) as described above for the preparation of **9.** Purification of the crude product on silica gel using EtOAc hexanes (1 1) as eluant gave pure benzimidazole derivative **8** (0 14 g, 80%) as a white solid m p 200°(d), R_f = 0 42 (EtOAc/hexanes 1 1), ^1H NMR ($CDCl_3$) δ 1 44 (s, 9H, 3xCH_3 Boc), 1 70 (d, 3H, CH_3, J = 5 81 Hz), 5 09 (m, 1H, H^{α}), 5 40 (d,1H, NH), 7 25 (m, 2H, aromatic), 7 54 (m, 2H, aromatic) MS (FAB, thioglycerol, m/e) 262 (M+1), HRMS m/e Calcd for $C_{14}H_{20}N_3O_2$ 262 1558 Found 262 1580

The Synthesis of Thiotuftsin in Solution

General Procedure for Coupling of Protected Amino Acids

Deprotection of Boc Peptides To Boc peptide (2 8 mmol) was added cold trifluoroacetic acid (TFA, 10 ml)) under N_2 The resultant solution was allowed to warm to room temperature and was stirred for an additional 1 5 hours The excess acid was removed under reduced pressure The oily residue was solidified by addition of hexanes (15 ml) The product was obtained as a white powder in quantitative yield

Generation of the Free Amine of Protected Amino Acids The protected peptideTFA salt previously prepared (2 mmol) was dissolved in dichloromethane (25 ml) To this was added an aqueous solution of 5% $NaHCO_3$ (20 ml) and the mixture was stirred vigorously at ambient temperature for thirty minutes The organic layer was then separated and dried over anhydrous $MgSO_4$ The solvent was removed under reduced pressure to give the free amine which was treated with the thioacylating reagent or coupled with the next amino acid without further purification

Coupling with the Adjacent Amino Acid Free amino or thioamino acid benzyl ester (2mmol) was dissolved in DMF (5 ml) together with Boc amino acid (2 2 mmol) and HOBt (2 2 mmol) After the addition of DCC (2 2 mmol) at 0°C , the solution was stirred overnight at this temperature then diluted with ethyl acetate DCU was filtered off and washed with EtOAc (20 ml) The solution was then washed successively with 5% $NaHCO_3$ (20 ml), brine (15 ml), 5% citric acid (20 ml), and brine (15 ml) The EtOAc layer was dried over anhydrous $MgSO_4$ and chromatographed on silica gel using EtOAc/hexanes 2 3 for di- and tripeptides or on alumina using pure EtOAc or 6% methanol/chloroform for tetra- and pentapeptides as eluent This afforded the expected peptide or thiopeptide in a 76-87% yield

Coupling of Thioacylating Reagents with Amino Acids or Peptides Free amino acid benzyl ester (2 mmol) was dissolved in dry DMF (0 5 ml) at 0°C under a stream of N_2 To this, thioacylating reagent (2 2 mmol) was added in portions over a period of 20 minutes The resulting mixture was stirred at this temperature for 2 hours and then at room temperature overnight Insolubles were removed by filtration and the solvent was evaporated under reduced pressure The remaining residue was dissolved in EtOAc (20 ml) and washed successively with 5% $NaHCO_3$ (25 ml), brine (25 ml), 5% citric acid (25 ml) and brine (25 ml) The organic layer was dried over anhydrous $MgSO_4$, evaporated and chromatographed on silica gel using EtOAc/hexanes 2 3 for di- and tripeptides or on alumina using pure EtOAc or 6% methanol/chloroform for tetra- and pentapeptides as the eluent This gives pure thioamide derivatives in a 72-78% yield

General Procedure for Removal of Protecting Groups

The fully protected peptides (0 5 mmol) were deblocked with 90% HF (5 ml) in the presence of anisole, ethyl methyl sulfide and thioanisole (1 1 1 v/v, 0 5 ml) in an ice bath for one hour The excess HF was removed by evaporation in vacuo at 0°C, the residue was treated with 10% acetic acid, washed with ether (30 ml) and then dissolved in water (10 ml) The resulting solution was then lyophilized The crude peptide thus obtained was dissolved in 12% acetic acid (25 ml) and purified by HPLC using the conditions described above Fractions of 5 ml were collected every fifteen minutes and the absorption at 268-270 nm was determined Fractions corresponding to the front main peak were combined and lyophilized The yields of the pure deprotected thiopeptides were approximately 49-71%

Boc-Arg(Tos)-OBn (14) This compound was prepared by the general procedure reported in the literature[25] Boc-Arg(Tos)-OH (3 g, 7 00 mmol) was dissolved in MeOH (28 ml) and water (3 ml) was added The solution was titrated to pH 7 0 with a 20% aqueous solution of Cs_2CO_3 (8 ml) The mixture was evaporated to dryness and the residue reevaporated twice from DMF (17 ml) The white cesium salt obtained was stirred with benzyl bromide (1 28 g, 7 49 mmol 0 9 ml) in DMF (17 ml) for 8 h On evaporation to dryness and treatment with a large volume of water (20 ml) the product solidified It was taken into EtOAc (25 ml), washed with water (10 ml), dried over Na_2SO_4, evaporated to a solid mass, and crystallized from pure EtOAc Yield (3 0 g, 5 79 mmol 85%), amorphous, R_f = 0 53 (EtOAc hex 2 3), IR ν_{max} ($CHCl_3$) 1705 (urethane), and 1735 cm^{-1} (ester)

Boc-Proψ [CSNH]Arg(Tos)-OBn (15) (0 55 g of **14**, 1 31 mmol) yield (0 60 g, 72%), m p 64-66° C, R_f = 0 47 (10 % MeOH / $CHCl_3$), UV λ_{max} ($CHCl_3$) 270 nm

Boc-Pro-Arg(Tos)-OBn (16)[26] (2 00 g of **14**, 3 86 mmol) yield (1 81 g, 76 %), R_f = 0 41 (10 % MeOH/$CHCl_3$)

Boc-Lys(2Cl,Z)-Proψ [CSNH]Arg(Tos)-OBn (17). (0 44 g of **15,** 0 84 mmol) yield (0 69 g, 87 %), m p 67-69°C, R_f =0 32 (10 % MeOH/$CHCl_3$), UV λ_{max} ($CHCl_3$) 271

Boc-Lys(2Cl,Z)ψ [CSNH]Pro-Arg(Tos)-OBn (18). (0 99 g of **16**, 1 60 mmol) yield (1 08 g, 72%), R_f = 0 50 (10% MeOH/$CHCl_3$)

Boc-Lys(2Cl,Z)-Pro-Arg(Tos)-OBn (19)[26]. (1 67 g of **16**, 2 71 mmol) yield (2 00 g, 81 %), R_f = 0 45 (pure EtOAc)

Boc-Thr(OBn)-Lys(2Cl,Z)-Proψ [CSNH]Arg(Tos)-OBn (20). (0 63 g of **17**, 0 67 mmol) yield (0 60 g, 80%), m p 78-80°C, R_f = 0 75 (pure CH_3CN), IR ($CHCl_3$) 1758, 1380 cm^{-1}, UV λ_{max} ($CHCl_3$) 271 nm, MS(FAB, thioglycerol, m/e) 1084 (M^+-Cl), Anal Calcd for $C_{55}H_{71}ClN_8O_{11}S_2$ hexane C, 60 75, H, 7 10, N, 9 30, Found C,60 34, H,7 07, N, 9 65

Boc-Thr(OBn)-Lys(2Cl,Z)ψ [CSNH]Pro-Arg(Tos)-Obn (21). (0 87 g of **18**, 0 93 mmol) yield (0 88 g, 84%), R_f = 0 68 (pure EtOAc)

Boc-Thr(OBn)ψ [CSNH]Lys(2Cl,Z)-Pro-Arg(Tos)-OBn (22). (1 24 g of **19**, 1 36 mmol) yield (1 18 g, 78%), m p 73-75°C, R_f = 0 65 (pure EtOAc), IR ($CHCl_3$) 1748, 1500 cm^{-1}, UV λ_{max} ($CHCl_3$) 271 nm, MS(FAB, thioglycerol, m/e) 1119 (M^+), 1028 (M^+-$C_6H_5CH_2$), Anal Calcd for $C_{55}H_{71}ClN_8O_{11}S_2$ C, 59 00, H, 6 40, N, 10 00, S, 5 72, Found C,59 66, H,6 78, N, 9 84, S, 5 70

Boc-Thr(OBn)-Lys(2Cl,Z)-Pro-Arg(Tos)-OBn (23)[26] (0 45 g of **19**, 0 49 mmol) yield (0 45 g, 83%), R_f = 0 66 (5% MeOH/EtOAc)

H-Thr-Lys-Proψ [CSNH]Arg-OH (24) (0 6 g of **20**, 0 53 mmol) yield (0 19g, 71%), m p 169-171°C, R_f = 0 25 (n-BuOH/AcOH/pyridine/H_2O 4 1 1 2), UV λ_{max} (50% aqueous ethanol) 268 nm, Amino acid analysis Thr (1 08), Lys (1 04), Pro (0 82), Arg (1 00), HPLC R_t = 18 36 min , MS m/e 517 (M^+), HRMS m/e Calcd for $C_{21}H_{40}N_8O_5S$ 517 2924 Found 517 2944, Anal calc for $C_{21}H_{40}N_8O_5S$ CF_3COOH $5H_2O$ C 38 32, H 6 64, N 15 54, found C 38 05, H 6 99, N 15 50

H-Thr-Lysψ [CSNH]Pro-Arg-OH (25). (0 3 g of **21**, 0 27 mmol) yield (0 068 g, 49%) m p 230°C (d), R_f = 0 24 (n-BuOH/AcOH/pyridine/H_2O 4 1 1 2), UV λ_{max} (50% aqueous ethanol) 268 nm, HPLC R_t = 18 36 min , MS m/e 517 (M^+)

H-Thrψ [CSNH]Lys-Pro-Arg-OH (26). (0 42 g of **22**, 0 37 mmol) yield (0 14 g, 73%), m p 187°C (d), R_f = 0 28 (n-BuOH/AcOH/pyridine/H_2O 4 1 1 2), UV λ_{max} (50% aqueous ethanol) 267 nm, Amino acid analysis Thr (0 90), Lys (0 99), Pro (1 02), Arg (1 00), HPLC R_t = 15 38 min , MS m/e 517 (M^+), HRMS m/e Calcd for $C_{21}H_{40}N_8O_5S$ 517 2924 Found 517 2960, Anal Calcd for $C_{21}H_{40}N_8O_5S$ $2CF_3COOH$ $4H_2O$ C 36 76, H 6 17, N 13 72, Found C 36 21, H 5 92, N 14 17

H-Thr-Lys-Pro-Arg-OH (27)[26] (0 33 g of **23**, 0 29 mmol) yield (0 086 g, 59%), R_f = 0 13 (n-BuOH/AcOH/pyridine/H_2O 4 1 1 2)

The Synthesis of Thiopeptides in Solid Phase

Preparation of Leuψ [CSNH]Phe-OH (28) or (29). Boc-Phe copolymer Merrifield Resin (0 632 m eq/g, 0 422 g, 0 28 mmol, BioChem Pharma) was treated with 55% TFA (10 ml) in CH_2Cl_2 (30 ml)for one hour at 25°C Solvent was then removed and the resin was washed successively with CH_2Cl_2 (4x10 ml), isopropanol (4x10 ml) and 10% Et_3N (1 ml) in MeOH under N_2 The presence of the free amino group was confirmed by Kaiser test To the resin was added a solution of D- or L-Boc-Leu thioacylating reagents (**1**, R = $CH_2CH(CH_3)_2$, R^1 = Boc) (0 15 g, 0 413 mmol, 1 5 eq) in dry DMF (7 ml) After sixteen hours of stirring at 25°C, another portion of the thioacylating reagents (0 15 g, 0 143 mmol, 1 5 eq) in dry DMF (7 ml) was

added The suspension was then stirred for eighty hours at 25°C The peptide was cleaved from the resin and deprotected with HF at 0°C The supernatant was filtered off and the resin was washed with methanol (2x10 ml) The organic layer was evaporated under reduced pressure and purified by HPLC using the system described above to afford the dipeptide (L)-Leu ψ[CSNH]Phe-OH (**28**, 0 054 g, 95%) or (D)-Leu ψ[CSNH]Phe-OH (**29**, 0 035 g, 90%) as a white solid material

Preparation Of thiothymopentin H-Arg-Lysψ [CSNH]Asp-Val-Tyr-OH (30)

Deprotection Of The Boc Group Boc-Tyr(2,6-Cl_2-Bn) Merrifield resin (0 794 mmol/g, 1 55 g, 1 19 mmol, Biochem Pharma) was treated with 55% TFA (10 ml) in CH_2Cl_2 (30 ml) for one hour at 25°C Solvent was removed and the resin was washed with CH_2Cl_2 (42x10 ml) and isopropanol (4x10 ml) Kaiser test was positive

Coupling with Boc-Val-OH. To the amino resin was added a mixture of Boc-Val -OH (0 78 g, 3 59 mmol, 3 eq) and HOBt (0 55 g, 3 58 mmol, 3 eq) in DMF (10 ml) at 25°C A solution of DCC (0 738 g, 3 58 mmol, 3 eq) in DMF (5 ml) was then added and the suspension was stirred for sixteen hours at 25°C A second portion with the same amounts of the amino acid, HOBt and DCC was added and the reaction was stirred for an additional sixteen hours Acylation of the peptide resin was carried out by using a 20% solution of acetic anhydride in CH_2Cl_2 (15 ml) The suspension was stirred for one hour at 25°C, solvent was removed and the resin was washed with DMF (3x10 ml) and isopropanol (3x10 ml)

Coupling With Fmoc-Asp(OtBu)-OH. The Boc resin was treated with TFA as described above The same procedure of coupling mentioned above was followed with the quantities of the starting materials and time of reactions as follows Fmoc-Asp(OtBu)-OH (1 47 g, 3 58 mmol), HOBt (0 55 g, 3 58 mmol), DCC (0 738 g, 3 58 mmol) were added after nine hours, four hours and nine hours respectively at 25°C for each coupling Acylation was done in the same manner and deprotection of the peptide was carried out with a solution of 20% piperidine in DMF (3x15 ml)

Addition of the Thioacylating Reagent. To the resin was added a solution of Fmoc-Lys thioacylating reagent (**1**, R = $(CH_2)_4$-NH(Boc), R' = Fmoc) (1 64 g, 2 38 mmol, 2 eq) in dry DMF (7 ml) After sixteen hours of stirring at 25°C, a second portion of thioacylating reagent (1 64 g, 2 38 mmol) in dry DMF (5 ml) was added, followed by a third portion after the same period of time The solvent was then removed

Coupling with Boc-Arg(Tos)-OH We followed the same procedure for deprotection and coupling Boc-Arg(Tos)-OH (1 53 g, 3 58 mmol), HOBt (0 55 g, 3 58 mmol) and DCC (0 738 g, 3 58 mmol) was added three times at the beginning, after nine hours and after eleven hours respectively at 25°C

Removal of the Peptide from The Resin. The thiopeptide was stirred with 90% HF (10 ml) in the presence of anisole and thioanisole in an ice bath for one hour The excess HF was distilled and the resin was washed with dry ether (2x30 ml) The peptide was then extracted with a solution of 10% acetic acid and lyophilized Purification of the crude material was done by HPLC using the system described above to afford pure **30** (0 06 g, 15% yield) as a white solid This material was characterized by ^1H NMR, MS (FAB) spectrum and amino acid analysis

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REFERENCES AND NOTES

1 Fridkin, M , Gottlieb, P *Mol Cell Biochem* **1984**, *41*, 73-97 (b) Najjar, V A , Fridkin, M *Ann N Y Acad Sci* **1983**, *419*, 1-273

2 Verdini, A S , Silvestri, S , Becherucci, C , Longobardi, M G , Parente, L , Peppolon, S , Perretti, M , Pileri, P , Pinori, M , Viscomi, G C , Nencioni, L *J Med Chem* **1991**, *34*, 3372-3379 and references cited therein

3 (a) *WO Patent* 91/02747 (b) For a review on the biological activivities of Tuftsin see *Drugs Fut* **1992**, *17*, 646

4 (a) Galasik-Bartoszek, U , Konopinska, D , Plech, A , Najjar V A , Brus, R *Int J Pept Protein Res* **1991**, *38*, 176-180 (b) O'Connor, S D , Smith, P E , Al-Obeidi, F , Pettitt, B M *J Med Chem* **1992**, *35*, 2870-2881 (c) Paulesu, L , Di Stefano, A , Luzzi, E , Bocci, V , Silvestri, S , Nencioni, L *Immunol Lett* **1992**, *34*, 7-12 (d) Suda, H , Harada, J , Tanaka, S *EP* 0454302A, Banyu Pharm Co

5 (a) Michel, A G , Ameziane-Hassani, C , Boulay, G , Lajoie, G *Can J Chem* **1989**, *67*, 1312-1318 (b) Balaji, V N , Profeta, S , Jr , Dietrich, S W *Biochem Biophys Res Commun* **1987**, *145*, 834-841 (c) La Cour, T F M , Hansen, H A S , Clausen, K , Lawesson, S -O *Int J Pept Protein Res* **1983**, *22*, 509-512

6 Sherman, D B , Spatola, A F *J Am Chem Soc* **1990**, *112*, 433-441 and references cited therein

7 Sherman, D B , Spatola, A F , Wire, W S , Burks, T F , Ngugen, T M -D , Schiller, P W *Biochem Biophys Res Commun* **1989**, *162*, 1126-1132

8 (a) Clausen, K , Thorsen, M , Lawesson, S -O , Spatola, A F *J Chem Soc Perkin TransI* **1984**, 785-798 (b) Thorsen, M , Yde, B , Pedersen, U , Clausen, K , Lawesson, S -O *Tetrahedron* **1983**, *39*, 3429-3435

9 Jurayj, J , Cushman, M *Tetrahedron* **1992**, *48*, 8601-8614

10 (a) Brown, D W , Campbell, M M , Walker, C V *Tetrahedron* **1983**, *39*, 1075-1083 (b) For a recent review Cava, M P , Levinson, M I *Tetrahedron* **1985**, *41*, 5061-5087

11 Lajoie, G , Lépine, F , Maziak, L , Belleau, B *Tetrahedron Lett* **1983**, *24*, 3815-3818

12 Guziec, Jr , F S , Wasmund, L M *J Chem Research (s)* **1989**, 155, (M) **1989**, 1301-1309

13 Kessler, H , Geyer, A , Matter, H , Kock, M *Int J Pept Protein Res* **1992**, *40*, 25-40

14 Høeg-Jensen, T , Jakobsen, M H , Olsen, C E , Holm, A *Tetrahedron Lett* **1991**, *32*, 7617-7620

15 (a) Yamashiro, D , Blake, J *Int J Pept Protein Res* **1981**, *18*, 383-392 (b) Mitin, Y V , Zapevalova, N P *Int J Pept Protein Res* **1990**, *35*, 352-356

16 Zacharie, B , Martel, R , Sauvé, G , Belleau, B *BioMed Chem Lett* **1993**, *3*, 619

17 *US Patent* # 005138061, BioChem Therapeutic Inc

18 The addition of racemisation-suppressing additives i e Copper(II) chloride is desirable For a recent study see Miyazawa, T , Otomatsu, T , Fukui, Y , Yamada, T , Kuwata, S *Int J Pept Protein Res* **1992**, *39*, 237

19 Saha, A K , Schultz, P , Rapoport, H *J Am Chem Soc* **1989**, *111*, 4856

20 Rebek, J , McCready, R , Wolf, S , Mossman, A , *J Org Chem* **1979**, *44*, 1485

21 Eckert, H , Forster, B *Angew Chem Int Ed Engl* **1987**, *26*, 894

22 Burk, R M , Roof, M B *Tetarhedron Lett* **1993**, *34*, 395

23 For NMR studies of Tuftsin see (a) Blumenstein, M , Layne, P P , Najjar, V A *Biochemistry* **1979**, *18*, 5247 (b) Siemion, I Z , Lisowski, M , Sobczyk, K *Ann N Y Acad Sci* **1983**, *419*, 56

24 Heavner, G A , Audhya,T , Doyle, D , Tjoeng, F-S, Goldstein, G *Int J Pept Protein Res* **1991**, *37*, 198

25 Wang, S-S, Gisin, B F , Winter, D P , Makofske, R , Kulesha, I D , Tzougraki, C , Meienhofer,J *J Org Chem* **1977**, *42*, 1286

26 For the synthesis of Tuftsin and its analogs see (a) Yajima, H , Ogawa, H , Watanabe,H , Fujii, N , Kurobe, M , Miyamoto,S *Chem Pharm Bull* **1975**, *23*, 371 (b) Nozaki, S , Hisatsune,K , Muramatsu, I *Bull Chem Soc Japan* **1977**, *50*, 422 (c) Dagan, S , Gottlieb, P , Tzehoval, E , Feldman, M , Fridkin, M , Yasumura, K , Okamoto, K , Yajima, H *J Med Chem* **1986**, *29*, 1961 (d) Drugs fut **1984**, 9, 532